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Mutagenicity of N-OH-MOCA (4-amino-4′-hydroxylamino-bis-3,3′-dichlorodiphenylmethane) and PBQ (2-phenyl-1,4-benzoquinone) in human lymphoblastoid cells

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Abstract

The genotoxic potential of two occupationally significant chemicals, 4,4′-methylene-bis-2-chloroaniline (MOCA) and 2-phenyl-1,4-benzoquinone (PBQ), was explored by monitoring the induction of mutations at the HPRT locus of AHH-1 human lymphoblastoid cells. Exposure of AHH-1 cells to the putative carcinogenic metabolite of MOCA, N-OH-MOCA, induced a 6-fold increase in mutant frequency and resulted in base pair substitutions primarily at A:T base pairs. In contrast, exposure to PBQ did not result in an increased mutant frequency although this compound was significantly more cytotoxic than N-OH-MOCA at equimolar doses. The induction of mutations at A:T sites by N-OH-MOCA is consistent with the type of DNA damage known to be produced by MOCA and provides a specific marker of genotoxic damage for exposed populations.

Keywords: HPRT mutations; AHH-1 cells; N-OH-MOCA; PBQ

1. Introduction

The polyurethane extender 4,4′-methylene bis(2-chloroaniline) (MOCA) has been demonstrated to be carcinogenic in a number of experimental animals (Russfield et al., 1975; Stula et al., 1975, 1977; Kommineni et al., 1979) and is similar in structure to a number of other arylamine carcinogens (IARC, 1971). In 1993, IARC classified MOCA as a probable human carcinogen (IARC, 1993). While no longer produced in the United States, approximately 2 million pounds were imported in 1991; there are estimated to be approxi-

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mately 100 industrial users in the United States (ATSDR, 1994). It is believed that MOCA is first *N*-hydroxylated in the liver and the metabolite N-OH-MOCA is transported to the bladder as the proximate or ultimate carcinogen (Morton et al., 1988; Butler et al., 1989). Two MOCA-DNA adducts have been characterized, *N*-(deoxyadenosin-8-yl)-4-amino-chlorobenzyl alcohol and *N*-(deoxyadenosin-8-yl)-4-amino-chlorotoluene (Segerback and Kadlubar, 1992). *N*-(deoxyadenosin-8-yl)-4-amino-chlorobenzyl alcohol has been detected in rat liver DNA (DeBord et al., 1996), DNA from a human uroepithelial cell line following exposure to N-OH-MOCA (DeBord et al., 1996; Swaminathan et al., 1996), and in the DNA of exfoliated bladder cells of a worker occupationally exposed to MOCA (Kaderlick et al., 1993). MOCA has been shown to be mutagenic in Salmonella and in hamster V79 cells (McQueen and Williams, 1990). In the Salmonella system the presence of a rat liver metabolizing system was required and the mono- and diacetyl derivatives of MOCA were less mutagenic than the parent compound.

PBQ (2-phenyl-1,4-benzoquinone) is an oxidative metabolite of ortho-phenylphenol (OPP), which along with its sodium salt, sodium ortho-phenyl-phenate (NaOPP), are broad spectrum fungicides and germicides (IARC, 1983). These compounds have widespread agricultural and industrial usage. NIOSH has estimated that over 650000 non-agricultural workers in the United States may be exposed (NIOSH, 1990).

OPP has been reported to cause bladder cancer in rodents (Hiraga and Fujii, 1981, 1984; Fujii et al., 1987), but the mechanisms by which tumors are induced have not been established. Several lines of evidence suggest that PBQ is the carcinogenic metabolite of OPP. This occurs following liver metabolism of OPP to phenylhydroquinone (PHQ; 2,5-dihydroxy-biphenyl) and transport of PHQ to the bladder where it is converted to PBQ via prostaglandin (H) synthase (Kolachana et al., 1991). PBQ has been shown to form DNA adducts in vitro in human HL-60 cells (Horvath et al., 1992). Previous studies on the genotoxicity of PHQ have shown an increase in micronuclei formation in hamster V79 cells only in the presence of arachidonic acid supplementation, consistent with a prostaglandin (H) synthase mediated effect (Lambert and Eastmond, 1994). No increase in mutant frequency at the HPRT locus was detected following treatment of these cells with PHQ or PBQ.

Given their considerable usage, potential hazardous exposures to these chemicals may still exist. As a means of identifying possible biological markers of exposure and effect, we have explored the genotoxic potential of MOCA and PBQ in human lymphoblastoid cells and determined the specificity of mutations at the HPRT locus.

2. Materials and methods

2.1. Chemicals

PBQ was obtained from Sigma and used without further purification. MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine) was a generous gift of Dr Ching Wang (Karamanous Cancer Institute, Detroit, MI); N-OH-MOCA (4-amino-4′-hydroxylamino-bis-3,3′-dichlorodiphenylmethane) was prepared as previously described (DeBord et al., 1996).

2.2. Cell culture

AHH-1 human lymphoblastoid cells were obtained under license (Gentest Corporation, Woburn, MA). Cells were maintained at a density of $2–5 \times 10^5$ cells/ml in RPMI 1640 (Gibco, Grand Island, NY) containing 10% Fetal Clone II bovine serum (Hyclone, Logan, UT), 1% penicillin-streptomycin (Gibco) and 2 mM L-glutamine (Gibco).

2.3. Mutation assay

Prior to mutagen treatment, cells were grown in medium containing 2×10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin and 3.5×10^{-5} M thymidine (HAT medium) to remove pre-existing mutants. Following this treatment cultures were scaled up in normal medium to provide sufficient quantities of cells for exposure. For exposure to

mutagens, 3×10^7 cells were suspended in 60 ml of medium in T-75 flasks. Test compounds were dissolved in DMSO at the desired concentrations immediately prior to addition to the AHH-1 cells. The final DMSO concentration in the culture medium was 0.5%. Cells were treated for 24 h and then resuspended in fresh medium and maintained at a density of $2-5 \times 10^5$ cells/ml for 7 days to allow expression of the mutant phenotype. Cells were then transferred to medium containing 0.6 μg/ml 6-thioguanine (6-TG) and 0.2 ml seeded into 96-well plates (2×10^4 cells per well). Cells were also plated at 2 cells per well (0.2 ml), without 6-TG, to determine the plating efficiency of each treated culture. Cells were incubated for 14 days without further manipulation and then scored for the presence of colonies using the criteria of Penman and Crespi (Penman and Crespi, 1987). Mutant frequencies were calculated by application of the Poisson distribution (Furth et al., 1981). Relative survival was determined as described (Penman and Crespi, 1987). A single dose of 0.1 μM of MNNG, a direct acting mutagen, was used as a positive control. This dose was selected based on preliminary cytotoxicity experiments. Control cultures exposed to 0.5% DMSO were plated as described above.

2.4. Statistical analysis

Differences in mean mutant frequencies between control and treatment groups were evaluated using a one-tailed t-test. In order to be considered significantly mutagenic, the criteria established by Crespi and Thilly (1984) and Penman and Crespi (1987) was utilized. Using these criteria a treatment was considered significantly mutagenic if the mean mutant frequency of a treated culture was greater than the concurrent negative controls at the 95% confidence limit and also greater than the 99% upper confidence limit of the mean mutant frequency of all the control cultures collectively.

2.5. RT-PCR

Mutant colonies were expanded in medium containing 6-TG in 6-well plates. Approximately 500 cells were used to prepare *hprt* cDNA using the method of Yang et al. (1989). The cDNA was amplified by PCR in a 50 μl reaction containing 15 mM Tris (pH 8.8), 2.75 mM $MgCl_2$, 60 mM KCl, 400 μM each dNTP, 50 ng of each primer and 0.5 U of Taq DNA polymerase (Promega, Madison, WI). The primers were as described by Yang et al. (1989). The PCR reaction was carried out with a PTC-100-60 thermocycler (MJ Research, Watertown, MA) using a 5 min denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. A second amplification was carried out using 1 μl of a 1:20 dilution of the initial PCR reaction and nested primers as described (Yang et al., 1989). Otherwise the reaction conditions were identical to those used in the initial PCR amplification. To determine the yield and specificity of the PCR reactions, 10 μl of the second PCR amplification was analyzed by electrophoresis through 2% agarose gels.

2.6. DNA sequence analysis

Hprt cDNA was sequenced using a Licor Model 4000L automated DNA sequencer (Licor, Lincoln, NE). Sequencing primers containing an infra-red label (IRD41) attached at the 5′ end were obtained from Licor. The sequence of these primers is identical to the nested primers used for the secondary PCR amplification described above. Prior to sequencing PCR products were purified with a Wizard PCR Prep Kit (Promega, Madison, WI). DNA sequencing reactions were carried out utilizing the Long-Read Cycle Sequencing kit (Epicentre Technologies, Madison, WI). Approximately 100 ng of template DNA was used in each sequencing reaction along with 1 pmol of IRD41-labeled primer. For most samples the entire PCR product ($\sim$700 bp) was sequenced using only a single primer.

3. Results and discussion

The relative survival of AHH-1 cells exposed to PBQ and N-OH-MOCA is shown in Fig. 1. PBQ was much more toxic to the AHH-1 cells than

N-OH-MOCA at equimolar doses. Interestingly, the survival data are very similar to that shown for PBQ in hamster V79 cells (Lambert and Eastmond, 1994) and human uroepithelial cells (Swaminathan et al., 1996). The reason for the toxicity of PBQ may be due to the production of oxidative metabolites. PBQ is known to produce superoxide radicals although in relatively low amounts (Nagai et al., 1990). However, PBQ can be converted non-enzymatically in aqueous medium to PHQ which is much more efficient at producing the superoxide ion and producing DNA strand breaks (Nagai et al., 1990). As shown in Fig. 2, PBQ did not induce mutations in AHH-1 cells at doses up to 10 μM. This is in agreement with previous work showing PBQ to be ineffective at producing mutations at the HPRT locus in V79 cells (Swaminathan et al., 1996). Due to the toxicity of this compound doses higher than 10 μM were not tested.

In contrast to PBQ, exposure of AHH-1 cells to N-OH-MOCA resulted in a significant increase in mutant frequency at each dose. The increase in mutant frequency was approximately 6-fold at the highest dose tested. This is similar to the effect seen in rat fibroblasts where MOCA was moderately genotoxic (Kugler-Steigmeier et al., 1989). The overall increase in mutant frequency was linear with respect to dose ($r^2 = 0.997$). Exposure of AHH-1 cells to a single dose of 0.1 μM MNNG, used as a positive control, induced a 12-fold increase in mutant frequency (data not shown).

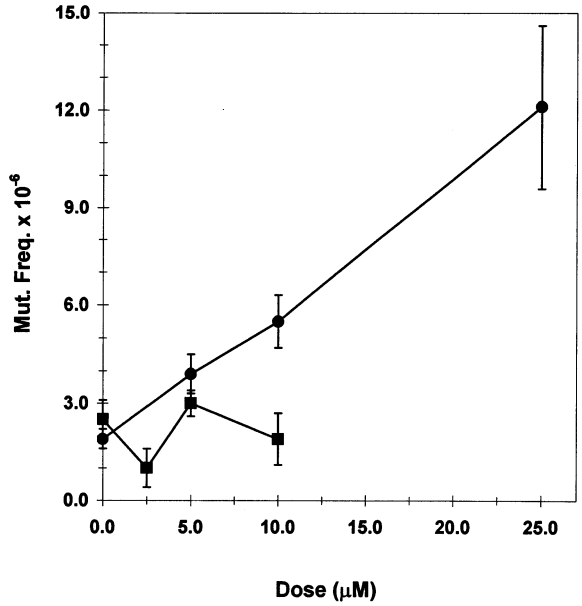

Fig. 2. Mutant frequency ($\pm$SEM) in AHH-1 cells following a 24 h incubation with N-OH-MOCA (●) or PBQ (■). Calculation of mutant frequency is as described in Section 2. Points represent duplicate (PBQ) or triplicate (N-OH-MOCA) experiments.

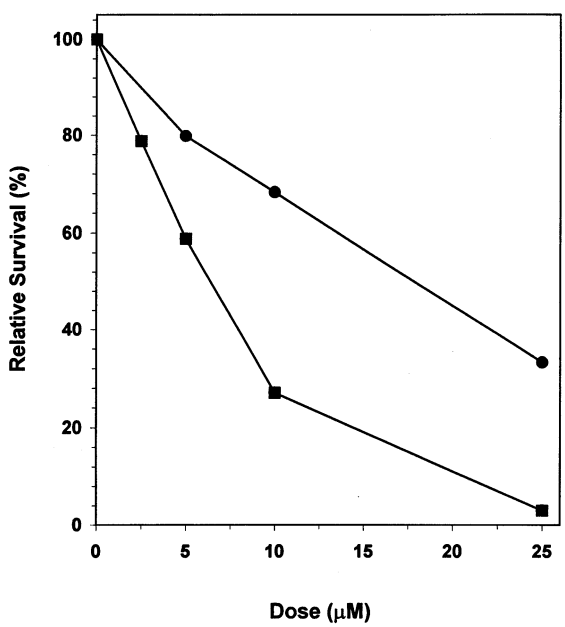

Fig. 1. Relative survival of AHH-1 cells following incubation with N-OH-MOCA (●) or PBQ (■). Incubation was at 37°C for 24 h. Calculation of survival is as described in Section 2.

The type of mutations induced by N-OH-MOCA are shown in Table 1. In this set of mutants, base pair mutations represented approximately 54% of the total; these occurred most often (5/7) at A:T base pairs. The remainder of the mutations were small deletions or loss of exons within the cDNA, possibly due to splicing errors (Andersson et al., 1992; Steingrimsdottir et al., 1992). Identification of deleted exons was determined by size comparison of amplified cDNAs following gel electrophoresis and an analysis of cDNA sequences corresponding to exon boundaries (Cariello and Skopek, 1993). Al-

though a majority of the large deletions resulted in the loss of exon 7, these mutants were isolated from different treatment groups from different treatment dates so presumably are independent mutants. Interestingly, the two small deletions both occurred at intron/exon boundaries suggesting that these also occurred due to splicing errors. Point mutations at intron/exon boundaries can cause incorrect mRNA splicing (Cariello and Skopek, 1993) and at least one of the affected exons in each of the small deletion or loss of exon mutations induced by N-OH-MOCA in this study contains a terminal A:T base pair, a target for MOCA-induced DNA damage. No base pair substitutions at A:T base pairs or similar splicing mutations were observed in the control samples (data not shown).

The occurrence of mutations at A:T base pairs is consistent with the type of damage that MOCA produces in DNA. While similar in structure to other arylamine carcinogens such as benzidine and 4-aminobiphenyl, MOCA does not form predominantly C-8 guanine adducts that are a general feature of arylamine-induced DNA damage (Singer and Grunberger, 1983). Instead, the most abundant MOCA-DNA adducts thus far identified are single-ring modifications of adenine residues (Segerback and Kadlubar, 1992). These unique adducts are not known to be formed by other genotoxic aromatic amine compounds. The major MOCA-DNA adduct, *N*-(deoxyadenosin-8-yl)-4-amino-chlorobenzyl alcohol, has been detected in rat liver DNA and DNA from a human uroepithelial cell line following in vitro exposure to N-OH-MOCA (DeBord et al., 1996; Swaminathan et al., 1996). This same adduct was also detected in the DNA of exfoliated bladder cells of a worker occupationally exposed to MOCA (Kaderlick et al., 1993). Thus, mutations at A:T base pairs are highly specific for MOCA-induced DNA damage. In populations with potential for exposure to MOCA the appearance of such mutations would be a reliable indicator of genotoxic damage attributable to this carcinogen.

Table 1
Summary of mutations induced by N-OH-MOCA in AHH-1 cells

Mutant	Base change	Positiona	Change in cDNA
M7	Deletion	709–717	
M12	A:T–>C:G	252	
M37	—	—	Loss of exon 7
M40	—	—	Loss of exon 4
M45	A:T–>G:C	141	
M75	—	—	Loss of exon 7
M76	G:C–>A:T	616	
M87	A:T–>C:G	398	
M92	A:T–>G:C	595	
M94	G:C–>A:T	424	
M97	A:T–>T:A	313	
M98	—	—	Loss of exon 7
M99	Deletion	127–130	

[a] Numbering according to Jolly et al. (1983).

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