Mutagenicity of N-hydroxy-4-aminobiphenyl in human TP53 knock-in (Hupki) mouse embryo fibroblasts

Lisa Hözl-Armstrong | Jill E. Kucab | Edwin P. Zwart | Mirjam Luijten | David H. Phillips | Volker M. Arlt

1Department of Analytical, Environmental and Forensic Sciences, MRC-PHE Centre for Environment and Health, King’s College London, London, UK
2Center for Health Protection, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
3Toxicology Department, GAB Consulting GmbH, Heidelberg, Germany

Correspondence
Volker M. Arlt, Toxicology Department, GAB Consulting GmbH, 69126 Heidelberg, Germany.
Email: volker.arlt@gabconsulting.de

Funding information
Cancer Research UK Grand Challenge Award, Grant/Award Number: C98/A24032; MRC Centre for Environment and Health, Grant/Award Number: PhD studentship Lisa Hözl-Armstrong

Accepted by: G. Johnson

Abstract
TP53 harbors somatic mutations in more than half of human tumors with some showing characteristic mutation spectra that have been linked to environmental exposures. In bladder cancer, a unique distribution of mutations amongst several codons of TP53 has been hypothesized to be caused by environmental carcinogens including 4-aminobiphenyl (4-ABP). 4-ABP undergoes metabolic activation to N-hydroxy-4-aminobiphenyl (N-OH-4-ABP) and forms pre-mutagenic adducts in DNA, of which N-(deoxyguanosin-8-yl)-4-ABP (dG-C8-4-ABP) is the major one. Human TP53 knock-in mouse embryo fibroblasts (HUFs) are a useful model to study the influence of environmental carcinogens on TP53-mutagenesis. By performing the HUF immortalization assay (HIMA) TP53-mutant HUFs are generated and mutations can be identified by sequencing. Here we studied the induction of mutations in human TP53 after treatment of primary HUFs with N-OH-4-ABP. A total of 6% TP53-mutants were identified after treatment with 40 μM N-OH-4-ABP for 24 hr (n = 150) with G>C/C>G transversion being the main mutation type. The mutation spectrum found in the TP53 gene of immortalized N-OH-4-ABP-treated primary HUFs was unlike the one found in human bladder cancer. DNA adduct formation (~40 adducts/10^8 nucleotides) was detected after 24 hr treatment with 40 μM N-OH-4-ABP, but lacZ mutagenicity was not observed. Adduct levels decreased substantially (sixfold) after a 24 hr recovery period indicating that primary HUFs can efficiently repair the dG-C8-4-ABP adduct possibly before mutations are fixed. In conclusion, the observed difference in the N-OH-4-ABP-induced TP53 mutation spectrum to that observed in human bladder tumors do not support a role of 4-ABP in human bladder cancer development.

KEYWORDS
4-aminobiphenyl, bladder carcinogen, DNA adducts, mutation, TP53

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1 | INTRODUCTION

Bladder cancer is the fourth most common cancer in the United Kingdom and smoking remains the best characterized risk factor for it (Ferlay et al., 2018; Ferlay et al., 2019). One of the many harmful compounds found in tobacco smoke is the aromatic amine 4-aminobiphenyl (4-ABP), which has been classified as a Group 1 human carcinogen by the International Agency for the Research on Cancer (IARC) and has been linked to bladder cancer in many studies (IARC, 1972; IARC, 1987; IARC, 2010). In addition to inhalation of tobacco smoke, humans are exposed to 4-ABP by its presence in cooking fumes and as a contaminant in the dye industry (IARC, 2010). 4-ABP is N-hydroxylated to N-hydroxy-4-ABP (N-OH-4-ABP) by cytochrome P450 (CYP) 1A2 in the liver as well as by CYP1A1, CYP1B1, and CYP2A13 in extrahepatic tissues (Butler et al., 1989; Shimada et al., 1996; Nakajima et al., 2006). Newer studies have further suggested an involvement of CYP2E1 in the hydroxylation of 4-ABP (Wang et al., 2015a; Wang et al., 2015b). N-OH-4-ABP can be further metabolized by N-acetyltransferases and sulfotransferases to unstable esters, which will undergo heterolytic cleavage and form an electrophilic nitrenium ion that can bind to DNA (Turesky and Le Marchand, 2011; Wang et al., 2019). Three DNA adducts are formed in the reaction of N-OH-4-ABP with calf thymus DNA with the major adduct N-(deoxyguanosin-8-yl)-4-ABP (dG-C8-4-ABP) accounting for 80% of quantified adducts. This is followed by 15% N-(deoxyadenosin-8-yl)-4-ABP (dA-C8-4-ABP) and 5% N-(deoxyguanosin-N2-yl)-4-ABP (dG-N2-4-ABP) (Beland et al., 1983; Beland and Kadlubar, 1985) (Figure S1). dG-C8-4-ABP has been detected in human bladder tumors and exfoliated urothelial tissue (Talaska et al., 1991; Talaska et al., 1993; Zayas et al., 2007) and genomic changes resulting from its formation have been shown to be substitution mutations at G:C base pairs (Verghis et al., 1997; Besaratinia et al., 2002; Yoon et al., 2012).

TP53 is commonly mutated in human tumors and specific mutation spectra linked to environmental carcinogens have been observed (Hölzl-Armstrong et al., 2019). For instance, aristolochic acid I (AAI) induces A>T/T>A transversion mutations at a variety of codons and this pattern is also found in urothelial carcinomas associated with aristolochic acid I exposure. In contrast, the polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP) leads to G>T/C>A transversions amongst TP53 codons 157, 158, 175, 245, 248, 273, which is well reflected in smokers’ lung cancer (Kucab et al., 2010). The effect of environmental carcinogens on TP53 mutagenesis can be modeled using the Human TP53 knock-in (Hupki) mouse embryo fibroblast (HUF) immortalization assay (HIMA), where TP53 mutations can be created and selected experimentally by carcinogen treatment. The Hupki mouse has human exons 4–9 in place of the corresponding mouse exons, including the codons where most mutations are found in human tumors (Hölzl-Armstrong et al., 2019). The information acquired in the HIMA can be compared to the TP53 mutation database curated by IARC that currently lists around 30,000 mutations (www.p53.iarc.fr). Some characteristic mutations of environmental carcinogens (e.g., BaP and AAI) were successfully replicated in the HIMA (Liu et al., 2005; Feldmeyer et al., 2006; Nedelko et al., 2009). Bladder tumors often harbor TP53 mutations, which have three distinct characteristics: mutations are (a) distributed evenly across exons 5–8 of TP53; (b) not more frequent at methylated CpG sites; and (c) distributed among five hotspot codons, namely R175, R248, R273, R280, E285, with R280 and E285 being unique to bladder tumors (Feng et al., 2002a). Mutations at these hotspots are responsible for 2.9, 5.8, 3.5, 4.8 and 5.6% of all mutations in bladder cancer, respectively. Proximate metabolites of 4-ABP have been shown to bind to all bladder tumor TP53 hotspots except R273 in human bladder cells suggesting an involvement of 4-ABP in the TP53 mutation distribution in bladder cancer (Feng et al., 2002a; Feng et al., 2002b). No study has yet utilized an experimental in vitro model to analyze TP53 mutagenesis after 4-ABP exposure. In order to understand if 4-ABP is responsible for the codon distribution and mutation pattern found in TP53 in human bladder tumors the present study used primary HUFs to study N-OH-4-ABP-induced TP53 mutagenesis. It was hypothesized that immortalized clones created in the HIMA would carry TP53 mutations like those found in human bladder tumors and that these mutations would be found mainly at the hotspots described above. To find the optimal treatment conditions, cell viability, DNA adduct formation, induction of the DNA damage response (DDR) and lacZ reporter gene mutagenicity were assessed in primary HUFs exposed to N-OH-4-ABP.

2 | MATERIALS AND METHODS

2.1 | Carcinogens

N-OH-4-ABP was purchased from Toronto Research Chemicals (#H767500, Toronto, Ontario, Canada). Stock solutions were prepared at 100 mM by dissolving carcinogen in water-free DMSO and aliquots were stored under nitrogen gas at −80°C. 3-Nitrobenzanthrone (3-NBA) was synthesized as described previously (Arlt et al., 2002) and stored in aliquots at −20°C as a 2 mM stock solution in DMSO.

2.2 | Isolation and culture of primary mouse embryo fibroblasts

Primary HUFs were isolated from day 13.5 embryos of Hupki+/−; lacZ−; Xpa+/+ (Arg/Arg codon 72) mice of a 129/Sv and C57B1/6 background according to a published procedure (Hölzl-Armstrong et al., 2019). The Hupki allele is a knock-in allele with exons 4–9 of the human TP53 gene in place of the corresponding mouse exons and the lacZ mutation marker gene is contained in the pUR288 plasmid. The mice were bred by crossing homozygous Hupki mice with transgenic Xpa+/− mice containing the pUR288 plasmid. Primary HUFs were genotyped where necessary as described previously (Kucab et al., 2015) and grown in Dulbecco’s Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, #31966047) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific #10270106), 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific #15140122) at
37°C, 5% CO2 and 3% (primary HUFs) or 20% O2 (immortalized HUFs). When confluence was reached, cells were detached with 0.05% trypsin–EDTA (Thermo Fisher Scientific #25300054) for 2–10 min and, if not otherwise specified, reseeded at 16,000 cells/cm² into flasks or multiwell plates.

2.3 Crystal violet staining assay for cell survival

Cell viability was assessed by crystal violet staining as described previously (Hölzl-Armstrong et al., 2020) for concentrations up to 150 μM N-OH-4-ABP diluted in growth medium. In addition to a 24 hr time-point a 24 hr + 24 hr time-point was included in which the treatment medium was replaced by fresh growth medium after 24 hr. Following treatment cells were washed with 180 μl PBS and stained with 30 μl 0.1% (wt/vol) crystal violet dye (Sigma #C3886) in 10% ethanol (Sigma-Aldrich #32221) for at least 10 min. After removal of excess dye by washing twice with PBS, plates were then air-dried at room temperature. At the time of measurement, 100 μl 50% ethanol per well were added and absorbance determined at 595 nm using a plate reader. Data are expressed as the percentage of absorbance per well relative to control cells and are representative of at least three independent experiments.

2.4 DNA adduct analysis by 32P-postlabelling

For DNA adduct analysis primary HUFs were seeded into 75-cm² flasks and exposed the next day to cytotoxic and sub-cytotoxic concentrations of N-OH-4-ABP with and without a 24 hr recovery period in fresh medium or solvent control (DMSO) diluted in growth medium. After treatment cells were harvested and stored as pellets at −20°C until DNA was isolated using a standard phenol-chloroform extraction method as described previously (Kucab et al., 2015). DNA adducts were determined using the butanol enrichment version of the thin-layer chromatography (TLC) 32P-postlabelling method as described previously (Phillips and Arlt, 2020). Briefly, samples were digested using 4 μl micrococcal nuclease (288 μU/sample; Sigma–Aldrich #N3755) and bovine spleen phosphodiesterase (1.2 μU/samples; Worthington Biochemical Corp. #LS003603) and enriched by 1-butanol extraction. Adducts were then labeled with 50 μCi γ-32P]ATP (Hartmann-Analytic #HP601PE), followed by TLC as previously reported (Phillips and Arlt, 2020). The TLC sheets were scanned and adduct spots visualized using an Amer sham Typhoon Biomolecular Imager (GE Healthcare). The signal intensity measured is proportional to the level of radioactivity present on an area of the TLC plates. Levels of DNA adducts were calculated as relative adduct labeling (RAL), which is the ratio of signal intensity of adducted nucleotides (adducts) over signal intensity of total (adducted plus normal) nucleotides in the DNA samples analyzed. A blank area on the TLC plates was used to determine the background level of radioactivity. A deoxyadenosine-3’-monophosphate (dAP) standard was labeled in each experiment to estimate the signal intensity of total nucleotides (normals).

2.5 Western blotting

To evaluate the expression of DDR proteins whole cell lysates were prepared and Western blotting performed as described previously (Hölzl-Armstrong et al., 2020). The following antibodies were utilized: anti-phospho-p53 (Ser15; 1:2,000; Cell Signalling, Danvers, MA, #9284s), anti-p53 (1:500; Cell Signalling, 2524S), anti-p21 (1:2,000; BD Biosciences, Franklin Lakes, NJ, #BD556431), anti-γ-H2ax (Ser139; 1:1,000; Cell Signalling #9718), anti-phospho-Chk1 (Ser345; 1:1,000; Cell Signalling #2348), glyceraldehyde 3-phosphate dehydrogenase (Gapdh; 1:25,000; Chemicon International, Temecula, CA, #MAB374), anti-Mdm2 (1:750; Abcam, Cambridge, United Kingdom #ab16895) and anti-β-actin (1:25,000; Abcam #ab6276).

2.6 LacZ mutation assay

To assess lacZ mutagenicity pellets of treated HUFs were prepared and the mutation assay performed as described previously (Hölzl-Armstrong et al., 2020). Briefly, primary HUFs were seeded into 75-cm² flasks and treated for 24 hr. After 48 hr cells 175-cm² flasks were reseeded with 2 × 10⁶ cells and allowed to grow for 4 days. Pellets were prepared and DNA isolated using a standard phenol-chloroform extraction. DNA was digested using HindIII, incubated with magnetic beads coated with lacI fusion protein and eluted from the beads using isopropyl-β-D-thiogalactopyranoside (IPTG). Plasmids were then circularized with T4 DNA ligase and electroplated into Escherichia coli lacking β-galactosidase (lacZ⁰) and galactose epimerase (galE⁰). One-thousand of the transformed bacteria were plated on nonselective, titer plates containing 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) and the remainder on mutant selective plates containing the lactose analogue phenyl-β-D-galactosidase (P-Gal). The ratio of the number of mutant colonies on the selective plates to the number of colonies formed on the nonselective titer plate (× dilution factor 1,000) equals the mutant frequency.

2.7 HUF immortalization assay (HIMA) and TP53 mutation analysis

The HIMA, Nutlin-3a counter-screen and TP53 mutation analysis were performed as described previously (Hölzl-Armstrong et al., 2019). Exons 4–9 of Nutlin-3a-selected cultures were amplified and subjected to Sanger sequencing and mutations analyzed as described previously (Hölzl-Armstrong et al., 2020). Mutations were confirmed by sequencing DNA from an independent sample of cells from the same culture.

2.8 Statistical analysis

Results are shown as mean values ± SD. The sample size is indicated in each section. Using GraphPad Prism version 8.2.0 (GraphPad
FIGURE 1  Optimization of treatment conditions for the HIMA. (a) Cell viability assessment: Primary HUFs were treated with indicated concentrations of \( N\)-OH-4-ABP for 24 hr and cell viability (% control) was assessed by staining with crystal violet. 24 hr + 24 hr refers to treatment for 24 hr followed by 24 hr in fresh growth medium. Cells treated with 0.1% DMSO served as controls. Shown are mean values ± SD \((n > 3)\). AUCs were calculated and significance was determined using unpaired \( t \) test assuming unequal variances (n.s., nonsignificant \( p > .05 \)).

(b) Western blot analysis of various DDR proteins (p-p53, p-Chk1, p21, and \( \gamma\)-H2ax) in primary HUFs exposed to indicate concentrations of \( N\)-OH-4-ABP for 24 hr with or without a 24 hr recovery period in fresh medium. Gapdh was used as a loading control and 3-NBA \((2 \mu M, 48 \text{ hr})\) included as positive control. Representative images of Western blot analysis are shown. Analysis was performed in duplicate from independent experiments.

(c–e) DNA adduct analysis: (c) Primary HUFs were treated with \( N\)-OH-4-ABP as indicated for 24 hr + 24 hr and formation of the dG-C8-4-ABP adduct was quantified using \( 32\)P-postlabelling. Control cells were treated with 0.1% DMSO only. Shown are mean values ± SD \((n = 4)\). n.d. (nondetected). (d) Representative autoradiographic profiles of DNA adducts formed in primary HUFs after \( N\)-OH-4-ABP exposure. The arrow indicates the dG-C8-4-ABP adduct. The bottom left corners (=origins) were cut off prior to imaging. (e) Induction of lacZ mutants in primary HUFs treated with \( N\)-OH-4-ABP for 24 hr + 24 hr. Cells treated with 0.1% DMSO served as controls. After a total of 48 hr cells were passaged to a new flask and allowed to double six times to fix DNA mutations. LacZ mutant frequencies were calculated as the number of mutant colonies per number of recovered transformants. Shown are mean values ± SD \((n > 3)\).
Software Inc., La Jolla, CA), groups of two were compared by two-sample t-test assuming unequal variances (**p < .001). For cell viability data, the area under the curve (AUC) and half-maximal inhibitory concentration (IC50) values were calculated.

3 | RESULTS

3.1 | Optimization of treatment conditions for the HIMA

3.1.1 | Cell viability in primary HUFs after treatment with N-OH-4-ABP

Before initiating the HIMA, treatment conditions were optimized. First, primary HUFs were treated with various concentrations of N-OH-4-ABP (0–150 μM) for 24 hr. As the HIMA was optimized for 48 hr treatments, a recovery period of 24 hr was included after treatment (24 hr + 24 hr) to better understand the viability of cells at the end of the exposure when cells are first passaged during the HIMA. Once all treatments were completed, cells were stained with crystal violet and cell viability relative to controls (i.e., untreated cells) calculated. N-OH-4-ABP treatment decreased cell viability in a concentration-dependent manner: the AUCs were not significantly different between the 24 hr and 24 hr + 24 hr timepoints (p > .05) and the IC50 values of 54 μM (24 hr) and 60 μM (24 hr + 24 hr) were very similar (p > .05). Based on the 24 hr + 24 hr crystal violet results, three concentrations, 30, 40, and 80 μM, which reflected 80–60, 60–40, and 20–40% cell viability, respectively, compared with the controls, were chosen for subsequent experiments.

3.1.2 | DDR in primary HUFs after N-OH-4-ABP treatment

Induction of DDR proteins (p-p53, p-Chk1, γ-H2ax, and p21) in primary HUFs was assessed by Western blotting in response to N-OH-4-ABP treatment for 24 hr or 24 hr + 24 hr. As shown in Figure 1b most DDR proteins investigated were induced by N-OH-4-ABP in a concentration-dependent manner, to a similar extent at both timepoints. The highest N-OH-4-ABP concentration tested (80 μM) resulted in similar levels of protein expression to those seen in HUFs treated with 3-NBA as positive control. Generally, high constitutive levels of p21 were found. Gapdh expression shows equal loading of samples.

3.1.3 | Formation of DNA adducts in primary HUFs after N-OH-4-ABP treatment

For DNA adduct analysis, cells were treated with the three N-OH-4-ABP concentrations selected above for 24 hr + 24 hr; additionally cells were treated for 24 hr with 40 μM reflecting 60–40% cell viability. One major DNA adduct spot was detected by 32P-postlabelling analysis in primary HUFs after exposure to N-OH-4-ABP (Figure 1c). Based on previous studies this adduct spot was tentatively identified as dG-C8-4-ABP (Beland et al., 1999; Torino et al., 2001). No DNA adducts were detectable in untreated (control) HUFs (data not shown). Formation of the dG-C8-4-ABP adduct was concentration-dependent, with levels starting at ~2 adducts/10⁸ nucleotides after treatment with 30 μM N-OH-4-ABP (Figure 1d); this value tripled to ~7 adducts/10⁸ nucleotides when cells were treated with 40 μM N-OH-4-ABP and finally was 12-fold higher compared to the lowest treatment concentration at ~24 adducts/10⁸ nucleotides after treatment with 80 μM N-OH-4-ABP. In addition, adduct levels were also examined after 24 hr exposure to 40 μM N-OH-4-ABP, in order to observe the extent of DNA damage at an earlier time-point and the ability of primary HUFs to repair pre-mutagenic DNA adducts. Approximately 44 adducts/10⁸ nucleotides were detected at 24 hr, which was six-fold more adducts than detected following the 24 hr recovery period (Figure 1e). This indicates that the HUF cells were able to repair the DNA adducts (dG-C8-4-ABP) extensively within 24 hr.

3.1.4 | Determination of lacZ mutant frequency in primary HUFs after N-OH-4-ABP treatment

Before initiating the HIMA the induction of lacZ mutants by N-OH-4-ABP was assessed in an E. coli host system (Figure 1f). Background mutant levels of ~9 × 10⁻⁵ mutants were calculated, which did not change at any of the treatment conditions used, with lacZ mutant levels in the range ~ 9–10 × 10⁻⁵ after exposure to N-OH-4-ABP. Thus, no increase in mutant frequency was identified in the lacZ reporter gene.

3.2 | TP53 mutations induced by N-OH-4-ABP

3.2.1 | Choice of treatment conditions and number of cultures for the HIMA

Based on these experiments it was decided to initiate the HIMA with 40 μM N-OH-4-ABP for 24 hr. A total of 150 cultures of primary HUFs were exposed to N-OH-4-ABP to evaluate the TP53 mutation pattern, with six cultures as controls (0.1% DMSO). The chosen treatment conditions are summarized in Table 1.

3.2.2 | Nutlin-3a counter-screen and sequence analysis in N-OH-4-ABP-treated HUFs

Following the Nutlin-3a counter-screen 9 out of 150 cultures (~6%) were classified as TP53-mutant HUFs. No TP53 mutations were found in spontaneously immortalized cultures (i.e., controls) which is
consistent with historic controls (Hölzl-Armstrong et al., 2020). TP53-mutant cultures were expanded into cell lines from which DNA was isolated. Exons 4–9 of the TP53 gene were amplified by PCR and subjected to Sanger dideoxy sequencing. There was a distinctive pattern of 56% G>C/C>G and 33% G>T/C>A transversions with 22% (2/9) based at CpG sites. The distribution of mutations on the transcribed and non-transcribed strand was the same. The observed mutant frequency and pattern are summarized in Table 1. It was not possible to establish the mutation type for one culture (i.e., NOA-59), which has been classified as “complex”. It was still included as a definite TP53-mutant for several reasons: (a) the Nutlin-3a counter-screen showed a strong resistant response; (b) p53 and its related pathway proteins p21 and Mdm2 were not expressed after treatment with Nutlin-3a (see Figure 2); and (c) after PCR amplification, sequencing of exon 7 was not successful. Therefore, it is likely that clone NOA-59 harbors a complex deletion in exon 7 that prevented primer annealing during sequencing.

3.2.3 | Exon and codon distribution of N-OH-4-ABP-induced mutations

A detailed overview of all N-OH-4-ABP-induced TP53 mutations can be found in Table 2. All but one (clone NOA-59) of the identified mutations were single-base substitutions that induced amino acid changes and each culture harbored only one mutation. Most of mutations were in either exon 8 (4/9) or exon 7 (3/9), with one mutation each within exon 5 and 6. Additionally, three mutations were found at one of the six mutation hotspot codons of TP53: NOA-36 at R248, NOA-54 at R273 and NOA-113 at G245. Mutations harbored in clones NOA-36 and -54 were also the only mutations to occur at CpG sites or at sites in p53 that make direct contact with the DNA. Further, the mutation identified in clone NOA-113 was at a site involved in the structure of the DNA binding surface of p53. Only one codon (D281) was targeted twice in culture NOA-109 and -114.

3.2.4 | Zygosity, Nutlin-3a response and p53 functionality in N-OH-4-ABP-induced TP53 mutants

Mutations were further classified as homo-/hemizygous and heterozygous. Interestingly, all clones but one (NOA-59) classified as fully

| TABLE 1 | Overview of treatment conditions, TP53 mutation frequency and pattern detected in N-OH-4-ABP-treated immortalized HUFs |
|-----------------|----------------------------------|
| N-OH-4-ABP     | Treatment concentration 40 μM   |
| Treatment time  | 24 hr + 24 hr                  |
| Total HUF cultures | 150                        |
| TP53-mutant immortalized clones | 9                    |
| Frequency of TP53-mutant clones | 6% (9/150) |
| Mutation type   | G>T/C>A 33% (3/9)             |
|                 | G>C/C>G 56% (5/9)             |
|                 | G>A/C>T 0% (0/9)              |
|                 | A>T/T>A 0% (0/9)              |
|                 | A>G/T>C 0% (0/9)              |
|                 | A>C/T>G 0% (0/9)              |
|                 | Complex 11% (1/9)             |
| Mutations on the transcribed strand | 50% (4/8) |
| Mutations on the nontranscribed strand | 50% (4/8) |

Note: Mutations were detected by Sanger dideoxy sequencing. Brackets show the number of TP53 mutant clones versus the total clones analyzed.

FIGURE 2  Expression and induction of p53 pathway proteins after treatment with 10 μM Nutlin-3a in N-OH-4-ABP-induced TP53-mutant clones. Protein expression of p53, Mdm2, and p21 was assessed by Western blot analysis of TP53-mutant HUFs treated without (−N) or with (+N) 10 μM Nutlin-3a for 24 hr. Gapdh and β-actin were used as loading controls. A confirmed TP53-WT clone was included to represent a WT response. Representative images of the Western blot analysis are shown.
resistant to Nutlin-3a (NOA-14, -109, -113, -114) harbored homo-/hemizygous mutations, while the mixed response clones (NOA-36, -37, -54 and -141) had heterozygous mutations. It should be noted that it is not possible to distinguish between homozygous (same mutation on both alleles) and hemizygous (one mutated allele and one allele with loss of heterozygosity) mutations. Six mutations (cultures NOA-14, -36, -54, -109, -113, and -114) were found to render p53 non-functional as assessed in a yeast promotor assay (Kato et al., 2003) while culture NOA-37 was found to have a TP53 mutation that produced a partially functional protein. No data were available for clone NOA-141. Further, seven mutations were of the missense type (cultures NOA-14, -36, -37, -54, -109, -113, -114) with only one nonsense mutation in clone NOA-141 identified.

### 3.2.5 | Expression and induction of p53 pathway proteins in N-OH-4-ABP-treated TP53 mutants following treatment with Nutlin-3a

To further understand the cellular response towards Nutlin-3a, TP53-mutant cultures were exposed to 10 μM Nutlin-3a for 24 hr and analyzed by Western blotting for the expression of p53 and its pathway proteins p21 and Mdm2. The effect of the mutation and Nutlin-3a on p53, p21 and Mdm2 expression is shown in Figure 2 and summarized in the supporting materials (Table S1). A TP53-WT culture was included to demonstrate a typical response towards Nutlin-3a treatment, which is reflected by an induction of all three proteins. In TP53-mutant cultures constitutive p53 levels are usually high and p21 as well as Mdm2 are not expressed. However, WT-like responses are observed in some TP53-mutant cultures (Kucab et al., 2017; Hözl-Armstrong et al., 2020). All TP53-mutants classified as resistant in the Nutlin-3a counter-screen (cultures NOA-14, -109, -113 and -114) responded to Nutlin-3a treatment like a usual TP53 mutant, that is, a strong constitutive expression of p53, but no induction due to Nutlin-3a treatment was observed, and neither p21 nor Mdm2 were expressed. Additionally, clone NOA-59 expressed none of the p53 pathway proteins. In TP53 mutants classified as mixed in the Nutlin-3a counter-screen (cultures NOA-36, -37, -54, and -141) all proteins were induced after Nutlin-3a treatment. As all those cultures carry heterozygous mutations it can be hypothesized that the mutation is only carried on one allele while the second allele retained the WT sequence. In most cultures except clones NOA-109, -114, and -141 Gapdh expression was affected by the TP53 mutation, which has been observed previously (Hözl-Armstrong et al., 2020). β-Actin was included as a second loading control and its expression was constant for all samples, indicating accurate gel loading.

### 3.2.6 | Comparison of TP53 mutations induced by N-OH-4-ABP with TP53 mutations found in human tumors

All N-OH-4-ABP-induced mutations in exons 4–9 of the TP53 gene were compared with TP53 mutations of human tumors listed in the
IARC TP53 mutation database (R20, July 2019) and are summarized in Table 3. The seven different mutations identified in N-OH-4-ABP-treated HUFs were found in a total of 155 human tumors with numbers ranging from 14–43 tumor samples. Further, all codons and splice sites targeted by N-OH-4-ABP were found to be mutated in other human cancers. As 4-ABP is a known bladder carcinogen (IARC, 2010), the occurrence of each mutation and targeted codon was further compared specifically with TP53 mutations found in human bladder cancer (Table 3). The exact mutations have been reported in 10 human bladder tumors.

As discussed previously, there are specific TP53 mutation hotspots in bladder cancer, so the codon distribution in bladder cancer of non-smokers and smokers was compared, with the historical HUF control, and all cancer types (Figure 3a–f). The codon distribution of the historical HUF control shows the most mutations at codon C135, R245 and D281 (Figure 3b). While the six mutation hotspots that occur across the TP53 gene of all tumor types are in codons R175, G245, R248, R249, R273, R282 (Figure 3f), TP53 of bladder tumors has mutation hotspots in three of these (R175, R248, and R273) plus two other hotspots, codons R280 and E285 (Figure 3c), with E285 being specific to smokers (Figure 3d). In bladder tumors of non-smokers R282 is more often mutated than E285 (Figure 3e), however, the sample number available for analysis was quite low. Immortalized N-OH-4-ABP-treated HUFs carried mutations within two (bladder) hotspot codons (R248 and R273) as well as on codons C135, Y236, R245, G262, and D281. The unique bladder tumor codons R280 and E285 were not mutated in primary HUFs treated with N-OH-4-ABP (Figure 3a).

Further, the mutation pattern of bladder cancer was compared with the pattern induced by N-OH-4-ABP in primary HUFs, the historical HUF controls and all cancer types (Figure 3g–l). The predominant mutation type in the TP53 gene of all bladder tumors independent of smoking status is G>A/C>T, followed by G>C/C>G and G>T/C>A (Figure 3i–k). In contrast, while in all cancer cases the G>A/C>T type is also the predominant type the second most common mutations are G>T/C>A transversions and not G>C/C>G as observed in bladder cancer (Figure 3l). In immortalized HUFs treated with N-OH-4-ABP no G>A/C>T transitions were detected. However, the hallmark mutations were identified as 56% G>C/C>G and 33% G>T/C>A transversions, which are also common TP53 mutation types in bladder tumors (Figure 3g). The predominant mutation type in historical HUF controls is 50% G>C/C>G transversions followed by 24% A>C/T>G transitions (Figure 3h).

### 3.2.7 | Comparison of TP53 mutations induced by N-OH-4-ABP with TP53 mutations found in previous HIMAs

Mutations induced by N-OH-4-ABP were further compared with those found in previous HIMAs (Table S2). The three mutations in clones NOA-14 (G262), -36 (R248) and -141 (Y236) have not been identified before in immortalized HUFs. The mutation identified within C135 of NOA-37 was found to be mutated many times before: most notably in seven untreated spontaneously immortalized HUF cultures but also after treatment with environmental carcinogens including N-hydroxy-3-aminobenzanthrone (N-OH-3-ABA) or BaP-7,8-diol-9,10-epoxide (BPDE). Further, mutations in the hotspot codons G245 and R273 found in clones NOA-113 and -54 were also identified after treatment with several other compounds (e.g., 3-NBA, N-OH-3-ABA, BPDE). G245 was often identified in untreated spontaneously immortalized HUFs prior to this study. Interestingly, D281, which was mutated in cultures NOA-109 and -114, was previously only mutated in untreated, spontaneously immortalized HUFs.

### 3.2.8 | Mutations induced by 4-ABP and its metabolites in the literature

Finally, the mutation pattern observed in the TP53 gene of N-OH-4-ABP-exposed HUFs was compared with mutations observed in other studies examining 4-ABP or its metabolites. As shown in Figure 4, studies found mutations predominantly at G:C base pairs (Besaratinia et al., 2002; Chen et al., 2005; Yoon et al., 2012). In the cll

| NOA– | Codon # | Mutation type | All tumors | Bladder tumors |
|------|---------|---------------|------------|---------------|
|      |         |               | Occurrence | Codon mutant frequency | Occurrence | Codon mutant frequency |
| 37   | 135     | G>C/C>G       | 28         | 268            | 1           | 17                       |
| 141  | 236     | G>T/C>A       | 14         | 182            | 1           | 9                        |
| 113  | 245     | G>C/C>G       | 15         | 868            | 2           | 38                       |
| 36   | 248     | G>C/C>G       | 19         | 1,891          | 1           | 86                       |
| 14   | 262     | G>T/C>A       | 17         | 52             | 0           | 3                        |
| 54   | 273     | G>T/C>A       | 19         | 1,823          | 1           | 52                       |
| 109, 114 | 281 | G>C/C>G       | 43         | 207            | 4           | 12                       |

Note: Occurrence refers to the number of human tumors harboring the exact indicated mutation. In addition, the total number of times the respective codon is mutated in human tumors is listed. Total count of mutations is 28,866 in all tumors and 1,522 in bladder tumors (IARC TP53 mutation database, R20, July 2019). Studies recommended to be excluded by IARC were not considered.
gene of Big Blue® MEFs treated with N-hydroxy-4-acetylaminobiphenyl (N-OH-4-ABP) 43% were G>T/C>A mutations, followed by 21% G>A/C>T and 9% G>C/C>G (Figure 4c) (Besaratinia et al., 2002). Another study examined the cII gene in livers of Big Blue® mice treated by intra-peritoneal injection with 4-ABP and found no difference in the mutation spectrum between treated and untreated (i.e., controls) tissues; both spectra mostly carried G>A/C>T transitions. However, when the liver cII gene of neonatal mice treated in the same way was sequenced, mainly G>T/C>A transversions (41%) and an equal amount of 16% each G>A/C>T and G>C/C>G substitutions were found (Figure 4a) (Chen et al., 2005). The mutation pattern of the cII gene in bladders of Big Blue® mice exposed for 6 weeks to various doses of 4-ABP administered by intraperitoneal injection was defined by 42% G>T/C>A and 25% G>A/C>T substitutions. In addition, 12% G>C/C>G transversions were induced in the bladder cII gene (Figure 4b) (Yoon et al., 2012).
**DISCUSSION**

The objective of this study was to assess the mutagenicity of N-OH-4-ABP in primary HUFs. It was hypothesized that N-OH-4-ABP induces a specific mutation spectrum in the human TP53 gene of immortalized HUFs reflecting the mutation spectrum observed in human bladder cancer. Human bladder cancer shows an even distribution of mutations amongst exons 5–8 of TP53 that is characterized by five hotspot codons (R175, R248, R273, R280, E285), while mutations are not biased towards CpG sites (Feng et al., 2002a). When the results acquired in the HIMA are compared with human bladder cancer, the first difference is that the codon distribution of TP53 mutations in N-OH-4-ABP-treated HUFs does not correlate with that found in human bladder cancer. No TP53 mutations were harbored in the unique bladder cancer codons R280 and E285 in immortalized HUFs despite previous studies showing that both codons are targets of DNA adduct formation by N-OH-4-ABP (Feng et al., 2002a). However, codon D281, which has also been shown to be a target for dG-C8-4-ABP adduct formation in human uroepithelial cells (Feng et al., 2002a), was mutated in two immortalized HUF clones after N-OH-4-ABP treatment. Further, TP53 mutations in immortalized HUFs were harbored in other bladder hotspot codons in this study, namely R248 and R273. In previous HIMAs TP53 mutations in R273 have been observed (Table S2), but finding a mutation at this codon after N-OH-4-ABP treatment was unexpected as dG-C8-4-ABP adduct formation did not occur at this codon in previous work (Feng et al., 2002a; Feng et al., 2002b). Thus, it could be speculated that the observed differences between HUFs and human uroepithelial cells are due to variations of enzymatic capabilities (e.g., N-acetyltransferases or sulfotransferases) and/or differences in DNA repair. Furthermore, while in human bladder tumors mutations are evenly distributed amongst the TP53 sequence, most TP53 mutations in HUFs were harbored within exons 7 and 8. However, only ~20% of TP53 mutations occurred at CpG sites, which agrees with lack of bias for CpG mutations in bladder cancer (Feng et al., 2002a).

The fact that G>C/C>G transversions are amongst the predominant mutation types in spontaneously immortalized HUFs (Figure 3h) could lead to the conclusion that the mutations found in the present study are induced spontaneously and not due to N-OH-4-ABP treatment. Importantly, three mutations (C135, G245 and D281) have previously been predominantly observed in untreated spontaneously immortalized HUF clones, and it cannot be ruled out that these mutations are unspecific to N-OH-4-ABP treatment but rather happened due to spontaneous mutation events. In addition, even though the spontaneous TP53 mutation frequency is very low (0–3.7%) in primary HUFs prepared and treated at 3% oxygen (Kucab et al., 2015; Kucab et al., 2016; Hölzl-Armstrong et al., 2020), some mutations could be caused spontaneously and not due to N-OH-4-ABP treatment. However, several reasons argue that most of the observed mutations are indeed caused by N-OH-4-ABP and not spontaneous: First, three of the TP53 mutations induced by N-OH-4-ABP have not been observed previously in carcinogen-treated or spontaneously immortalized HUFs, so they appear to be related to N-OH-4-ABP exposure. Secondly, guanine adducted with 4-ABP (i.e., dG-C8-4-ABP) can,
observed G>C/C>G transversions as the hallmark mutation, this type in this study was G>C/C>G transversion (56%). While no study versions as the hallmark mutation, while the predominant mutation
c persone found mutations predominantly at G:C base pairs (Besaratinia
could also be linked to oxidative damage. (Marnett, 2000). Thus, the high number of G>T/C>A transversions
which can result in the induction of G>T/C>A mutations
TP53
0
7,8-dihydro-2
et al
Marnett, 2000). Thus, the high number of G>T/C>A mutations
TP53
genotoxicity of tobacco smoke-
resulting in oxidative damage to DNA, including 8-oxo-
7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) (Murata et al., 2001),
which can result in the induction of G>T/C>A mutations
(Marnett, 2000). Thus, the high number of G>T/C>A transversions
could also be linked to oxidative damage.

Additionally, most other studies examining 4-ABP or its metabo-
lites found mutations predominantly at G:C base pairs (Besaratinia
et al., 2002; Chen et al., 2005; Yoon et al., 2012), which agrees with the
TP53 mutation pattern observed in HUFs, where all mutations are
based at G:C base pairs (Figure 4). Others (Besaratinia et al., 2002;
Chen et al., 2005; Yoon et al., 2012) have identified G>T/C>A trans-
versions as the hallmark mutation, while the predominant mutation
type in this study was G>C>C>G transversion (56%). While no study
observed G>C>C>G transversions as the hallmark mutation, this type
was present in each study.

The results of the lacZ mutation assay (Figure 1f), in which muta-
tions were not induced in N-OH-4-ABP- treated primary HUFs, were
surprising. dG-C8-4-ABP adducts were clearly formed in primary
HUFs and other studies have observed mutagenicity after exposure
to 4-ABP or its metabolites in reporter gene assays (e.g., cII, Tk,
HGPRT) in other cultured mammalian cells (Bookland et al., 1992;
Besaratinia et al., 2002; Guo et al., 2016). In other studies lacZ muta-
genicity has been a good predictor of TP53 mutagenicity (Kucab
et al., 2016; Hözl-Armstrong et al., 2020). It is puzzling that there is a
lack of lacZ mutants after treatment with N-OH-4-ABP, although the
TP53 mutant frequency of 6% obtained in this study is also low com-
pared to the mutant frequencies induced by other agents in previous
HIMAs, ranging from 9 to 33% (Feldmeyer et al., 2006; vom Brocke
et al., 2009; Kucab et al., 2015; Hözl-Armstrong et al., 2020). It seems
likely that the dG-C8-4-ABP adduct levels in N-OH-4-ABP-treated
primary HUFs were not high enough to induce mutagenicity in the
lacZ reporter gene or that the majority of adducts were repaired
before mutation fixation, as suggested by the greatly decreased dG-
C8-4-ABP adduct levels in HUFs after adding a 24 hr recovery period.
Other studies have also shown that the dG-C8-4-ABP adduct can be
repaired. For instance, in human urinary bladder transitional cell carci-
noma cell lines treated with 15 μM N-OH-4-AABP for 8 hr, 75% of
adducts were removed within 25 hr (Torino et al., 2001). Another
explanation for the lack of lacZ mutants and low rate of TP53
mutants could be efficient error-free translesion synthesis past the
dG-C8-4-ABP adduct. In fact it has been shown that the efficiency of
translesion synthesis is affected by the sequence surrounding the
dG-C8-4-ABP adduct (Yagi et al., 2017), which could explain why
mutations were still induced in the TP53 gene. This observation could
also be linked to differences in the timing of the two assays. As shown
by the DNA adduct experiments many adducts were repaired within
24 hr. Thus, remaining unrepaired adducts resulting in mutations are
very rare and the 6 days allowed for mutation fixation in the lacZ
assay might not be sufficient for recognition because lacZ mutant cells
are too diluted amongst lacZ WT cells. In contrast, as the HIMA
cultures are sometimes grown for up to 3 months the TP53-mutants
HUFs have a chance to appear amongst the WT cells due to clonal
expansion.

In summary, we have performed the first comprehensive in vitro
study assessing N-OH-4-ABP-induced mutagenesis in the human
TP53 gene, which is often mutated in human bladder cancer. N-OH-
4-ABP induced a characteristic TP53 pattern in immortalized HUFs,
which agrees with the formation of the main dG-C8-4-ABP adduct
that leads to mutations at G:C base pairs. However, the observed
mutation spectrum of TP53 mutations was different to that observed
in human bladder tumors, which is mostly likely due to other factors
also playing a role in human bladder cancer development. High repair
efficiency together with a possible error-free translesion synthesis
could be responsible for the absence of lacZ mutants and low TP53
mutation frequencies.

ACKNOWLEDGMENTS
Lisa Hözl-Armstrong was supported by a PhD studentship from the
MRC Centre for Environment and Health. Work at King’s College
London was supported by the Cancer Research UK Grand Challenge
Award “Mutographs of Cancer” (grant C98/A24032).

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Hözl-Armstrong L, Kucab JE, Zwart EP, Luijten M, Phillips DH, Arlt VM. Mutagenicity of N-hydroxy-4-aminobiphenyl in human TP53 knock-in (Hupki) mouse embryo fibroblasts. Environ Mol Mutagen. 2021;62:252–264. https://doi.org/10.1002/em.22429