

AN ABSTRACT OF THE THESIS OF

Anneka Wickramanayake for the degree of Honors International Baccalaureate of Science in Biology presented on May 18, 2010. Title: Mismatch Repair-Dependent Cellular Responses to Polycyclic Aromatic Hydrocarbons: An Investigation of Research Methods and Materials.

Abstract Approved: _____

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Polycyclic aromatic hydrocarbons (PAHs) are carcinogenic chemical compounds found in the environment, largely as a result of partial combustion of organic compounds. PAHs are present in the atmosphere in populated cities worldwide. Because of the health risks they create, PAHs are a global health concern. PAHs are introduced into the body, primarily through diet. Cells metabolize PAHs into highly reactive diol epoxides, which react with DNA, forming PAH-DNA adducts. These adducts can cause mutations in DNA, contributing to the increased risk of cancer associated with PAH exposure. DNA mismatch repair (MMR) is a cellular mechanism that protects the genomic integrity of a cell. Mismatch repair deficiencies are associated with human cancer, particularly colorectal cancer. It is hypothesized that MMR status will influence cellular responses to PAH exposure; specifically, MMR-deficient cells will show decreased apoptosis and increased mutations induced by exposure to PAHs. The goal of this project is to determine how to use a cell culture model to measure mutations induced by PAH exposure in MMR proficient and deficient human cells. We conclude that Alamar Blue is an appropriate, efficient reagent and method for cell density and viability measurements in 96-well plate cultures and we demonstrate the conditions for the phenotypic selection of HPRT mutants with 6-thioguanine.

Key Words: DNA Mismatch Repair, Polycyclic Aromatic Hydrocarbons, Colorectal Cancer, Environmental Pollution

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Mismatch Repair-Dependent Cellular Responses to Polycyclic Aromatic Hydrocarbons:

An Investigation of Research Methods and Materials

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Mismatch Repair-Dependent Cellular Responses to Polycyclic Aromatic Hydrocarbons: An Investigation of Research Methods and Materials

PAHs

Polycyclic aromatic hydrocarbons (PAHs) are large aromatic planar compounds formed largely by the partial combustion of organic compounds. They are readily found in the environment. The ability of PAH-containing mixtures to induce human cancer is well-known. Humans are exposed to PAHs in a variety of ways, but the primary route of exposure is through diet (Baird, 2005). Many of these environmental pollutants are metabolically activated in mammalian cells to form highly reactive PAH-diol epoxide derivatives which can covalently bind to cellular macromolecules, including native DNA (Wu, 2003). This mechanism of activation, with modifications in certain cases, has been found to occur in all carcinogenic PAHs that have been studied (Phillips, 1999).

There are many different PAHs found in the environment. They each have a different prevalence and a different toxicity. The US Environmental Protection Agency (EPA) has proposed a system of ranking the relative toxicity of individual PAHs. Their system assigns a Toxicity Equivalency Factor (TEF) to each PAH. Its basis is to separate all PAHs into carcinogenic and non-carcinogenic compounds. The PAH, Benzo[a]pyrene (B[a]P), is used as a standard, set to a TEF of 1. All other PAHs are ranked relative to B[a]P. A non-carcinogenic PAH would be assigned a TEF of 0. Carcinogenicity is measured by rate of tumorigenesis. The TEFs of several PAHs are listed in Figure 1. However, there are several other proposed methods of measuring TEFs , and the validity of this ranking scheme is debated (Nisbet 1992).

PROPOSED TOXICITY EQUIVALENCY FACTORS (TEFS) FOR INDIVIDUAL PAHS

Compound	TEF
Dibenzo[<i>a,h</i>]anthracene	5 ^a
Benzo[<i>a</i>]pyrene	1
Benzo[<i>a</i>]anthracene	0.1
Benzo[<i>b</i>]fluoranthene	0.1
Benzo[<i>k</i>]fluoranthene	0.1
Indeno[123- <i>c,d</i>]pyrene	0.1
Anthracene	0.01
Benzo[<i>g,h,i</i>]perylene	0.01
Chrysene	0.01
Acenaphthene	0.001
Acenaphthylene	0.001
Fluoranthene	0.001
Fluorene	0.001
2-Methylnaphthalene	0.001
Naphthalene	0.001
Phenanthrene	0.001
Pyrene	0.001

^a A TEF of 1 appears to be appropriate for high doses of DBA but the TEF of 5 is considered more likely to be applicable to environmental exposures (chemical-related tumor incidence rate of less than about 25%).

Figure 2- Proposed Toxicity Equivalency Factors for Individual PAHs (Figure from Nisbet 1992)

Benzo[*a*]pyrene is a known carcinogenic PAH that is metabolically activated to form benzo[*a*]pyrene diol epoxide (BPDE). BPDE is a highly reactive electrophilic metabolite that covalently binds to DNA forming PAH-DNA adducts. Unrepaired DNA-adducts may result in mutations during cellular replication (Schiltz, 1999).

In this study, B[*a*]P is used as a representative PAH, thus BPDE is used as a representative for PAH diol epoxides. BPDE is used instead of B[*a*]P because it is the reactive form that would be found *in vivo* as a product of metabolism. Benzo[*a*]pyrene was the selected as a representative because it is the most studied PAH and it is known to be a potent carcinogen in mammals (Phillips, 1999). B[*a*]P is also present in nearly all

environmental samples of PAHs. Therefore this isolated PAH was used to minimize any influence of differential metabolism in this study. It should be noted that it does not take into account the effects of other chemical components of polluted ambient air and their effects when combined with B[a]P.

PAHs and Cancer

PAH adducted DNA undergoes errors in replication causing mutations that initiate the carcinogenic process. Associations have been observed between DNA adduct formation, mutagenesis, and tumorigenesis (Farmer, 2003). The mutagenic potential of PAH-DNA adducts are determined by several factors: the specific PAH structure, the site of the epoxide formation, the targets on DNA that are attacked by the PAH and the location of the base that is targeted (Boffetta 1997). Studies have also shown that the efficiency of DNA polymerases and the efficiency of repair mechanisms such as nucleotide excision repair and mismatch repair affect the mutagenicity of PAH-DNA adducts (Binkova 2007).

There are many types of cancer that are thought to arise due to PAH exposure. Occupational exposure to PAHs is associated with increased rates of lung cancer, skin cancer and bladder cancer. In the general population, PAHs are linked to gastric, colorectal pancreatic and cervical cancers (Boffetta 1997).

Human exposure to PAHs

PAHs are introduced into the human body by inhalation, ingestion, and skin contact. Non-occupational respiratory exposure is mainly from tobacco smoke and urban air, while the major source of ingested PAHs is cooked food. The main route of occupational exposure is inhalation, and in some cases, skin exposure (Bofetta 1997). The primary route of human exposure, however, is through diet.

There are several ways that PAHs can become introduced into the diet. PAHs can be present in uncooked food. Atmospheric PAH contamination results in the compounds becoming deposited in the aquatic environment where they can be taken up by aquatic organisms, and thus enter the food chain, ending up in humans. The presence of PAHs in plants has been widely demonstrated. There are three possible sources of contamination of plants: uptake from atmospheric exposure, uptake from the soil and endogenous biosynthesis. Leafy vegetables can also be a significant source of PAHs in the human diet. The main source of contamination in this case is the deposition of small airborne particles containing the compounds. According to Grasso, levels of 2.85-24.5 ppb benzo[a]pyrene are typical in fresh vegetables (Phillips, 1999)

PAHs are also found in processed or smoke-cured food. Smoke curing of meat and fish is often done by treating with wood smoke. The smoke is produced by incomplete combustion, which therefore generates PAHs. In a study of smoked food, total PAH concentrations in smoked meat ranged from 2.6-29.8 ppb and 9.3-86.6 ppb in fish (Phillips, 1999).

Cooked food may also contain high levels of PAHs. When food, particularly meat is cooked over an open flame, PAHs are formed. If the meat is in direct contact with the flame, pyrolysis of the fats generates PAHs that can become deposited on the meat. Even when not in direct contact, fat dripping on to the flame or hot coals creates PAHs which can then be deposited on the food. Charred food of almost any composition will contain PAHs. Some of the highest levels of PAHs reported in foods have been detected in food cooked over open flames. For example, in barbequed meat, total PAHs were found to be present at levels up to 164 ppb, with benzo[a]pyrene being present at levels as high as 30ppb (Phillips, 1999).

Several studies have been carried out to determine the level of intake associate with a normal or average human diet. In an analysis of PAHs in the typical diet of people living in the UK it was found that about one-third of PAHs came from cereals and another one-third came from oils and fats. Fruits, vegetables and sugars contributed much of the remainder. The contribution of meat, fish, milk and beverages were relatively small. It is of note that barbecued food consumption in the UK is infrequent for most of the population. Based on a total daily consumption of 1.46kg of food and beverages, the total daily dietary load of PAHs was calculated to be 3.70 μ g. A similar study of the Dutch diet estimated an average daily intake of PAHs at between 5 and 17 μ g per day (Phillips, 1999).

Global Prevalence of PAHs

PAHs are present worldwide, especially in densely populated areas. The majority of PAHs in the environment come from incomplete combustion of carbonaceous material during energy and industrial production processes. Natural processes, such as forest fires and volcanic eruptions also produce PAHs. The major anthropogenic atmospheric emission sources of PAHs are biomass burning, coal and petroleum combustion and coke and metal production (Zhang, 2009 B).

In developing countries, emissions of PAHs have decreased as the efficiency of energy utilization has improved over the past few decades. Prior to this, however, evidence suggests that PAH emission have been increasing in developing countries due to rapid population growth and the associated energy demand. Because of the close relationship between PAH emissions and energy consumption, a strong correlation is anticipated between PAH emissions and some social and economic parameters. Additionally a strong correlation exists between atmospheric PAH concentrations and population demographics (Zhang, 2009 B).

Globally, biomass burning, including both biofuel combustion and wildfires, dominated the PAH emission sources with contributions of 56.7% and 17.0% of the total global PAH emissions, respectively, as can be seen in Figure 2 (Zhang, 2009 B). Other important global PAH sources included consumer products, traffic oil combustion and domestic coal combustion which contributed 6.9%, 4.8% and 3.7 %, respectively. The

major industrial activities contributed less than 10% to the total global PAH emissions, with coke production contributing to the most, at 3.6% (Zhang, 2009 B).

The relative contribution of different PAH sources in the different countries depended on the energy structure, status of development, population density and vegetation cover of the country. For example, in India, biofuel accounted for over 90% of the total emissions because the country relies heavily on firewood, straw, animal dung and petroleum for domestic energy, while PAH emission from petroleum combustion were much lower than biomass. According to the International Energy Agency, in India, in 2004, biofuel and petroleum products accounted for 82.6% and 11.5% of the total energy consumption of PAH emissions in the domestic sector (Zhang, 2009 B).

In China, coal is used extensively for coke production and domestic cooking and heating. In 2004, coal combustion accounted for 60% of the energy consumption in China, according to the International Energy Agency. Although biomass ranks first in PAH emission sources in China, its relative contribution was 66.4% which is significantly lower than in India. The coke industry and domestic coal combustion ranked second and third among PAH emission sources in China. According to an emission inventory in China, the PAH emissions from the coke industry were primarily from small-scale coke ovens run by local farmers adjacent to coal mines. These small-scale ovens have been phased out by a relatively newly issued Coal Law. However, according to Yanxu Zhang, because of the combustion of biomass and coal for energy in China is not expected to

change in the upcoming decades, severe PAH contamination is expected to continue (Zhang 2009 A).

In Brazil and Sudan, forest fires and savanna fires surpassed biofuel sources in PAH emission due to the high vegetation cover and low population density. Fire events were also serious contributors in the Amazon region and in many African countries. Similar to other developed countries, consumer product use and traffic oil combustion were the major PAH emission sources in the United States, followed by waste incineration, biofuel combustion and petroleum refining (Zhang, 2009 B).

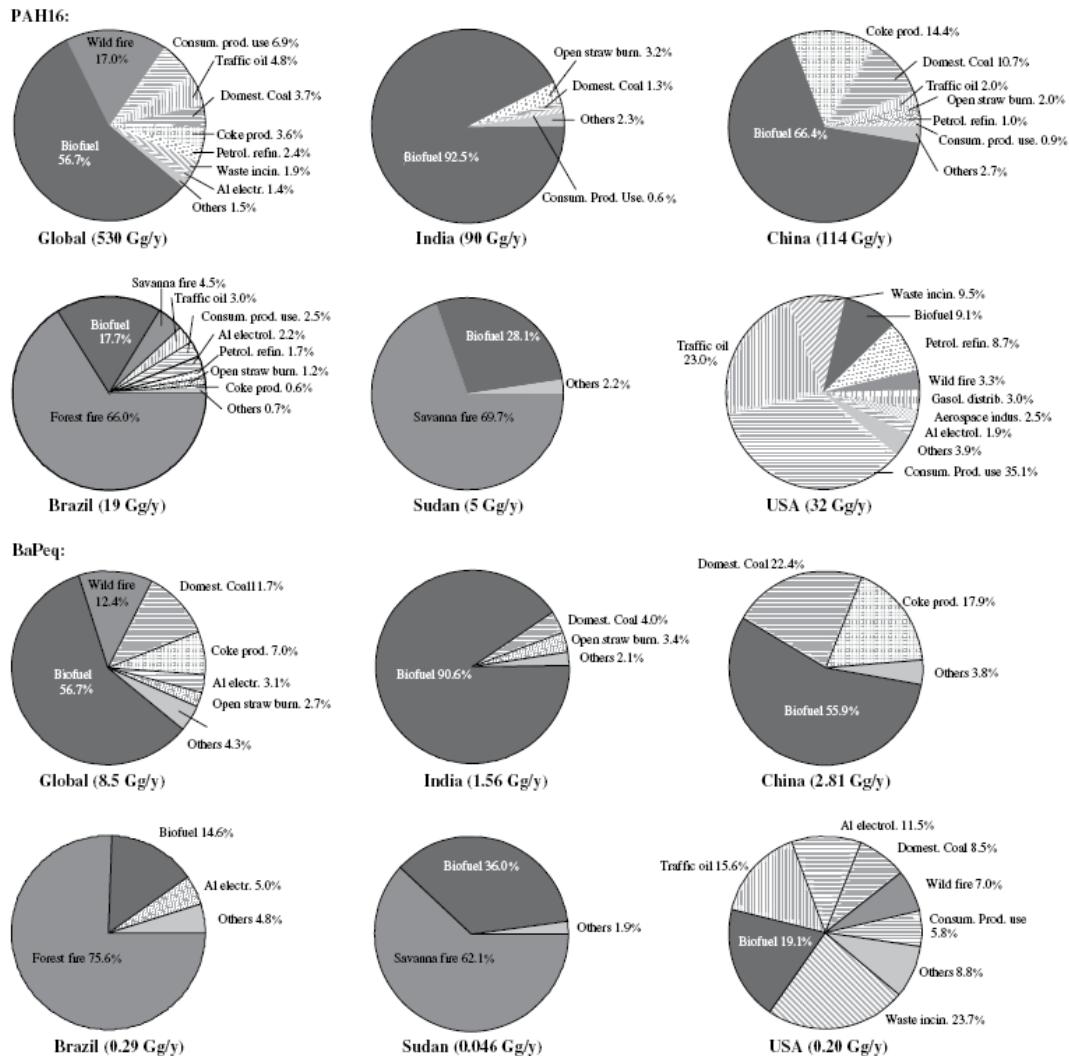


Figure 2: Global PAH Sources- The relative contributions of various sources to PAH emissions in several countries. Includes emission of a combination of 16 PAHs (PAH16) and emission of Benzo[a]pyrene (BaP eq). (Image from Zhang 2009)

A few countries have adopted guidelines or regulations in order to limit the levels of PAHs in the air. In July of 2003, the European Commission presented a Proposal for a Directive relating to arsenic, cadmium, mercury, nickel and polycyclic aromatic hydrocarbons in ambient air as one of several daughter directives of the Directive of the Air Quality Framework Directive. The Commission decided to set a ‘target value’ for benzo[a]pyrene, as an indicator for PAHs, which should be attained ‘as far as possible

and without entailing excessive costs.' The target value of benzo[a]pyrene was determined to be 1ng/m³ (Bostrom 2002).

In Sweden, the Governmental Commission on Environmental Health (1996) proposed objectives as well for indicators of volatile and non volatile carcinogens. For PAHs the proposal was that by 2002, the long-term mean level of benzo[a]pyrene should not exceed 0.1ng/m³. This level is equivalent to a theoretic excess lifetime cancer risk of 1 in 100,000 for benzo[a]pyrene as an indicator of PAH, based on a risk assessment by the World Health Organization. This long-term objective was later adopted by Government Bill 2000/01:130 (Bostrom, 2002). In Sweden, residential burning of wood is regarded as the largest source of PAHs, and in cities, mobile sources, including working machinery contribute to the major part of PAH emissions.

There is evidence that the levels of contamination of crops are lower now than they were in the last century. An analysis of stored crop samples from Rothamsted Experimental Station has shown that the highest levels of PAHs in herbage and wheat grain are found in samples from 1879-1881, while those from the 1980s had the lowest levels. The decline in contamination may be attributed to a reduction in coal burning in the US since the Clean Air Act of 1956 and a conversion to cleaner burning fuels for power generation and domestic heating, such as natural gas (Phillips, 1999).

However, in several countries, PAHs are still an abundant pollutant. In 2001, samples were taken in Hong Kong and the concentrations of 16 selected PAHs were quantified.

Samples were taken according to particle size (PM₁₀ and PM_{2.5}). Low molecular weight PAHs tend to be more concentrated in the vapor-phase while the ones with higher molecular weight are often associated with particulates. The sum of the 16 PAHs in PM_{2.5} at roadside ranged from 3 to 330 ng/m³, and in PM₁₀ between 5 and 297 ng/m³, whereas in a residential/industrial/commercial site, the total PAHs in PM_{2.5} ranged from 0.5 to 122 ng/m³, and 2 to 269 ng/m³ in PM₁₀ (Guo, 2003). These values are significantly higher than the maximum levels established by the European Commission.

The use of combustion for domestic activities, especially in developing countries is also a significant source of PAHs. Industrial combustion, which is large scale, is normally better controlled, and more complete, thus resulting in lower formation of PAHs than small-scale combustion such as the use of small, domestic cookstoves. For example, the emission factor of benzo[a]pyrene from cookstoves can exceed that from coal on an energy equivalent basis by a factor of 100. And in eastern North America, residential wood combustion alone, was estimated to account for >30% of anthropogenic PAH emissions in the area (Oanh, 1999).

Also, intensive use of biofuels for domestic combustion causes emission of high levels of PAHs. This is due to the volatile content of biofuels, which leads to a higher possibility of incomplete burning. It is estimated that 50% of the world's households use biofuels for daily cooking and heating. And according to the World Health Organization, the current economic trends indicate that dependence on biofuels is likely to continue. In developing countries, combustion of biofuel, such as wood, agricultural residues, cattle dung, etc,

accounts for more than 90% of the total fuel consumption in rural areas (Oanh, 1999). These sources of PAH emissions are difficult to regulate, thus this form of pollution is still a global concern.

Because of the global prevalence of PAHs and the human exposure to them, this study has important implications for understanding the incidence of human cancer worldwide.

MMR

DNA mismatch repair (MMR) is a protein system conserved from bacterial to human cells that is essential for maintaining the fidelity of DNA replication. The MMR pathway targets base-substitution mismatches and insertion-deletion mismatches that arise as a result of replication errors that escape the proofreading mechanism of DNA polymerases. DNA polymerases already ensure a high level of accuracy in replication, with the probability of an erroneous base being incorporated being only on the order of 10^{-7} per base pair, per replication (Iyer, 2006). MMR contributes an additional 50-1000-fold to the overall fidelity of DNA replication. (Hsieh, 2008).

MMR repair in human cells is not fully understood, but the study of this mechanism in *Escherichia coli* (diagrammed in Figure 3) has contributed significantly to our understanding of the pathway. In prokaryotes, MMR is dependent on three highly conserved proteins: MutS, MutL, and MutH. MMR is initiated when mismatches are recognized by MutS, which acts as an ATPase. MutS acts together with MutL to trigger the excision repair pathway by activating endonucleolytic cleavage by MutH. Mut H

nicks the unmethylated DNA strand on either the 5' or 3' side of the mismatched base pair. This nick, with the help of Mut L, acts as an entry point for helicase II and single-stranded binding protein (SSB) to unwind the strand so that it is now exposed to digestion by one of four single-stranded exonucleases. These exonucleases are ExoI, ExoVII, ExoX, and RecJ. Once, the exonuclease has excised past the mismatch in either the 3'-5' or 5'-3' direction, depending on its polarity, the DNA in the gap is resynthesized by DNA pol III and ligase (Hsieh, 2008).

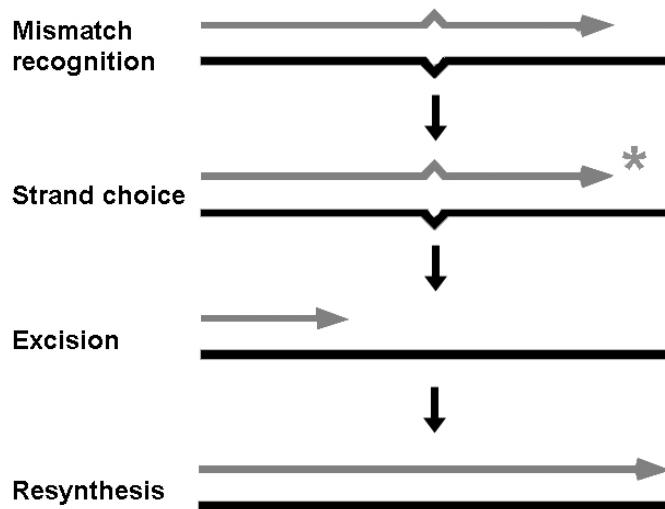


Figure 3: Mismatch Repair Mechanism- The general repair mechanism as seen in prokaryotes. Mismatch is recognized by MutS, Mut L aids in choosing the strand which MutH nicks and endonucleases excise, and DNA is resynthesized by DNA pol III and ligase (Figure by Andrew Buermeyer, reprinted with permission).

Studies have shown that the MMR pathway is highly conserved in prokaryotes and eukaryotes, but with a few key modifications. There are several homologs of MutS and MutL that reflect this. While MutS proteins function as homodimer in prokaryotes, they function as a heterodimer in eukaryotes. There are two heterodimeric complexes: MutS α and MutS β , which are made up of MutS homologs (Kolodner 1996). Both complexes contain MSH2 as a subunit. In MutS α , MSH2 is paired with MSH6, while in MutS β ,

MSH2 is paired with MSH3 pairs MSH2 with MSH3. MutS α recognizes base pair mismatches and insertion-deletion loops of 1-2 nucleotides in length and MutS β recognizes insertion-deletion loops of 2-10 nucleotides (Hsieh, 2008).

Once MutS α or MutS β has recognized and bound to mismatched DNA, a MutL homolog, known as MutL α , is recruited (Jiricny 2006). There are three MutL homologs represented in eukaryotes: MutL α , MutL β , and MutL γ . Mut α is the primary protein involved in MMR, however recent findings show that MutL γ is also active in MMR (Cannavo 2005). MutL α is a heterodimer consisting of MLH1 and PMS2. Once it has been recruited to the mismatched DNA, the complex of MutS α and MutL α translocates along the DNA until it reaches a strand break, when it loads the appropriate exonuclease, specifically EXO1, to degrade the error-containing strand. Studies have demonstrated that MutL α , itself, has latent endonuclease activity. When it is activated, it is able to cause single-strand breaks near the mismatch in DNA. This creates new sites for the exonuclease to excise the mismatch-containing strand (Jiricny 2006).

There are several other proteins involved in eukaryotic MMR, although their function is not yet understood. For example, the MutL α complex interacts with proliferating cell nuclear antigen (PCNA). The precise role of PCNA in MMR has not been fully elucidated, but it is thought that it acts as a scaffold for protein interactions, thus aiding in the coordination of events in the MMR pathway. Additionally, DNA polymerases Pol δ and Pol ϵ have been implicated in the synthesis process of eukaryotic MMR (Lee 2006). Studies which reconstitute eukaryotic MMR *in vitro* have shown that RPA, HMGB1, RFC, and ligase I are also essential for MMR (Zhang 2005).

There are other similarities between MMR in prokaryotes and eukaryotes. The repair mechanism functions bi-directionally and the efficiency of repair is similar. Also, both prokaryotes and eukaryotes have strand-specific MMR, although the mechanism of strand differentiation may be different between the two (Hsieh, 2008).

MMR-Dependent Responses to DNA Damage

MMR also influences cellular responses to damaged DNA. In addition to directly repairing DNA lesions as described, MMR may also trigger cell-cycle delay so that other DNA repair pathways can be employed to remove the lesion. In very severe cases, the MMR pathway can also reduce the potential negative consequences for a multicellular organism by signaling cells that have an irreparable level of DNA damage to undergo apoptosis (Wu 1999).

It has been demonstrated that cell lines that are defective in MMR show resistance to the cytotoxic effects of various DNA-damaging agents. This increase in viability is associated with an induced hypermutability (Glaab, 2000). Hypermutability in response to DNA-damaging exposure in MMR-deficient cells is likely to be the result of failure to repair promutagenic DNA mismatches induced by misincorporation opposite damaged bases during DNA replication (Glaab, 1999). Thus, cells deficient in MMR are more resistant to the cytotoxic effects of various agents that are normally toxic to human cells. This means that MMR deficient cells are more likely to survive and proliferate. By increasing mutation associated with exposure to such agents, there is a higher probability of mutating genes that are essential in control of cell proliferation and cell death.

Therefore tumorigenesis is likely promoted by the combination of deficiencies in both of these control mechanisms. It is a logical conclusion that there are other compounds which humans are exposed to daily, such as PAHs, that will produce a similar response in MMR-deficient cells and that exposure to these compounds will have an effect on the risk of colorectal tumorigenesis (Glaab, 2000).

MMR Deficiency and Human Cancer:

MMR deficiencies and the associated loss of genomic stability have been well documented in human cancers, specifically, colorectal cancer (CRC). Inactivation of MMR creates a strong mutator phenotype, meaning that the rate of spontaneous mutation is greatly elevated (Hsieh, 2008). Significantly elevated DNA mutation levels are observed to spontaneously arise in MMR defective cells, both at endogenous loci and in microsatellite sequences. Eukaryotic cells that are deficient in MMR have greatly increased mutation rates, on the order of a 10-1,000-fold increase (Kunkel, 2005). This may be the cause of the increased risk of tumor formation in MMR-deficient individuals and a significantly increased risk of cancer (Glaab, 2000). It has been shown that a high level of genetic instability in microsatellite sequences is a characteristic of hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch Syndrome. It is also observed in approximately 15-20% of sporadic colonic cancers (Poulogiannis 2010).

Lack of appropriate MMR-dependent cellular responses to DNA damage generated by dietary carcinogens may also contribute to the risk of CRC in MMR-deficient individuals. Several studies suggest that MMR responds to PAH-derived DNA lesions by

specifically recognizing and binding to PAH-adducted DNA, resulting in an induction of MMR-dependent apoptosis. MMR deficient mice show an increase in PAH-induced lymphoma compared to wild-type individuals (Zienoldin, 2005). These facts, together, suggest that MMR suppresses mutations induced by PAHs and that the frequency of mutations would increase in MMR-deficient individuals, perhaps increasing the risk of cancer.

Materials and Methods

Cell Lines

Human lymphoblast cell lines, TK6 and MT1, were used. The TK6 line of cells is the parental line for MT1. TK6 cells are known to be sensitive to the toxic and mutagenic effects of alkylating agents (Goldmacher, 1986). The MT1 line was derived from TK6 cells by selecting for MNNG-resistant clonal derivatives of TK6 cell culture. MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) is an alkylating agent which gives rise to substantial amounts of O⁶-methylguanine DNA adducts. Normal TK6 cells are unable to remove these cytotoxic lesions from their DNA because they do not express the methylguanine methyl transferase (MGMT) gene (Szadowski, 2005). Goldmacher et al. generated the MT1 cell line by treating TK6 cells with the mutagen, ICR-191, and then selecting with MNNG (Goldmacher, 1986). MT1 cells are highly resistant to killing by MNNG and are hypermutable by this agent, indicating that they did not reactivate the MGMT gene. Their resistance is linked to a deficiency in MMR which allows MT1 cells to tolerate the mutagenic mispairs between O⁶-methylguanine and thymine. This finding was substantiated, and the MMR defect in MT1 cells was found to be the result of two

mutations, one that inactivates the ATPase activity of hMutS α , and another that destabilizes the hMSH6 protein in these cells. Thus TK6 cells are used as a control for the MMR-deficient MT1 cell line (Szadkowski, 2005).

Additionally, it has been proven that the cell lines differ phenotypically even without the presence of an alkylating agent. MT1 has an increased rate of spontaneous mutation, increased approximately 60-fold at the HPRT gene locus (Goldmacher, 1986). The HPRT gene is used in purine biosynthesis through the salvage pathway (Glaab 1999).

Cell Culture

Both TK6 and MT1 cell lines were grown in RPMI + 10% FBS (Fetal Bovine Serum) + PenStrep + Non-essential amino acids + L-Glutamine. Cells were incubated in 5% CO₂ in an air-jacketed incubator at 37°C. The cells are non-adherent and were grown in suspension culture.

MTS Assay

MT1 cells were taken from culture and cell density was measured using a hemocytometer loaded with 10 μ l of cell culture + Trypan Blue in a 1:1 ratio. Cells were pelleted out of suspension by centrifugation at 2000RPM for 4 minutes. The media was aspirated and cells were resuspended in fresh 10% RPMI media in order to obtain a cell density of 1,000,000 cells/ml. This cell culture was then serially diluted to create cultures with cell densities ranging from 50,000 to 1,000,000 cells/ml. These cell cultures were plated in a 96-well clear-bottomed plates with 100 μ l of culture per well using an

Eppendorf 12-channel pipette. 20 μ l of MTS was added to each well using an Eppendorf 12-channel pipette. The absorbance for each well was read using a microplate reader (Molecular Devices SpectraMax 250) set at 490nm. Absorbance measurements were taken every 30 minutes for 4 hours after exposure to MTS.

Alamar Blue Assay

MT1 cells were taken from culture and cell density was measured using a hemocytometer loaded with 10 μ l of cell culture + Trypan Blue in a 1:1 ratio. Cells were pelleted out of suspension by centrifugation at 2000RPM for 4 minutes. The media was aspirated and cells were resuspended in fresh 10% RPMI media in order to obtain a cell density of 2,000,000 cells/ml. This cell culture was serially diluted to create cultures with cell densities ranging from 0 to 2,000,000 cells/ml. These dilutions were then plated in 96-well clear-bottomed plates, using an Eppendorf 12-channel pipette to add 100 μ l of culture per well. Alamar Blue was then added with the same Eppendorf multichannel pipette, at 10 μ l per well. Fluorescence measurements were then taken 4 hours and 24 hours after Alamar Blue treatment using a GeminiXPS Spectramax spectrophotometer (excitation: 535nm, emission: 590nm, temperature: 37°C, shake tray three times before measurement).

MNNG and 6-TG Cytotoxicity Assay

TK6 and MT1 cells were taken from cell culture and cell density for each cell line was measured using a hemocytometer loaded with 10 μ l of cell culture + Trypan Blue in a 1:1 ratio. Cells were pelleted out of suspension by centrifugation at 2000RPM for 4 minutes.

The media was aspirated and cells were resuspended in fresh 10% RPMI media in order to obtain a cell density of 100,000 cells/ml. The cell culture was then plated in four 96-well clear-bottomed plates using an Eppendorf 12-channel pipette to add 100 μ l of culture per well. Simultaneously, MNNG was serially diluted in 10% complete RPMI media to concentrations ranging from 0 to 10,000nM. Immediately after the MNNG was prepared, 10 μ l of it was added to each well containing cells using the 12-channel pipette. Then, using the 12 channel pipette, 10 μ l of Alamar Blue was added to each well in only one plate and the fluorescence was read 4 hours later. This was repeated in each of the 96-well plates, one plate per day, for 3 days.

This experiment was repeated using 6-thioguanine (6-TG) instead of MNNG. 6-TG was serially diluted in 10% complete RPMI media to concentrations ranging from 0 to 5,000nM. Immediately after the 6-TG media was prepared, 10 μ l of it was added to each well containing cells using the 12-channel pipette. Then, using the 12 channel pipette, 10 μ l of Alamar Blue was added to each well in only one plate and the fluorescence was read 4 hours later. This was repeated in each of the 96-well plates, one plate per day, for 4 days.

Plating Efficiency

TK6 and MT1 cells were taken from cell culture and cell density for each cell line was measured using a hemocytometer loaded with 10 μ l of cell culture + Trypan Blue in a 1:1 ratio. Cells were pelleted out of suspension by centrifugation at 2000RPM for 4 minutes. The media was aspirated and cells were resuspended in fresh 10% RPMI media in order

to obtain a cell density of 25 cells/ml. This cell density was then serially diluted in 10%RPMI media to obtain additional cultures with cell densities of 15 cells/ml and 5 cells/ml. Each cell culture was plated in a different 96-well clear-bottomed plate with 200 μ l of culture per well using an Eppendorf 12-channel pipette. The plates were incubated at 37° C for 14 days. Then the plates were examined visually, with the aid of a microscope. The number of surviving colonies was measured by counting the number of wells that contained live cells. Wells containing media that was yellow in color were labeled as having live cells, while wells containing media that was pink in color were labeled as not containing live cells.

Using the number of wells containing live cells, the plating efficiency (without selection) was calculated using the following formula:

$$PE = \frac{1}{N_{PE}} \times \ln \left[\frac{(T_{PE} - P_{PE})}{T_{PE}} \right]$$

PE = plating efficiency

N_{PE} = number of cells plated in each well (in this case 1, 3, or 5 cells)

T_{PE} = total number of wells plated with cells

P_{PE} = total number of wells with surviving colonies

Spontaneous Mutation

6-TG media was prepared by adding 6-TG to 10% RPMI media at a concentration of 1 μ g/ml. TK6 and MT1 cells were taken from cell culture and cell density for each cell line was measured using a hemocytometer loaded with 10 μ l of cell culture + Trypan Blue in a 1:1 ratio. Cells were pelleted out of suspension by centrifugation at 2000RPM for 4 minutes. The media was aspirated and cells were resuspended in the 6-TG media to a density of 40,000 cells/ml. This culture was then plated in 96-well clear-bottomed plates

with 200 μ l of culture per well using an Eppendorf 12-channel pipette. The plates were incubated at 37° C and observed for 3 weeks. The number of surviving colonies was measured by counting the number of wells that contained live cells. Wells containing media that was yellow in color were labeled as having live cells, while wells containing media that was pink in color were labeled as not containing live cells. The cells that survive are HPRT mutants and they were selected and frozen for later use.

Using the number of wells containing growth and the plating efficiency, the spontaneous mutant frequency (MF) was calculated using the following formula:

$$MF = P_{PE} / [T_c \times PE]$$

MF = mutant frequency

P_{PE} = total number of wells with surviving colonies

T_c = total number of cells plated

PE = Plating efficiency

It should be noted that plating efficiency is an estimate as it was calculated after two weeks of growth while mutant formation was analyzed after three weeks of growth.

Gel Electrophoresis

The surviving HPRT mutants from the induced mutation study were analyzed by gel electrophoresis. RNA was purified from these cells using a Quiagen RNeasy Mini Kit. Frozen cells were thawed, then 350 μ l of RLT buffer was added for cell lysis. Cells were vortexed for 1 minute, then pipetted into a QIAshredder spin column (Quiagen), placed in a 2ml collection tube, then centrifuged for 2 minutes at 14,000RPM. 350 μ l of 70% ethanol was added. 700 μ l of the sample was transferred to an RNeasy spin column (Quiagen) placed in a 2ml collection tube and centrifuged at 10,000RPM for 15 seconds.

700 μ l of buffer RW1 was added to the tube which was centrifuged at 10,000RPM for 15 seconds. The flow-through was discarded. 500 μ l of buffer RPE was added to the spin column and centrifuged for 15 seconds at 10,000RPM. The flow-through was discarded. Another 500 μ l of buffer RPE was added to the spin column and centrifuged for 2 minutes at 10,000RPM. The spin column was placed in a new collection tube and centrifuged at 14,000RPM for 1 minute. The spin column was placed in a new 1.5ml collection tube and 50 μ l of RNase-free water was pipetted onto the spin column membrane. This was then centrifuged for 1 minute at 10,000RPM to elute the RNA. The samples were analyzed on a NanoDropND-1000 Spectrophotometer for purity and for RNA concentration. Samples were used if they had a 260/280 and a 260/230 reading of 2.00 ± 0.25 . The samples were stored overnight at -20°C.

These samples were then used to synthesize cDNA using Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR. Approximately 1000-1500ng of RNA was used from each sample and combined with 1 μ l of 50 μ M oligo(dT)_{20'}, 1 μ l of 50ng/ μ l random hexamers, 1 μ l of 10mM dNTP mix and enough DEPC-treated water to make a final volume of 10 μ l. These samples were incubated at 65°C for 5 minutes, then placed on ice for at least 1 minute. cDNA synthesis mix was made for 14 reactions by mixing 28 μ l of 10X RT buffer, 56 μ l of 25mM MgCl₂, 28 μ l of 0.1M DTT, 14 μ l of RNaseOUT (40U/ μ l) and 14 μ l of SuperScript III RT (200U/ μ l). 10 μ l of cDNA synthesis mix was added to each RNA/primer mixture on ice. The samples were mixed gently and collected by brief centrifugation. They were then incubated at 25°C for 10 minutes, at 50°C for 50 minutes, 85°C for 5 minutes, and then chilled on ice. Reactions were collected by brief

centrifugation, then 1 μ l of RNaseH was added to each sample. Then they were incubated for 20 minutes at 37°C. The samples were stored over night at -20°C.

Next, PCR amplification was performed on the samples with Promega GoTaq Green Master Mix. Eight samples were prepared at a time. A reaction mix was prepared on ice by mixing 100 μ l of GoTaq Green Master Mix 2X, 20 μ l of forward primer (CCT GAG CAG TCA GCC CGC GC, 100pmol/ μ l), 20 μ l of reverse primer (CAA TAG GAC TCC AGA TGT TT, 100pmol/ μ l), and 36 μ l of nuclease-free water. Then 22 μ l of the master mix was added to each cDNA sample of 3 μ l. The samples were denatured, annealed and extended by incubation cycles as programmed in the thermal cycler. The samples were stored over night at -20°C.

The PCR products were then cleaned with USB ExoSAP-IT. 5 μ l of each PCR product was mixed with 2 μ l of ExoSAP-IT. The samples were incubated for 15 minutes at 37°C, then at 80°C for 15 minutes. These products were then stored at -20°C until use for gel electrophoresis.

The samples were then loaded and run on a 1.0% agarose gel with ethidium bromide for 50 minutes at 180V and 120mA. Two ladders were also run on the gel: Gene Ruler 100bp Plus (Fermentas) and TrackIt 100bp DNA Ladder (Invitrogen). The gel was imaged with the ChemiGenius (Synoptics) imaging station.

Results and Discussion

Research Goals

MMR proteins recognize PAH-DNA adducts and are necessary for normal cytotoxic responses to PAH exposure. We hypothesize that cells lacking MMR will show increased PAH-induced mutation. Exposure to these compounds may increase the risk of colorectal cancer for MMR-deficient individuals. This fact has potential implications for global health. To test this hypothesis, the ultimate goal of this research is to determine the response of MMR-deficient cells to PAH exposure. This project establishes the methods and conditions that are best suited for this study. It determines an effective assay for determining cell density, the conditions for measuring cytotoxicity, and for phenotypic selection of HPRT-mutant cells.

Cell Density-MTS Assay

The first goal of this study is to test the MT1 and TK6 cells' cytotoxic response to BPDE. Previous studies show that there is an increased rate of cell death among MMR deficient cells (Wu, 2003). In order to test this assumption, a method for measuring changes in cell density and viability was needed. First, an MTS assay was tested, which is a colorimetric assay using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). MTS, in the presence of phenazine methosulfate (PMS), is reduced by metabolically active cells to a formazan product soluble in tissue culture medium whose absorbance can be measured at 490nm. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number

of living cells in culture. Representative results of this assay with MT1 cells can be seen in Figure 4.

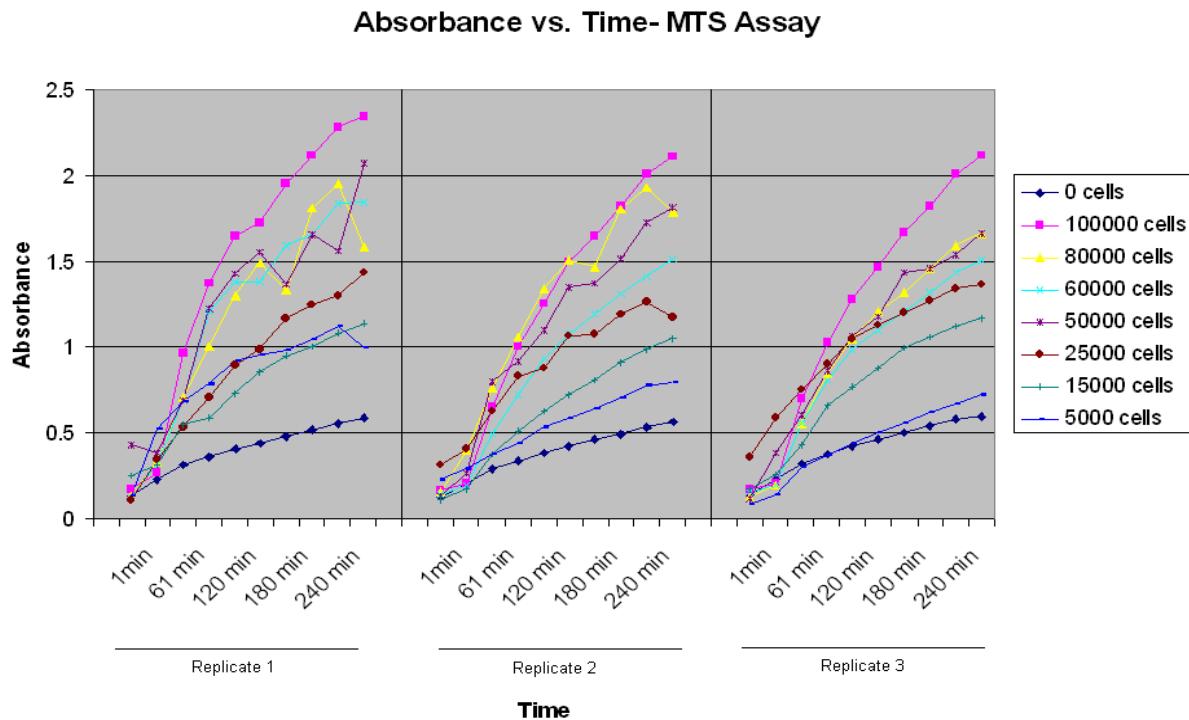


Figure 4: MTS Assay – 3 replicate plates of MT1 cells at varying concentrations and treated with MTS. Absorbance measured every 30 minutes for 4 hours.

This data generally show an increase in absorbance signal over time and with increasing cell density. However, with repeated trials, the data showed significant variation. This may be due to the relatively small dynamic range of signal relative to the number of cells plated. The range of absorbance signal was between 0 and 2.5, which is a very small range for initial cell concentrations varying between 5,000 and 100,000 cells per well. The assay was repeated several times without eliminating the variation in data. Therefore it was concluded that an MTS assay would not provide a reliable measurement of cell viability.

Cell Density-Alamar Blue

As an alternative to the MTS assay, Alamar Blue was evaluated as a method for measuring cell density. Alamar Blue contains resazurin which is reduced by metabolically active cells to the fluorescent molecule resorufin. Cell density and viability can thus be measured by measuring the fluorescence of a cell culture sample after treatment with Alamar Blue. A representation of the results of this assay can be seen in Figure 5.

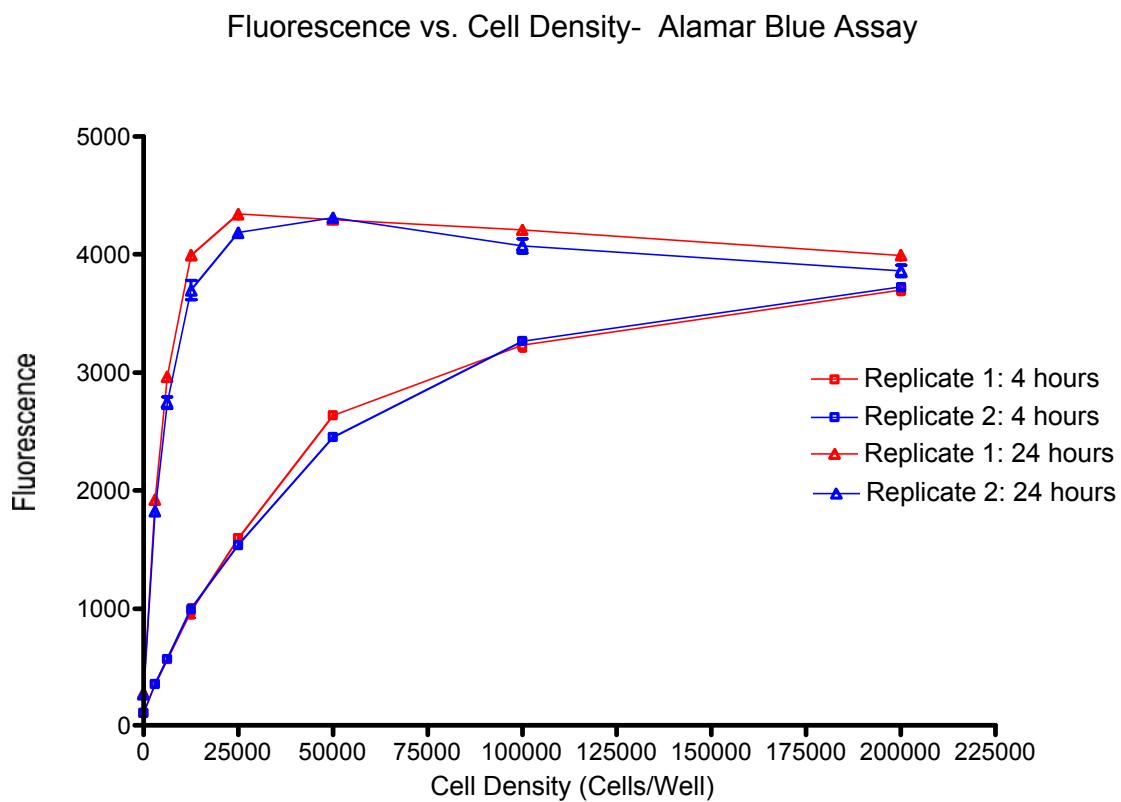


Figure 5: Representative Alamar Blue Assay- 2 replicates of MT1 cells at varying concentrations and exposed to Alamar Blue. Fluorescence measurements taken 4 hours and 24 hours after exposure.

Measurements taken 4 hours after the addition of Alamar Blue show increasing fluorescence with increasing cell density. Measurements taken 24 hours after the addition of Alamar Blue also demonstrate this; however the signal peaks at a cell density of approximately 25,000cells/well. For both time periods, the two replicates show very close fluorescence signals for the same cell density. The average background (the fluorescence of media with Alamar Blue, and no cells) was calculated to be approximately 500.0.

From this data we can conclude that fluorescence is proportional to the concentration of cells and that fluorescence signal can be used to measure cell density. The lowest cell density that can accurately read is 30,000cells/ml or 3000 cells per well. The highest cell density that can be accurately read is approximately 2,000,000 cells/ml, or 200,000 cells per well, which is generally a higher density than cells can reach in culture. Alamar Blue was most effective if the fluorescence measurement was taken 4 hours after cells were exposed to it. The amount of signal emitted by samples containing is well above the background level, and there is a large dynamic range of fluorescence signal. Thus it was established that the Alamar Blue assay can be used to monitor cell viability.

Cytotoxicity- MNNG

The next goal of this study was to determine the conditions for measuring cytotoxicity responses in TK6 and MT1 cells. MNNG was used as the cytotoxic agent since it has been proven that MNNG induces cell death in the TK6 line while the MT1 cells are more resistant to it. The Alamar Blue assay was used to measure surviving cell density as

previous experimentation established it as an effective assay. The results of this cytotoxicity assay are displayed in Figure 6.

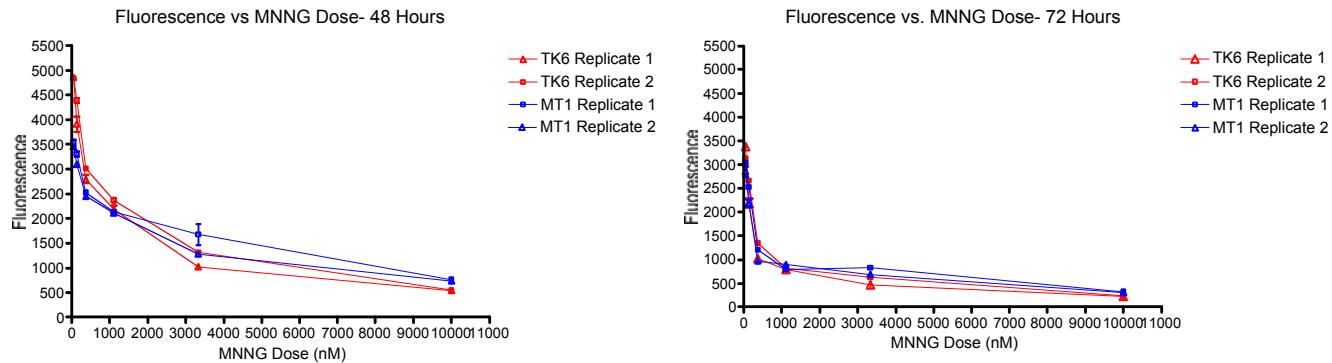


Figure 6a: MNNG Cytotoxicity- TK6 and MT1 cells at a concentrations of 10,000 cells per well were exposed to MNNG. Surviving cell densities were measured using Alamar Blue 48 hours after exposure to MNNG and 72 hours after exposure to MNNG

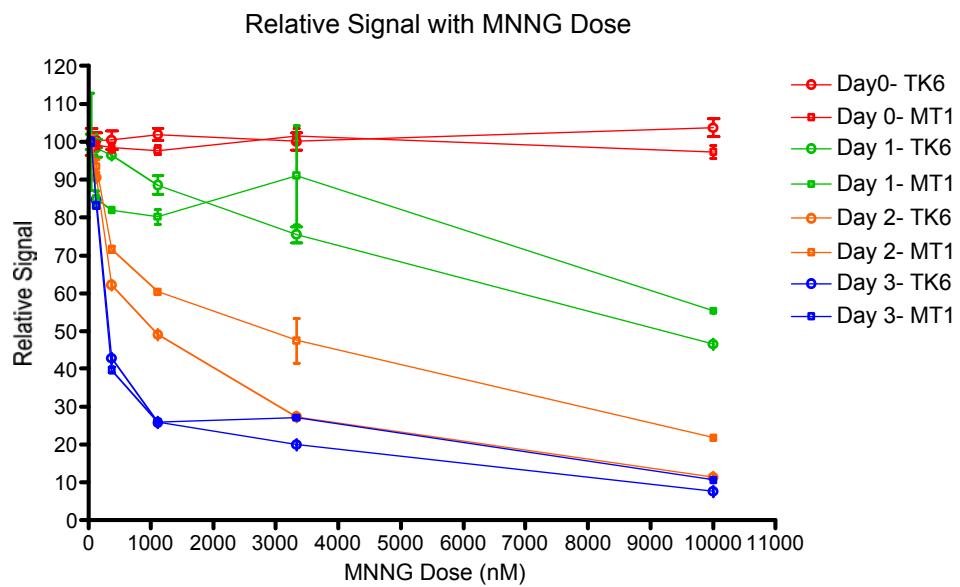


Figure 6b: MNNG Cytotoxicity- TK6 and MT1 cells at concentrations of 10,000 cells per well exposed to MNNG and treated with Alamar Blue 0,1,2, and 3 days after exposure to MNNG. Data transformed to eliminate background (media with Alamar Blue)

The data show that MNNG did induce a cytotoxic response in both cell lines, which increased with increasing concentrations of MNNG and with increased time of exposure to MNNG, as can be seen in Figure 6a. It was predicted that MT1 cells would show a lower rate of cell death, and thus a higher cell density than TK6 when both cell lines were treated with MNNG. Surprisingly, there was no significant difference between the responses of TK6 and MT1, as seen in Figure 6b, suggesting that the identity of the cell lines used is in question. Alternatively, there might be a problem with the MNNG used, such that cell killing observed was not via an MMR-dependent pathway.

Cytotoxicity- 6-thioguanine

In order to eliminate the possibility that the MNNG was the cause of these unexpected results, this experiment was repeated using 6-thioguanine (6-TG) as the cytotoxic agent. 6-TG is a base analog. It is a thiolated guanine, which has the ability to code ambiguously during replication and is unable to form a perfect base pair. Cells are able to salvage 6-TG via HPRT and to incorporate the base into their DNA. Replication of this DNA is aberrant, and discontinuities accumulate in the daughter strands. It has been suggested that MMR is able to recognize the 6-TG –containing base pairs. However some cells are tolerant, and have the ability to perform efficient replication of DNA that has been substituted with 6-TG (Griffin 1994). Therefore, we expect that the TK6 cell line will not be able to proliferate in the presence of 6-TG, while the MMR-deficient MT1 line will show a higher level of cell viability compared to TK6. The results of the 6-TG cytotoxicity assay, as measured by Alamar Blue, are displayed in Figure 7.

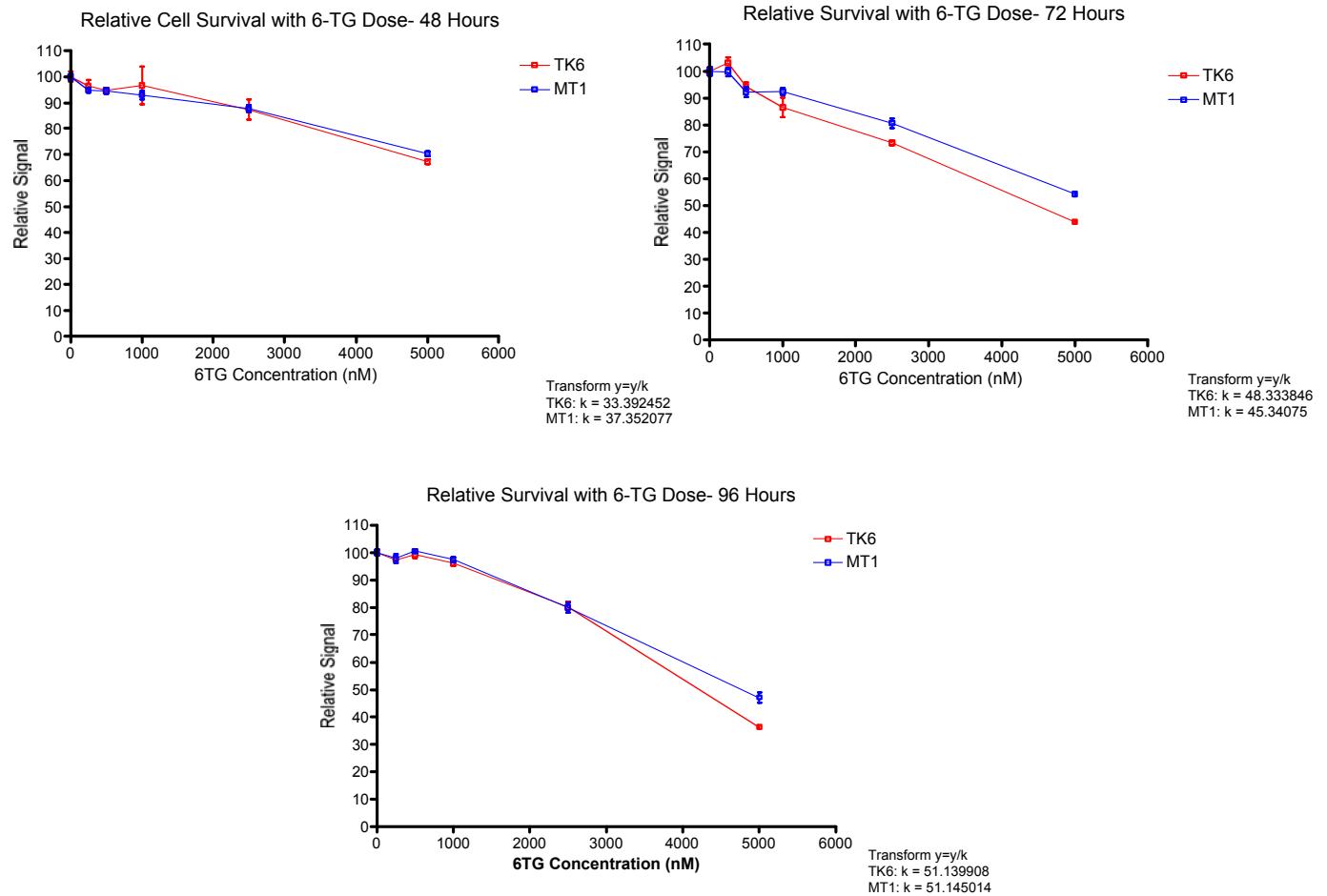


Figure 7: 6-thioguanine Cytotoxicity- TK6 and MT1 cells plated in 96 well plates at a concentration of 10,000 cells per well were exposed to 6-TG. Then Alamar Blue was added 48 Hours, 72 Hours, and 96 hours after exposure to 6-TG. Data transformed to eliminate background (media with Alamar Blue)

As seen in Figure 7, this experiment showed similar results to the MNNG cytotoxicity assay. TK6 and MT1 cells both showed decreased cell viability with increased 6-thioguanine concentrations and increased time, and there was no notable difference between the cell lines. Thus, it is likely that the cell lines are not what they are supposed to be. It is possible that there was an error in the laboratory which resulted in mislabeling. Alternatively, there could have been cross-contamination such that the two independent

MT1 and TK6 cell lines are now a mixed population. To resolve this issue, the cell cultures used can have their DNA sequenced to determine their identity. This experiment can be repeated with cells whose identities have been confirmed and that are free of cross-contamination.

Spontaneous Mutation

The second goal of this research is to induce mutation in the TK6 and MT1 cell lines and measure the frequency of mutation in order to determine whether MMR status influences mutagenic responses to PAH exposure. Ultimately, cell lines will be exposed to PAHs and the induced mutation frequency will be measured. This requires a method to distinguish mutants from non-mutants. Mutants can be identified phenotypically by performing a reporter gene assay using the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus. The HPRT gene is used in purine biosynthesis through the salvage pathway. Only cells with mutations inactivating the HPRT locus survive in the presence of the purine antimetabolite, 6-thioguanine (Barnett 2000). Exposure of mutated and non-mutated cells to 6-thioguanine selects for HPRT mutants.

Mutant frequencies are calculated as the proportion of surviving (mutated) cells to the total number of cells that were plated, adjusted based on the plating efficiency. The plating efficiency was calculated to be 0.462 for TK6 and 0.420 for MT1. The induced mutant frequency can then be calculated by subtracting the mutant frequency of a control group of cells that are not exposed to PAHs. The PAH-induced mutant frequency of MMR-proficient and deficient cells will then be compared.

Before this method can be employed, it is first necessary to demonstrate that mutant cells can be identified phenotypically, and to determine the conditions for selection that accurately identify HPRT mutants. We chose to measure the spontaneous mutant frequency in growing cultures of TK6 and MT1 cells. The spontaneous mutant frequency for TK6 was 0.000139% and for MT1 was 0.00119%. The surviving mutant cells were frozen for later use and for DNA analysis.

HPRT Transcript

In order to confirm that phenotypic selection actually distinguishes HPRT mutants from non-mutant cells, we analyzed RNA transcripts of the HPRT gene in cultured 6-TG clonal populations. We performed RT-PCR analysis in which RNA was purified, converted to cDNA and HPRT gene product was specifically amplified using PCR. MT1 and TK6 cell lines were analyzed and gel electrophoresis was performed on the samples. The results are shown in Figure 8.

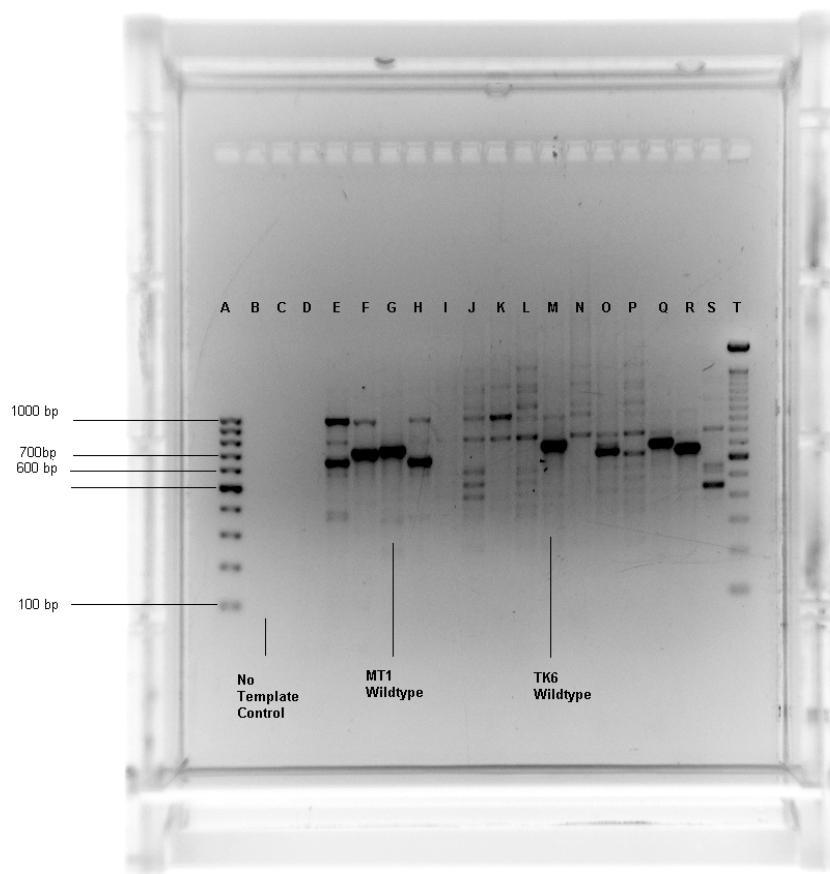


Figure 8: Gel Electrophoresis of TK6 and MT1 HPRT mutants- Lanes A and T contain a ladder, B is a no template control, and lane C is empty. Lane G contains MT1 wildtype as a positive control and lane M contains a TK6 wildtype control. Lanes D, E, F, H, I, J, N and P contain MT1 mutants. Lanes K, L, O and R contain TK6 mutants.

The apparent full-length message was amplified in reactions in lanes F, G, M, and Q.

Lanes G and M contain wildtype cells, while lanes F and Q contain supposed MT1 mutants. RT-PCR products from several other clones are shorter (lanes E, H, O, R, and S), which may indicate a deletion in the mRNA. Several of the lanes on the gel show complicated patterns with multiple faint product bands (lanes I, J, K, L, N, P, and S), and lane D appears empty altogether, indicating a poor RNA extraction or aberrant splicing which significantly altered the product.

Suggestions and Further Study

This study was successful in identifying Alamar Blue as an appropriate, efficient reagent and method for cell density and viability measurements in 96-well plate cultures. We also demonstrated the conditions for the phenotypic selection of HPRT mutants with 6-TG.

Technical Problems

There were some difficulties that arose in the technical aspects of this study. When using 96-well plates, there were inconsistencies in pipetting, which may have caused incorrect measurement of volume and estimation of cell densities. Well-functioning instruments need to be used in order to ensure a higher level of accuracy. Additionally, speed is important in these manipulations so the experimenter must take care to work efficiently. Several of the compounds used begin to degrade, so the time taken to plate and expose cells should be minimized and should be kept uniform.

Another problem that arose was the discovery that the cell lines TK6 and MT1 used may not have matched their description. This study needs to be repeated with new cells whose identity has been verified and care must be taken to properly label all samples and to avoid any cross-contamination.

Future Goals

The ultimate goal of this research is to demonstrate mismatch repair-dependent cellular responses to PAHs in order to provide information on this pressing global-health concern. Using the methods tested in this study, such as the Alamar Blue assay to measure cell

density and the 6-TG cytotoxicity assay to measure the induced mutant frequency of, the differential response of MMR-proficient and MMR-deficient cell lines to BPDE should be tested.

It also needs to be taken into consideration that humans are rarely exposed to just one form of PAH. They are exposed to complex mixtures in the environment. Thousands of chemicals, including PAHs, have been identified in polluted ambient air (Sevastynova 2007). Studies of exposure to a single chemical to not take into account the interactions of various components of polluted air which may produce synergistic, antagonistic or additive effects. It is not valid to assume that the total genotoxicity of a complex mixture is the weighted sum of the individual contributions of its components. This study should be performed with complex mixtures sampled from the environment in order to be more relevant to actual human exposure and to provide information about this global health issue.

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