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The genotoxicity of 3-nitrobenzanthrone and the nitropyrene lactones in human lymphoblasts

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Abstract

Polycyclic aromatic hydrocarbons (PAH) and nitrated polycyclic aromatic compounds (nitro-PAC) have been found to be mutagenic in bacterial and human cells as well as carcinogenic in rodents. In this investigation, the genotoxic effects of 3-nitrobenzanthrone (3NB) and a mixture of nitropyrene lactones (NPLs) were determined using forward mutation assays performed in two human B-lymphoblastoid cell lines, MCL-5 and h1A1v2, which are responsive to the nitro-PAC class of compounds. Mutagenicity of the compounds was determined at the heterozygous *tk* locus and the hemizygous *hprt* locus, thus, identifying both large-scale loss of heterozygosity (LOH) events as well as intragenic mutagenic events. Genotoxicity was also determined using the CREST modified micronucleus assay, which detects chromosomal loss and breakage events. Results indicate 3NB is an effective human cell mutagen, significantly inducing mutations at the *tk* and *hprt* loci in both cell lines, and inducing micronuclei in the h1A1v2 cell line. The NPL isomers are also mutagenic, inducing mutations at the two loci as well as micronuclei in both cell lines. Because of their mutagenic potencies and their presence in ambient air, further assessments should be made of human exposures to these nitro-PAC and the potential health risks involved.

Keywords: 3-Nitrobenzanthrone; Nitropyrene lactones; Nitro-PAC; Human cells; Mutagenicity; Micronucleus

1. Introduction

Many nitrated polycyclic aromatic hydrocarbons (nitro-PAH) and nitrated polycyclic aromatic compounds (nitro-PAC) (including, for example, nitro-PAH lactones and nitro-PAH ketones) are carcinogenic and mutagenic [1–14]. The role of genotoxic nitro-PAH in the development of lung cancer in humans exposed to diesel exhaust has been extensively studied but remains controversial [10,15]. Because the ambient concentrations of nitro-PAC are controlled by combustion emissions [8,14,15] and atmospheric formation reactions [7,16–19], it is important to determine the contribution of both atmospherically formed and

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directly-emitted nitro-PAC to the mutagenicity of ambient air. Improved understanding of the nitro-PAC contribution to ambient mutagenic activity is essential in evaluation of the risk associated with human exposure to ambient atmospheric mutagens.

Recent mutagenicity studies in the human B-lymphoblastoid cell lines, h1A1v2 and MCL-5, which focused on 2-nitronaphthalene (2NN), a two-ring nitro-PAH, and 2-nitrodibenzopyranone (2NDBP), a three-ring nitro-PAH lactone, identified some unexpected results [6,12,13]. These compounds were mutagenic only at the autosomal, heterozygous *tk* locus but not at the X-linked, hemizygous *hprt* locus. These data suggest that large-scale loss of heterozygosity (LOH) mechanisms, available only for heterozygous sequences, such as *tk*, were the principal or exclusive mutagenic pathway for 2NN and 2NDBP. Furthermore, several lines of evidence suggested an important role for oxidative rather than reductive metabolism in the mutagenicity of these compounds [6,13]. 2NN and 2NDBP have also recently been shown by our group [6,12,13] to induce micronuclei in the same cell lines.

The human B-lymphoblastoid cell line MCL-5 has facilitated the investigation of the mutagenicity of PAH [6,12,20] and nitro-PAC [1,2,6,12,13] since the cell line has been transfected with two plasmids encoding multiple cytochrome P450 and epoxide hydrolase genes, and also has inducible endogenous cytochrome P450 1A1 (CYP1A1) [21]. Therefore, exogenous metabolic activation of PAH is not required. A related cell line, derived from AHH-1, h1A1v2, which is transfected with a single cytochrome P450 enzyme, CYP1A1 [22], has also been employed in studies of the genotoxicity of PAH [3,6,23] and nitro-PAC [2,3,6,13]. The differing transfected enzymatic activities in the two cell lines can potentially result in differing mutagenic metabolites, and therefore, differing mutagenic profiles. The h1A1v2 and MCL-5 cell lines also differ in their *p53* status [24]. MCL-5 is wild type for *p53*, whereas h1A1v2 carries a heterozygous *p53* mutation [24], and exhibits a delayed apoptotic response and an increased recovery of chromosomal scale LOH mutants [25,26].

The studies presented here were designed to evaluate whether the findings for 2NN and 2NDBP of human cell mutagenicity at the heterozygous *tk* locus but not at the hemizygous *hprt* locus could be generalized to include other nitro-PAC. The nitro-PAC chosen for this study are potent bacterial mutagens that were identified through the use of bioassay-directed chemical analysis of ambient particles [5,7], diesel exhaust [5] and atmospheric chamber reactions [11]. The nitropyrene lactones (NPLs; Fig. 1(**I**)), the 4-ring analogues of 2NDBP, were identified in environmental chamber simulations of atmospheric reactions of pyrene as mutagenic reaction products [11] and have also been reported in ambient air, although the specific isomers involved have not yet been isolated [7,11]. Also chosen for study was 3-nitrobenzanthrone (3NB; Fig. 1(**II**)), identified in diesel exhaust and ambient air particles and representing a new chemical class of mutagenic nitro-PAC, a nitro-PAH ketone [5].

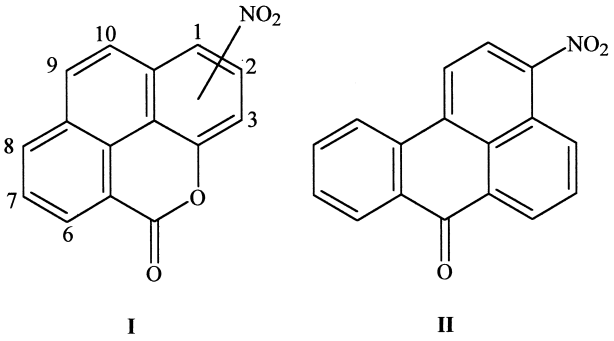

Fig. 1. Structure and numbered NO_2 positions of the nitropyrene lactones (**I**). Structure of 3-nitrobenzanthrone (**II**).

Researchers employing the h1A1v2 cell line have reported that urban airborne particles contain human cell mutagens, including unsubstituted PAH and, in the more polar liquid chromatographic fractions, nitro-PAH and PAH ketones [27,28]. However, a large portion of the mutagenicity attributed to semipolar and polar compounds remains unassigned [28]. Because of their strong bacterial mutagenicity and their presence in ambient air, the NPLs and 3NB are candidate contributors to the unassigned human cell mutagenicity of ambient particles. Here, the genotoxicity of 3NB and the NPLs was evaluated in two human cell lines using mutagenicity assays at *tk* and *hprt*, as well as the CREST modified micronucleus assay as an indicator of clastogenic or aneugenic properties [29,30]. The results suggest that these compounds induce a broader range of genotoxic effects than previously observed for 2NN and 2NDBP and their contribution to the human cell mutagenicity of ambient particles should be further assessed.

2. Materials and methods

2.1. Chemicals

Benzo[a]pyrene (BaP) [50-32-8] (98% stated purity), dimethyl sulfoxide [67-68-5] (99.5% stated purity), and melphalan [148-82-3] (95% stated purity) were purchased from Sigma (St. Louis, MO). Benzanthrone [82-05-3] (technical grade) was purchased from Aldrich (Milwaukee, WI), and pyrene lactone (>99% purity) was synthesized as described elsewhere [31].

2.2. Synthesis and purification of 3-nitrobenzanthrone and the nitropyrene lactones

Two convenient methods of synthesizing nitro-PAC are the nitration of a parent compound either with dinitrogen pentoxide (N_2O_5) in carbon tetrachloride solution at room temperature [32] or with dinitrogen tetroxide (N_2O_4) in methylene chloride (CH_2Cl_2) at room temperature [33]. The N_2O_5 reaction often produces isomer profiles different from those formed in the N_2O_4 reaction. In aprotic solution, the N_2O_5 undergoes dissociation to NO_3 and NO_2, allowing nitration by way of a radical reaction mechanism, whereas the N_2O_4 reaction in CH_2Cl_2 is believed to proceed via electrophilic nitration.

The synthesis of the NPLs was accomplished by reaction of the parent compound, pyrene lactone (PL), with both N_2O_4 and N_2O_5. Each reaction formed multiple mononitro-PL and dinitro-PL isomers. The NPLs were separated from the dinitro-PL isomers by flash chromatography and purified by recrystallization. Because, the specific NPL isomers found in ambient air extracts [7] and as pyrene atmospheric reaction products [11] are not yet known, rather than separate the mononitro-derivatives and test each individually, it was decided to assay the NPLs as a mixture. By mixing aliquots from the two nitrations after removal of the dinitro-PL isomers, a mixture was produced that contained the mononitro-PL isomers expected to be formed by electrophilic nitration and thus representative of NPLs emitted in combustion sources, plus additional isomers formed by a radical reaction mechanism, as often occurs in atmospheric reactions [16]. The NPLs purity as determined by gas chromatography was >99% and the six NPL chromatographic peaks had area percents (in order of their elution on a DB-1701 capillary column) of 7.0, 46.3, 27.5, 0.7, 17.4 and 1.1. The NPLs were identified by their characteristic mass spectra, which were similar to those reported for the nitrophenanthrene lactone isomers, including 2NDBP [34]. All the NPL isomers had a strong molecular ion peak at $m/z = 265$, a base peak at $m/z = 163$ which corresponds to the loss of C_2NO_4 (-102), and additional fragment ions from the loss of CNO_2 (-58) and CNO_3 (-74).

3NB was synthesized by reacting benzanthrone (Bz) with N_2O_4 in CH_2Cl_2 at room temperature [33]. This reaction formed 3NB as the major mononitro-Bz isomer, along with one minor mononitro-Bz isomer and several dinitro-Bz isomers. The 3NB was separated from the mononitro- and dinitro-Bz isomers by flash chromatography, and the fractions collected were analyzed by GC–MS. The fraction containing the desired 3NB was then further purified by recrystallization and quantified by GC–FID (>99% purity). 3NB was conclusively identified by HH-COSY and ^{1}H-NMR ($CDCl_3$): $\delta = 7.69$ (dt, $J = 7.89$, 1H), 7.84 (dt, $J = 8.63$, 2.01, 1H), 8.02 (dt, $J = 7.73$, 1.32, 1H), 8.38 (d, $J = 8.15$, 1H), 8.43 (d, $J = 8.09$, 1H), 8.52–8.57 (m, 2H), 8.87 (dd, $J = 7.35$, 1.47, 1H), 8.98 (dd, $J = 8.82$, 1.47, 1H), which was in general agreement with a previously reported spectra [35].

2.3. Cell cultures and genotoxicity assays

2.3.1. Derivation of the cell lines

AHH-1 is a clonal isolate derived from RPMI 1788 cells (a human B-lymphoblastoid cell line obtained from a healthy male donor), selected for sensitivity to BaP. The h1A1v2 cell line was derived by further transfecting AHH-1 with a plasmid encoding additional inducible CYP1A1 activity [22]. The L3 variant of the AHH-1 cell line was selected in benzo[*ghi*]perylene for elevated levels of endogenous CYP1A1 activity and has a lower spontaneous mutant fraction at the *tk* locus [36]. MCL-5 was derived from L3 by transfection with plasmids encoding epoxide hydrolase as well as CYP1A2, CYP2A6, CYP2E1, and CYP3A4 genes [21].

2.3.2. Maintenance and growth of cell cultures

The lymphoblastoid cell lines h1A1v2 and MCL-5 were maintained and grown as recently described

[25] in suspension cultures in Gentest RPMI 1640 media (without histidine and with 2 mM histidinol required for plasmid selection) supplemented with 9% horse serum, L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin sulfate. Cells were grown to a density of 10^6 cells/ml and subcultured to 2.5×10^5 cells/ml every other day. At this time, MCL-5 cells were further supplemented with 100 μg/ml hygromycin B for plasmid selection.

2.3.3. Chemical exposure

Prior to dosing the cells with the test chemicals, the cells were maintained for 3 days with HAT (containing 2×10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin and 3.5×10^{-5} M thymidine) to eliminate any pre-existing mutants. On day 3, the cells were centrifuged out of HAT media, and resuspended in fresh media containing 2×10^{-4} M hypoxanthine and 3.5×10^{-5} M thymidine.

Dosing solutions of the compounds were made up in dimethyl sulfoxide (DMSO) at 1000× the final desired concentration of the compound in cell culture. Approximately 3×10^7 cells were centrifuged out of maintenance media and resuspended in 40 μl of Cellgro RPMI 1640 media containing histidine without horse serum. Following a protocol previously evaluated using BaP, the chemical exposure was performed in the absence of serum to maximize the bioavailability of the test compound [6,12]. The cell culture exposure was then limited to 10 h at 37°C to minimize effects due to growth in serum-free conditions [6,12]. To enhance the solubility of test chemical in media, an aliquot of 120 μl DMSO was added to each cell culture prior to the addition of the 40 ml aliquot of test chemical, resulting in 0.4% DMSO in all treatments [6,12]. BaP (15 ng/ml, determined to be the minimum mutagenic concentration in h1A1v2 [3]) was used as the positive control, and DMSO (0.4%) was used as the negative control. At the completion of the exposure period, the cells were centrifuged out of chemical and resuspended in fresh maintenance media.

2.3.4. Clonogenic survival assay

At the completion of the dosing period, survival plates were prepared at 10 cells/ml (2 cells/well, 2 plates/culture) and scored for clonogenic survival after 14 days. The remaining cells were grown to a density of 10^6 cells/ml and subcultured to 5×10^5 cells/ml daily during the phenotypic expression period required for selection of $TK^{-/-}$ and $HPRT^-$ mutants.

2.3.5. Selection of $TK^{-/-}$ and $HPRT^-$ mutants

The cells were allowed a phenotypic expression period of 5 days prior to selection of $TK^{-/-}$ mutants and 7 days prior to selection of $HPRT^-$ mutants as determined previously by Sasaki et al. [6,12]. Selection was performed by preparing 96-well plates at a cell density of 10^5 cells/ml (20,000 cells/well, 4 plates/culture) in media containing 4 μg/ml trifluorothymidine and 9% heat-inactivated horse serum for $TK^{-/-}$ mutants, and a cell density of 5×10^4 cell/ml (10,000 cells/well, 4 plates/culture) in media containing 0.6 μg/ml 6-thioguanine and 9% horse serum not heat-inactivated for $HPRT^-$ mutants. Cloning efficiency plates were prepared in parallel at a cell density of 10 cells/ml (2 cells/well, 2 plates/culture) in non-selective media. Selection plates and cloning efficiency plates were scored 14 and 12 days after seeding for $TK^{-/-}$ and $HPRT^-$ mutants, respectively.

2.3.6. Preparation of cell cultures for the CREST modified micronucleus assay

The same cell lines and chemical exposure parameters used in the mutagenicity assay were employed for the CREST modified micronucleus assay. The CREST modified micronucleus assay employs the CREST stain, an antikinetochore antibody obtained from patients with the autoimmune disease scleroderma CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly and telangiectasia) syndrome [37–39]. Melphalan (4 μM) was used as the positive control, and DMSO (0.4%) was used as the negative control. After the 10 h chemical exposure period, $\sim 10^6$ cells were resuspended in fresh media with horse serum and 3 μg/ml cytochalasin B was added to each culture. After further incubation of the cells for 36 h at 37°C, allowing for cell recovery and the maximum number of cells to go through one cell division, the cells were harvested by cytocentrifugation. The slides were fixed in 100% methanol for 10 min and allowed to air dry. The slides were then stored in a nitrogen atmosphere at −20°C until later use. The CREST staining and scoring procedures were performed as previously described [29,30].

2.4. Statistical methods

The program StatView SE + Graphics was used to calculate the statistical values for the *tk* and *hprt* mutagenicity assays. A test for linear trend was performed using a 95% confidence interval.

The dose-related increases in the induction of micronuclei were tested for significance using the Cochran–Armitage test for trend in binomial proportions. Following the observation of a significant trend, a one-tailed Fisher exact test or Chi-square test was used as a post hoc test to determine the significance of each test concentration.

3. Results

3.1. Mutagenicity of 3-nitrobenzanthrone and the nitropyrene lactones at the tk and hprt loci

Clonogenic cytotoxicity was determined in parallel with mutagenicity and is presented in Table 1. In both cell lines, 3NB is more cytotoxic than the mixture of NPLs tested, and BaP, the positive control, was not toxic at the dose tested. At the highest dose tested, 3NB exposure resulted in 46 and 7% cell survival in h1A1v2 and MCL-5 cells, respectively, compared to 80 and 37% cell survival for the corresponding NPLs exposure. The h1A1v2 cell line sustained a lower amount of cytotoxicity, which may be due to its apoptotic deficient status [24].

The results of the mutagenicity assays indicate a general dose-dependent increase in mutation frequency for 3NB (Fig. 2) and the NPLs (Fig. 3), in each cell line at both the *tk* and *hprt* loci. A significant increase in the $TK^{-/-}$ mutant fraction was induced by 3NB in h1A1v2 (Fig. 2A, $P = 0.0085$) and MCL-5 (Fig. 2B, $P = 0.0001$). 3NB also induced a significant increase in the $HPRT^{-}$ mutant fraction in h1A1v2 (Fig. 2A, $P = 0.0001$) and MCL-5 (Fig. 2B, $P = 0.0001$). The NPLs induced a significant increase in the $TK^{-/-}$ mutant fraction in h1A1v2 (Fig. 3A, $P = 0.0383$) and MCL-5 (Fig. 3B, $P = 0.0058$). The NPLs also induced a significant increase in the $HPRT^{-}$ mutant fraction in h1A1v2 (Fig. 3A, $P = 0.0001$) and MCL-5 (Fig. 3B, $P = 0.0027$). BaP induced a significant increase in the $TK^{-/-}$ mutant fraction in MCL-5 ($P = 0.05$) but not in h1A1v2 ($P = 0.147$). BaP also induced a significant increase in the $HPRT^{-}$ mutant fraction in MCL-5 ($P = 0.0022$) but not in h1A1v2 ($P = 0.353$). Our results are in general agreement with previously determined mutation frequencies for BaP using the same protocol [12,13].

A comparison of the mutagenicity of 3NB and the NPLs within each cell line (Table 2) reveals a three- to four-fold higher mutation frequency at *tk* than *hprt* in the h1A1v2 cell line (Figs. 2A and 3A), although comparable levels of mutagenicity were observed at *tk* and *hprt* in the MCL-5 cell line (Figs. 2B and 3B). The

Table 1
Fraction of cell survival[a,b] after treatment with 3-nitrobenzanthrone and the nitropyrene lactones

Compound	Concentration (μM)	Fraction of cell survival ± S.E.M.	
		h1A1v2	MCL-5
DMSO[c]	–	1.00	1.00
3-Nitrobenzanthrone	0.364	0.92 ± 0.12	0.61 ± 0.12
	1.82	0.60 ± 0.09	0.21 ± 0.04
	3.64	0.46 ± 0.07	0.07 ± 0.05
Nitropyrene lactones	0.377	1.20 ± 0.18	1.06 ± 0.17
	1.89	0.92 ± 0.15	0.66 ± 0.11
	3.77	0.80 ± 0.10	0.37 ± 0.06
Benzo[a]pyrene[d]	0.060	1.08 ± 0.09	1.23 ± 0.12

[a] Fraction of cell survival was calculated with data from three independent experiments.
[b] Fraction of cell survival is given ± S.E.M.
[c] Negative control (0.4% DMSO; clonogenic survival defined as 1.00).
[d] Positive control.

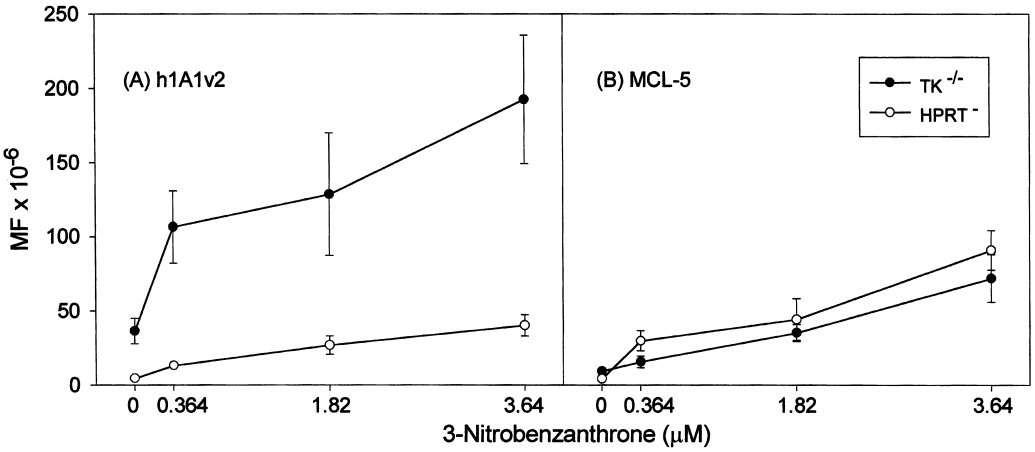

Fig. 2. $TK^{-/-}$ and $HPRT^{-}$ mutation frequency per 10^6 viable cells (MF $\times$ 10^{-6}) induced in (A) h1A1v2 and (B) MCL-5 cells treated with 3-nitrobenzanthrone. The mutation frequency was calculated with data from three independent experiments.

increased mutagenicity at *tk* in h1A1v2 cells may be due to the p53-deficient background in these cells [24] and a resultant increased recovery of recombinational LOH events [25,26].

Comparing the results between the two cell lines, the recovery of induced $TK^{-/-}$ mutants in h1A1v2 was consistently higher than the recovery of induced $TK^{-/-}$ mutants in MCL-5 cells exposed to equal concentrations of mutagen (Table 2). In contrast, for both 3NB and the NPLs, the recovery of induced $HPRT^{-}$ mutants was greater in MCL-5 than in h1A1v2 (Table 2), perhaps reflecting the production of additional mutagenic metabolites in MCL-5 associated with its broader metabolic capacity.

3.2. Micronuclei induced by 3-nitrobenzanthrone and the nitropyrene lactones

Table 3 lists the nuclear division index (NDI), which is indicative of cell growth or cell division, for h1A1v2 and MCL-5 cells after treatment with 3NB and the NPLs. Treatment of both cell lines with 3NB resulted in a linear decrease in NDI, indicating that 3NB inhibits cell growth in both cell lines. Treatment with

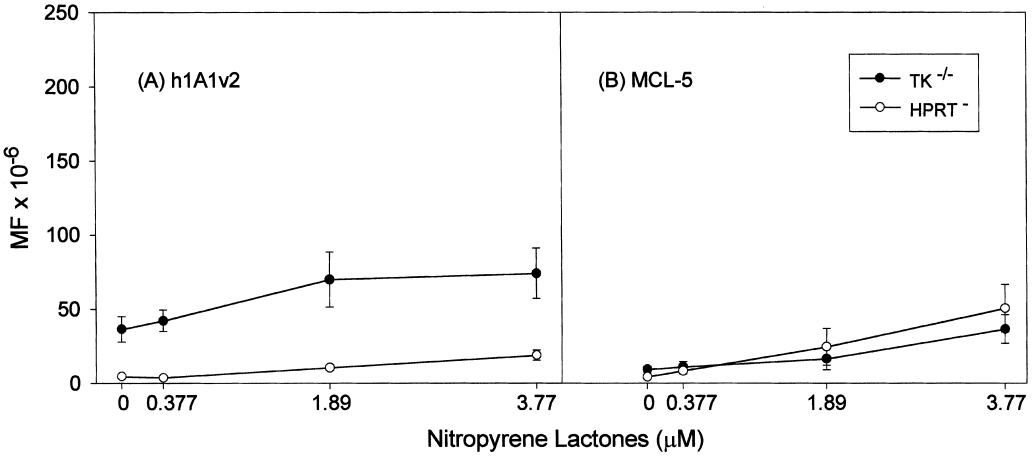

Fig. 3. $TK^{-/-}$ and $HPRT^{-}$ mutation frequency per 10^6 viable cells (MF $\times$ 10^{-6}) induced in (A) h1A1v2 and (B) MCL-5 cells treated with the nitropyrene lactones. The mutation frequency was calculated with data from three independent experiments.

Table 2
Induced mutation frequency[a] at *tk* and *hprt* at the highest concentration tested

Compound	Concentration (μM)	h1A1v2		MCL-5	
		tk	*hprt*	*tk*	*hprt*
3-Nitrobenzanthrone	3.64	156.1	35.6	62.6	86.7
Nitropyrene lactones	3.77	37.6	14.3	27.1	46.4
2-Nitrodibenzopyranone[b]	4.15	16.7	0	7.2	0
2-Nitronaphthalene[b]	462	96.7	0	83.6	0

[a] Induced mutation frequency is defined as the number of mutants per 10^6 viable cells minus the background mutants per 10^6 viable cells.
[b] Data from Grosovsky et al. [6].

Table 3
Nuclear division indexes (NDI)[a,b] for h1A1v2 and MCL-5 cells after treatment with 3-nitrobenzanthrone and the nitropyrene lactones

Test compound	Concentration (μM)	NDI ± S.E.M.	
		h1A1v2	MCL-5
3-Nitrobenzanthrone	0	1.97 ± 0.13	1.61 ± 0.04
	0.364	1.88 ± 0.05	1.54 ± 0.15
	1.82	1.78 ± 0.02	1.47 ± 0.07
	3.64	1.66 ± 0.06	1.36 ± 0.11
	5.45	1.47 ± 0.06	1.31 ± 0.07
	7.27	1.31 ± 0.07	1.18 ± 0.05
Nitropyrene lactones	0	1.96 ± 0.03	1.69 ± 0.05
	0.377	2.11 ± 0.08	1.58 ± 0.06
	1.89	1.97 ± 0.04	1.61 ± 0.04
	3.77	1.92 ± 0.09	1.63 ± 0.02
Melphalan[c]	4.00	1.13 ± 0.02	1.12 ± 0.03

[a] NDI = $\{[N1+(2\times N2)+(3\times N3)+(4\times N4)]/(N1+N2+N3+N4)\}$ where $N1$–$N4$ represent the number of cells with 1–4 nuclei, respectively [29], and was calculated with data from three independent experiments.
[b] NDI values are given ± S.E.M.
[c] Positive control. NDI was calculated with data from six independent experiments.

the NPLs, on the other hand, did not affect the NDI in either cell line, indicating that the NPLs did not cause growth inhibition in the cells at the doses tested. Treatment with melphalan, the positive control, resulted in substantial decreases in NDI at the dose tested in h1A1v2 and MCL-5 cells.

The data in Figs. 4 and 5 represent the percent of total micronuclei (total MN) induced by the exposure of h1A1v2 and MCL-5 cells to 3NB (Fig. 4) and the NPLs (Fig. 5). Of the total MN, MN− indicates the number due to chromosomal breakage and MN+ indicates the number due to chromosomal loss. A significant dose-related increase was observed in total MN in h1A1v2 (Fig. 4A) after treatment with 3NB, but only a weak significant increase was observed in MCL-5 (Fig. 4B). At the highest dose tested in h1A1v2, 3NB induced a significant amount of both chromosomal loss (MN+) and chromosomal breakage

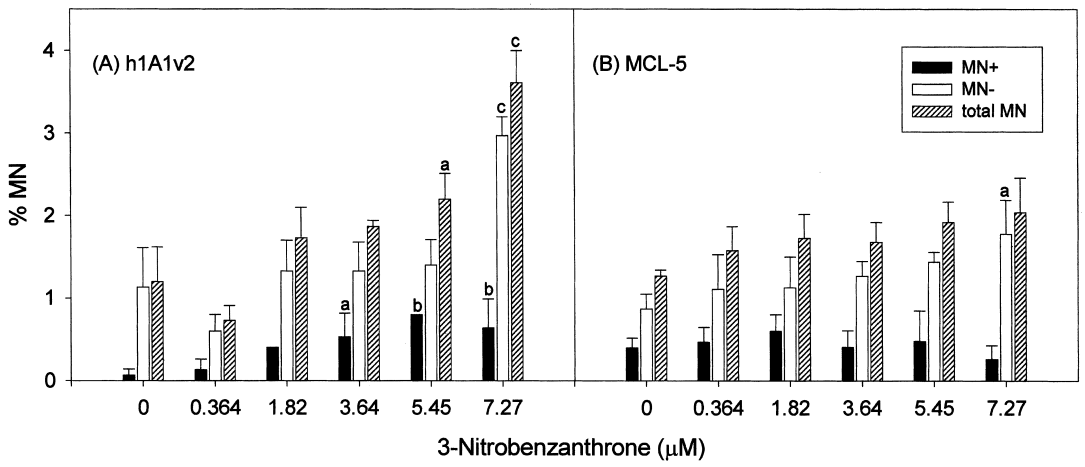

Fig. 4. Percent micronuclei (% MN) induced in (A) h1A1v2 and (B) MCL-5 cells treated with 3-nitrobenzanthrone. The percent micronuclei was calculated with data from three independent experiments. a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.001$.

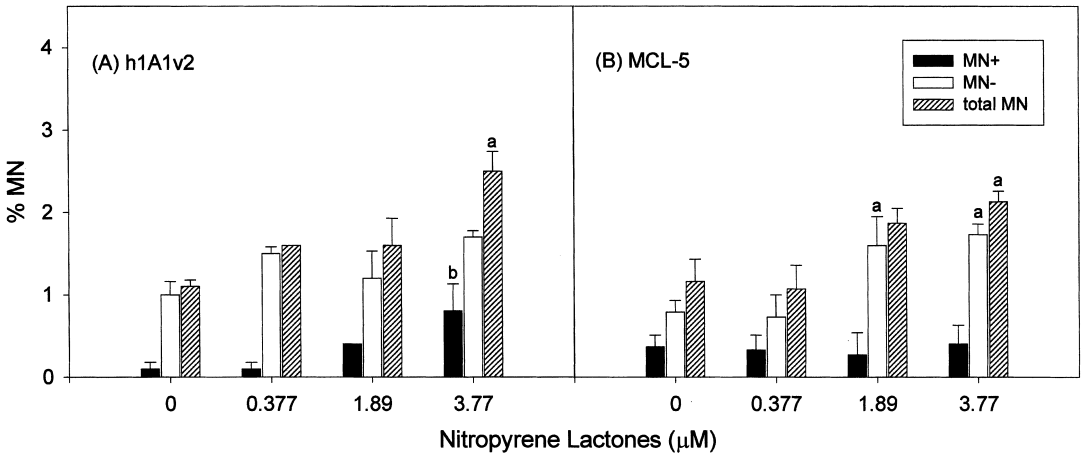

Fig. 5. Percent Micronuclei (% MN) induced in (A) h1A1v2 and (B) MCL-5 cells treated with the nitropyrene Lactones. The percent micronuclei was calculated with data from three independent experiments. a: $P < 0.05$; b: $P < 0.01$.

(MN−), although the significant increase in chromosomal loss (MN+) may be due to an abnormally low background frequency compared to historical values. In MCL-5, 3NB only induced a significant increase in chromosomal breakage (MN−). Fig. 5 illustrates micronuclei induced by the NPLs in h1A1v2 and MCL-5 cells. In both cell lines there was a significant dose-related increase in total MN, with comparable induction of total MN at the highest dose tested. Of the total MN induced, a significant amount were due to chromosomal loss (MN+) in h1A1v2 (Fig. 5A) and chromosomal breakage (MN−) in MCL-5 (Fig. 5B). Melphalan induced a significant amount of total MN in h1A1v2 ($8.90 \pm 0.68\%$, $P < 0.0001$). This induction was primarily due to chromosomal breakage (MN−) ($6.66 \pm 0.59\%$, $P < 0.0001$), although a significant increase was due to chromosomal loss (MN+) ($2.24 \pm 0.63\%$, $P < 0.0001$). Melphalan also induced a significant amount of total MN in MCL-5 cells ($7.67 \pm 0.90\%$, $P < 0.0001$). Again chromosomal breakage (MN−) ($6.33 \pm 0.95\%, P < 0.0001$) dominated, but chromosomal loss (MN+) ($1.34 \pm 0.41\%$, $P < 0.0001$) was also significant.

4. Discussion

Enya et al. [5] reported 3NB to be highly mutagenic in the Ames *Salmonella* assay in strains TA98 and YG1024, comparable to 1,8-dinitropyrene, one of the most mutagenic nitro-PAH reported to date [14], and these investigators also reported that 3NB induced micronuclei in mouse peripheral blood reticulocytes after intraperitoneal injections. Other mononitro-Bz isomers were found to be less mutagenic in TA98 [5]. Busby and co-workers evaluating the mononitro- and dinitro-pyrenes, demonstrated that potency in a bacterial assay is not necessarily a good predictor of activity in human cell assays [1]. However, we tested the 3NB isomer in human cells, because, in addition to its high bacterial mutagenicity, it has been identified as a component of both diesel exhaust particle extracts and ambient particle extracts [5].

While two NPL isomers are formed in simulated atmospheric reactions of pyrene [11], and two NPL isomers have been reported in analyses of ambient samples [7], the specific isomers have not yet been identified. Two specific nitropyrene lactone isomers, 1-NPL and 3-NPL, have been found to be highly mutagenic in the Ames *Salmonella* assay [4]. By testing a mixture of six of the eight possible NPL isomers, it is likely that isomers representative of both combustion emission and atmospheric formation were screened, allowing us to determine if NPLs are likely to be important human cell mutagens. Because NPLs are atmospheric reaction products of the abundant PAH, pyrene [11], human populations downwind of

sources of PAH such as vehicle emissions may be exposed to increasing levels of NPLs.

Our laboratory has previously shown that in both MCL-5 cells and h1A1v2 cells 2NN and 2NDBP were mutagenic at the *tk* but not the *hprt* locus, suggesting that the human cell mutagenicity of these compounds was exclusively attributable to LOH events not available at the hemizygous *hprt* locus [6,13]. In contrast, 3NB and the NPLs were strongly mutagenic at *tk* and *hprt* in both MCL-5 and h1A1v2, suggesting a significantly different mutational spectrum (Table 2, Figs. 2 and 3).

Previous reports [25,26] have demonstrated that higher mutation frequencies at *tk* but not *hprt* are characteristic of h1A1v2, probably due to the abnormal apoptotic response associated with a heterozygous mutation at p53 [24]. The human B-lymphoblastoid cell line MCL-5 is wild type for *p53*, whereas AHH-1 and its derivatives, h1A1v2 and h2E1v2, carry a heterozygous *p53* mutation [24], and exhibit delayed apoptotic responses and increased spontaneous and induced mutation frequencies [6,13,25,26]. MCL-5 and h2E1v2 have similar $HPRT^-$ mutation frequencies [25], but h2E1v2 has a four- to five-fold higher $TK^{-/-}$ mutation frequency [25,26]. Molecular analysis of the $TK^{-/-}$ mutants indicates the higher $TK^{-/-}$ mutation frequency in h2E1v2 can be entirely attributed to the recovery of large-scale LOH [25,26]. This pattern closely resembles the source of increased $TK^{-/-}$ mutation frequency in p53 deficient WIL2-NS human lymphoblasts and its derivative, WTK-1, as compared to related *p53* wild type TK6 cells [40–42]. Induced $HPRT^-$ mutation frequencies are similar in the cell lines [41], but induced mutation frequencies at the heterozygous *tk* locus are 20- to 50-fold higher in the WIL2-NS cell lines than in the TK6 cells [41,42]. These similar patterns in two independent cell line pairs suggests that the p53 deficiency, and the resultant abnormal apoptotic response, is responsible for the increased recovery of large-scale LOH mutants.

Summarized in Table 2 are the induced mutation frequencies for 3NB, the NPLs, and the atmospherically-formed [11] nitro-PAC, 2NDBP and 2NN, tested previously [6]. In the MCL-5 cell line, both 3NB and the NPLs induced comparable mutation frequencies at *hprt* and *tk* (Table 2; Figs. 2B and 3B). In contrast, 3NB and the NPLs were three- to four-fold more mutagenic at *tk* than *hprt* in the h1A1v2 cell line (Table 2, Figs. 2A and 3A), suggesting an increment of recoverable mutations attributable to large-scale LOH events. Clearly, although the mutations induced by 2NN and 2NDBP are restricted to large-scale LOH events, this cannot be generalized to all nitro-PAC. A comparison of the induced mutation frequency at *tk* and *hprt* of comparable test concentrations (Table 2) shows that 3NB is two- to four-fold more potent than is the mixture of NPLs, and almost an order of magnitude more potent than 2NDBP. The NPLs are two times more mutagenic than is the individual nitrophenanthrene lactone isomer, 2NDBP, at the *tk* locus in h1A1v2, and four times more mutagenic in MCL-5. 2NN (note higher test dose, Table 2) is the least potent mutagen of the group.

The results of the CREST modified micronucleus assay show that both compounds are inducers of micronuclei. The results are more significant in the h1A1v2 cell line than the MCL-5 cell line for both compounds. Again this may be attributed to the apoptotic deficiency in h1A1v2 cells, which may allow survival of a greater number of damaged cells. No conclusion can be made regarding which transfected enzymes are required for metabolizing the nitro-PAC, since both cell lines were responsive to both test compounds. Both compounds can be classified as clastogens as well as aneugens, since both compounds induced both chromosomal loss and chromosomal breakage. In the past, it has been found that alkylating agents are both clastogens and aneugens [29], indicating that the tested nitro-PAC may be alkylating agents.

Results presented here demonstrate that exposure of 3NB and the NPLs to two human B-lymphoblastoid cell lines resulted in induction of mutations at the *tk* and *hprt* loci. This pattern differs from previous studies of smaller nitro-PAC such as 2NN, which induced mutations only at the *tk* locus [12,13]. Thus, unlike 2NN genotoxicity, which was restricted to the induction of large-scale LOH events and micronuclei, 3NB and the NPLs appear to be capable of inducing a broader range of genetic alterations including smaller-scale chromosomal changes and intragenic mutations. The significant genotoxicity of 3NB and the NPLs in human cell assays verify that human risk from exposure to these atmospheric pollutants should be further assessed.

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