AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Targeted Gene Bisulfite Sequencing Identifies Changes In P21 Methylation by the Nephrotoxicant Bromate

Prakash Mahadeo Wadkar[1*]; Kailash Gupta[2], Sameer Mehra[3]

1.Research Scholar, Department of Toxicology, Preston University, Melbourne Campus, Melbourne VIC 3000 Australia

2.Research Supervisor, Department of Toxicology, Preston University, Melbourne Campus, Melbourne VIC 3000 Australia

3.Research Co-Supervisor, Department of Toxicology, Preston University, Melbourne Campus, Melbourne VIC 3000 Australia

ABSTRACT

This study aimed to evaluate site-specific changes in the methylation of the nephro-protective gene p21 (CDKN1a) after exposure to nephrotoxicants using a targeted bisulfite sequencing approach. A targeted gene bisulfite sequencing (TGBS) method was developed using the Illumina MiSeq platform and Bismark bisulfite mapper. Human embryonic kidney (HEK293) cells and freshly isolated human proximal tubular cells (hPT) were analyzed, and 5-aza-2'-deoxycytidine (5-Aza), a DNA methyltransferase inhibitor, served as a positive control. Methylation differences between human and rat p21 were also investigated. TGBS analysis identified a methylation-sensitive site in the p21 promoter region, sis-inducible element-1 (SIE-1), which regulates p21 expression via transcription factor binding. Treatment with 5-Aza altered methylation at this site, whereas nephrotoxicants cisplatin and bromate ($BrO3^-$) did not, despite increasing p21 protein expression. No SIE-1 site was detected in normal rat kidney cells (NRK), indicating species-specific differences. Cisplatin and $BrO3^-$ exposure did not decrease promoter methylation in either HEK293 or NRK cells. These findings reveal species-specific differences in basal p21 promoter methylation and indicate that nephrotoxicant-induced changes in p21 expression are independent of promoter DNA methylation. The study also demonstrates the utility of TGBS for rapid analysis of locus-specific DNA methylation.

Keywords: Targeted gene bisulfite sequencing, DNA methylation, p21 (CDKN1a), nephrotoxicants, 5-Aza, Cisplatin

*Corresponding Author Email: indpbk@gmail.com

Received 27 September 2025, Accepted 10 October 2025

Please cite this article as: Wadkar PM *et al*., Targeted Gene Bisulfite Sequencing Identifies Changes In P21 Methylation by the Nephrotoxicant Bromate. American Journal of PharmTech Research 2025.

INTRODUCTION

DNA methylation is a well-established epigenetic mechanism involving the addition of a methyl group to the 5-carbon position of cytosine residues, typically within CpG dinucleotides. Although it does not alter the DNA sequence, methylation plays a crucial role in regulating gene expression, with promoter hypermethylation commonly associated with transcriptional silencing. Aberrant DNA methylation patterns have been implicated in the onset and progression of numerous diseases, including various cancers. One key gene regulated by methylation is p21 (CDKN1a), a cyclin-dependent kinase inhibitor that serves as an important regulator of the cell cycle. Abnormal methylation of p21 has been associated with gene silencing in cancers such as prostate, lung, and lymphomas, highlighting its role in tumor suppression.

While the significance of p21 methylation in cancer biology has been extensively studied, its role in non-cancer contexts, particularly in response to chemical or environmental toxicants, remains less understood. In the kidney, p21 protein expression increases following exposure to nephrotoxicants such as cisplatin and bromate, where it can exert protective effects, sometimes independently of the classical regulator p53. The interplay between p53 and p21 expression may determine cellular outcomes, with co-induction often correlating with toxicity. These observations underscore the need to explore alternative molecular pathways regulating p21 during nephrotoxicant exposure.

Previous studies have suggested that DNA methylation may contribute to the regulation of p21 in response to toxicants. However, earlier approaches, including methylation-specific PCR, were limited in scope and accuracy, while large-scale genomic methods, although comprehensive, are more suitable for hypothesis generation rather than targeted mechanistic analysis. To overcome these limitations, targeted gene bisulfite sequencing (TGBS) offers a precise and efficient method for site-specific analysis of DNA methylation. This approach enables detailed examination of how nephrotoxicants influence p21 regulation and provides insights into potential epigenetic mechanisms underlying renal protection and toxicity.

MATERIALS AND METHOD

Materials

Human embryonic kidney (HEK293) and normal rat kidney (NRK) cells and penicillin and streptomycin were purchased from American Type Culture Collection (Manassas, VA). The freshly isolated human proximal tubule (hPT) cells were generously provided by Dr. Lawrence H. Lash at the Wayne State University (Detroit, MI). Potassium bromate (KBrO3), 5-aza-2'-deoxycytidine (5-Aza) and trypsin EDTA were purchased from Sigma-Aldrich (St. Louis, MO), DMEM media from

HyClone technologies (Logan, UT), 5- Aza was dissolved in dimethyl sulfoxide (DMSO) from Fisher Scientific (Pittsburg, PA), DNeasy blood and tissue extraction kit were purchased from Qiagen (Valencia, CA). The EZ-DNA methylation lightning kit and the Zyppy plasmid miniprep kits were purchased from Zymo research (Irvine, CA). Nucleospin gel and PCR clean-up kits were purchased from Macherey-Nagel (Düren, Germany). The MiSeq reagent v3 kit was purchased from Illumina Inc (San Diego, CA), the Strataclone PCR cloning kit from Agilent technologies (Santa Clara, CA), the Kapa HiFi PCR kit from Kapa Biosystems (Wilmington, MA), and the Maxima hot-start Taq polymerase and Sera-Mag magnetic speed beads were purchased from Thermo Scientific (Waltham, MA).

Cell culture and treatment

5-aza-2'-deoxycytidine (5-Aza) is a DNA methyltransferase inhibitor and is used in many studies for its demethylating properties. HEK293 cells (3 x 10^6) were seeded in T-175 tissue culture flasks and grown at 37^0C in a 5% CO2 incubator for 24 hrs. Cells were then treated with 0-100 ppm bromate ($BrO3^-$), 40 µM 5-Aza, DMSO (vehicle control for 5-Aza) or 1 µM cisplatin for 72 hrs. The total amount of DMSO was never above 0.5% of the total volume per flask. The rationale for these doses are explained in our recent studies. Cells were released from the plate following treatment using trypsin/EDTA and $5x10^6$ cells were collected for DNA isolation.

DNA extraction and bisulfite conversion

Cells ($5x10^6$) were pelleted at 1000 rpm for 5 min and the supernatant was discarded. Genomic DNA was extracted using the Qiagen's DNeasy blood and tissue kit following the manufacturer's protocol. DNA was eluted in two successive steps to obtain a maximum yield, using 120 µl followed by 40 µl of elusion buffer. Following quantification using a Nanodrop spectrophotometer, 2 µg of the extracted DNA was bisulfite treated using the Zymo Research's EZ-DNA methylation lightning kit following the manufacturer's protocol, and re-quantified.

Target amplification and purification

Bisulfite converted DNA (350 ng) was used to amplify different regions of the p21 promoter. The locus specific primers were designed using Methprimer and were synthesized by Integrated DNA Technologies Inc. (IDT, Coralville, IA). Partial TruSeqHT sequences corresponding to part of the Illumina Read1 (R1) and Illumina Read2 (R2) sequencing primer-binding sites were added 5' to the locus specific primers during primer synthesis. The locus specific primers and the partial TruSeqHT sequences are as given in Table 1. Fusion primers were synthesized by IDT where R1 was fused to forward primers and R2 was fused to reverse primers.

Table 1: TruSeqHT fusion stubs and locus-specific primers.

Locus	Forward Primer (5'→3')	Reverse Primer (5'→3')	T_m (°c)	Product size (bp)
TrueSeq HT Fusion	iTru R1: ACACTCTTTCCCTCACG ACGCTCTTCCGATCT	iTru R2: GTGACTGGAGTTCAGA CGTGTGCTCTTCCGAT CT		
hp21-TSS	ATAGTGTTGTGTTTTTTT GGAGAGTG	ACAACTACTCACACCT CAACTAAC	61.8	350
Hp21-SIE1	TTTTTTGAGTTTTAGTTT TTTTAGTAGTGT	AACCAAAATAATTTTT CAATCCC	61.8	335
Rp21-coding	TGTAATTAGTTATAGGTA TTAGTAGTGT	ACCCCTACAACAAAAC CGAA	54.2	326
rp21-TSS	TTTTTTATTTTTGGTTGT TTTTTTT	ACAAACAATTAACTCT CCTCAAATC	54.2	208

The iTru R1 fusion sequence was synthesized on the 5' end of each of the four forward primers and the iTru R2 fusion sequence was synthesized on the 5' end of each of the four reverse primers.

The first locus amplified was a 350 bp fragment of the human p21 promoter region adjacent to the transcription start site (TSS) termed as hp21-TSS. The second locus was a 335 bp fragment including the transcription factor binding site approximately 700 bp upstream of the TSS called the sis-inducible element (SIE-1) termed hp21-SIE1. The third site was the 250 bp fragment of the rat p21 promoter region near the TSS termed as rp21- TSS, and the fourth locus was the rat p21 coding region approximately 9 Kb downstream of the TSS, termed rp21-coding (Figure 1).

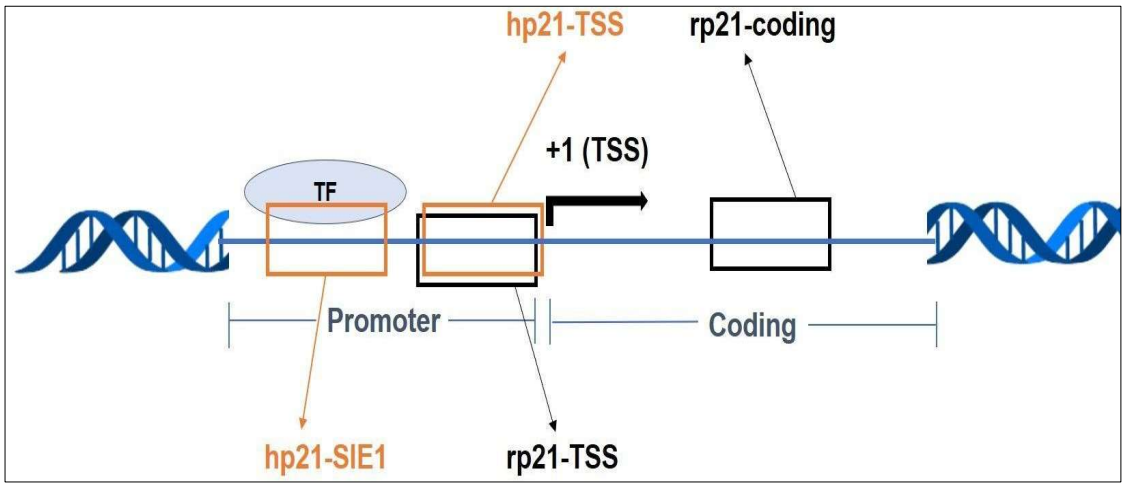

Figure 1: Schematic of p21 gene organization highlighting the loci of interest for DNA methylation analysis

This includes the human p21 promoter region adjacent to the transcription start site (hp21-TSS), the human transcription factor binding site called the sis-inducible element (hp21-SIE1), the rat

p21 promoter region starting near the start site (rp21-TSS) and the rat p21 coding region (rp21-coding).

The 25 µl PCR amplification reaction mix contained 3 mM MgCl2, 1X hot start buffer (Thermo Scientific), 0.2 mM of each deoxynucleoside 5'-triphosphate (dNTP), 0.4 µM each of the forward and reverse primers, 1.5 units HotStart Taq DNA polymerase (Thermo Scientific) and the 350 ng DNA template. PCR was performed under the following conditions: 95°C for 5 min, 40 cycles of 95°C for 30 sec, 61.8°C (hp21-TSS, hp21-SIE1) or 54.2°C (rp21-TSS or rp21-coding) for 45 sec followed by, 72°C for 45 sec and a final 72°C for 10 min. The PCR products were then separated by electrophoresis on a 1% (w/v) agarose gel and visualized with ethidium bromide under a UV trans- illuminator and the amplicons corresponding to the loci were extracted from the gel using Nucleospin gel and PCR clean-up kit (Macherey-Nagel) following the manufacturer's instructions. The sequences of the purified PCR products were confirmed using Sanger sequencing at the Georgia Genomics Facility (GGF) at the University of Georgia. All sequences obtained were verified for locus-specificity using the Basic Local Alignment Search Tool.

Sanger sequencing of bacterial clones

StrataClone PCR cloning kits (Agilent) were used for Sanger bisulfite sequencing. Briefly, 50 ng of gel extracted PCR products were cloned into *Escherichia coli* (*E. coli*) following the manufacturer's instructions. After cloning and plating, about 3 bacterial colonies (white or light blue) were picked and suspension cultures were prepared for plasmid minipreps. Plasmids from the cultures were isolated using Zyppy plasmid miniprep kit (Zymo Research) following the manufacturer's instructions. The plasmid inserts were sequenced at the GGF by Sanger sequencing. The sequences were analyzed using BiQ Analyzer DNA methylation analysis software following software instructions.

Library preparation and next-generation sequencing

Purified PCR amplicons from agarose gel extraction were normalized to 5 ng/µl. A limited cycle PCR was performed to attach the iTru5 and iTru7 primers with eight nucleotide indexes. The 25 µl limited cycle reaction contained 1X Kapa buffer, 0.3 mM of each dNTP, 0.3 µM of each primer, 25 ng template DNA and 0.5 U of HiFi hotstart DNA polymerase (Kapa Biosciences). The reaction conditions were: 98°C for 5 min, 11 cycles of 98°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec and a final 72°C for 1 min. Aliquots (10 µl) from each reaction were pooled together and cleaned up using Thermo Scientific's Sera-Mag magnetic speedbeads. An equal ratio of speedbeads to the sample pool were vortexed and placed on the magnet and incubated at room temperature for 10

min. Once the beads were drawn to the magnet, the supernatant was discarded. The beads were washed with 80% ethyl alcohol twice and the residual liquid was removed by absorption using a wooden toothpick. DNA was then eluted using TLE buffer (10 mM Tris pH 8 & 0.1 mM EDTA) and supernatant collected. The pooled and cleaned sample was then processed for sequencing on an Illumina MiSeq platform as described by Glenn *et al* 2016 using the Illumina's MiSeq 600 cycle v3 kit.

Sequence analysis

Read quality and trimming

The paired end 250-350 bp reads obtained from Illumina MiSeq were demultiplexed using Illumina software bcl2fastq. The sequence reads in fastq format were trimmed for better alignment using Babraham Bioinformatics' free software Trim Galore or Geneious. However, since the sequencing templates included mostly the uniformly sized PCR products, trimming did not affect read alignment (data not shown).

DNA methylation analysis using Bismark

Bismark bisulfite mapper is a Linux based free software from Babraham Bioinformatics Institute. Methylation analysis using Bismark was carried out in three steps listed below.

Genome preparation

A reference genome was prepared where an NCBI genome sequence for the target locus (p21 promoter or coding region) was downloaded as a fasta file. The reference genome was prepared using the following command from the software guide: "/bismark/bismark_genome_preparation --path_to_bowtie /usr/bin/bowtie2/ --verbose /data/genomes/homo_sapiens/GRCh37/". For example: "bismark_genome_preparation -/home/user/DNA/bowtie2-2.3.0/ -verbose/home/user/DNA/bowtie2- 2.3.0/bismark_v0.17.0/REF/", where the reference fasta file was saved in a directory or folder REF in the home folder of the user within the bismark folder. This created two folders within the genome folder REF, one with C ->T genome index and another with G->A for the reverse reads.

Read alignment

The second step was running Bismark using the command from the guide: "bismark –bowtie2 -n 1 -l 50 /data/genomes/homo_sapiens/GRCh37/ test_dataset.fastq". For example, read alignment for sequences in the folder named SampleSeq_R1 with a single-end approach was performed using: "bismark --bowtie2 /home/user/DNA/bowtie2- 2.3.0/bismark_v0.17.0/REF/ SampleSeq_R1.fastq.gz". This aligned the sequence reads to the reference genome and created a combined alignment or methylation call output in a binary representation of sequence alignment

map called BAM format, and yielded a run statistics report. Output files included a bam file and report.txt file (Appendix Datafile 1). The BAM file can only be opened in Bismark.

Methylation extraction

The third and final step was methylation extraction of the bam file generated in the second step. The command used was "bismark_methylation_extractor --gzip test_dataset.fastq_bismark.bam". For example: "bismark_methylation_extractor -s – comprehensive SampleSeq_R1.fastq_bismark_bt2.bam". This generated output files that included M-bias.txt file, M-bias_R1.png file, CpG (Appendix Data file 2-4), CHH and CHG context bt2.txt files, which contain information on strand specific methylation. The key information on CpG site specific percent methylation was obtained from the M-bias.txt file (Appendix Data file 2). The targeted bisulfite sequencing with short products allowed for manual extraction of methylation values for comparison across samples and treatments. A processing report was generated using the command "bismark2report" that summarized the process with a read alignment chart, methylation extraction report and an M-bias plot.

Virtual Box with ready-to-run Bismark package

Virtual Box is an open-source software that runs on various operating systems and supports various guest operating systems. The path to download a ready-to-run Virtual Box package containing all the tools and installations required for DNA methylation analysis of a given fastq sequence file is indicated below. The package includes step-by-step instructions for running Bismark, which is a Linux software, in a Virtual Box on Windows host system.

Samples isolated from a distinct cell passage represented one experiment (n=1). Data are represented as mean ± SEM (standard error of the mean) from at least three separate experiments (n=3). An unpaired Student's t-test was used to compare two groups using Graphpad PRISM considering $p<0.05$ indicative of a statistically significant difference between the mean values.

RESULTS AND DISCUSSION

Sanger's vs next-generation bisulfite sequencing

NRK cells were treated with BrO3-, 5-Aza (positive control), or DMSO for 72 hrs, and extracted DNA was bisulfite converted for rp21-coding region amplification. PCR products (350 bp) were gel purified, cloned, and sequenced using the Sanger method. Sequences were aligned manually or with BiQ Analyzer (Figure 1). Results showed variable methylation among clones, with at least one of three displaying inconsistency, requiring 8–10 clones per treatment for adequate analysis.

To overcome this limitation, the same region was analyzed on the Illumina MiSeq platform using TGBS. Purified PCR products were indexed with iTru5 and iTru7 primers, pooled, and sequenced

using 600 cycle v3 kits, yielding ~10,000 reads per sample. This provided far greater statistical power than Sanger's sequencing and ensured consistency across replicates. Data were processed with Bismark and methylation percentages visualized by heat-maps (Figure 2). Results indicated no significant methylation changes in the coding region following BrO3-, cisplatin, or 5-Aza treatment, suggesting this region is not central to p21 epigenetic regulation. Importantly, NGS provided reproducible results across three independent experiments.

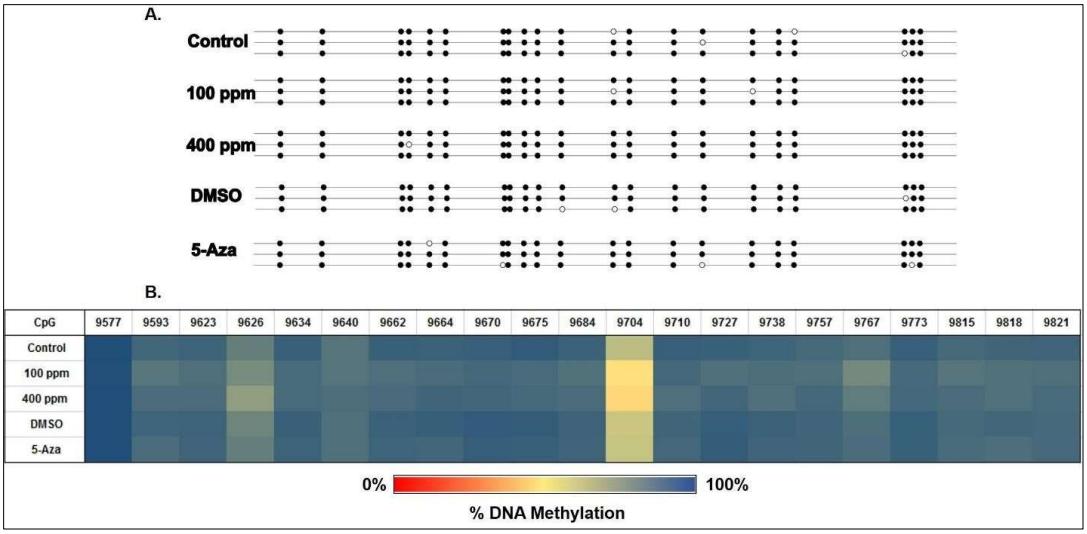

Figure 2: DNA methylation status of the rat p21 coding region

A) Methylation of the rat p21 coding region as determined using Sanger's bisulfite sequencing: Data are shown as a lollipop plot generated using BiQ Analyzer. Each treated group includes three random clones and each line represents sequence from a clone. Black indicates methylated CpGs and the white represents unmethylated CpGs. **B)** Methylation of the rat p21 coding region as determined by TGBS using Illumina next-generation sequencing: Data are represented as a heat-map with average DNA methylation increasing from red (0%) to blue (100%). The position indicates the CpG dinucleotide site in the sequenced fragment.

Differential methylation analysis

Difference in basal DNA methylation of the p21 promoter region between human and rat kidney cells

We used TGBS to assess differences in basal DNA methylation between human and rat p21 promoters isolated from HEK293 and NRK cells. This included analysis of a 350 bp upstream fragment of the human p21 promoter adjacent to the transcription start site (hp21-TSS) and a 250 bp upstream fragment of the rat p21 promoter near the transcription start site (rp21-TSS). The data showed differences in methylation between the two cell lines with an average of 16.4% at the

rp21-TSS site and 0.8% at the hp21- TSS site (Figure 3), suggesting species-dependent differences in basal methylation of the p21 promoter region at the TSS.

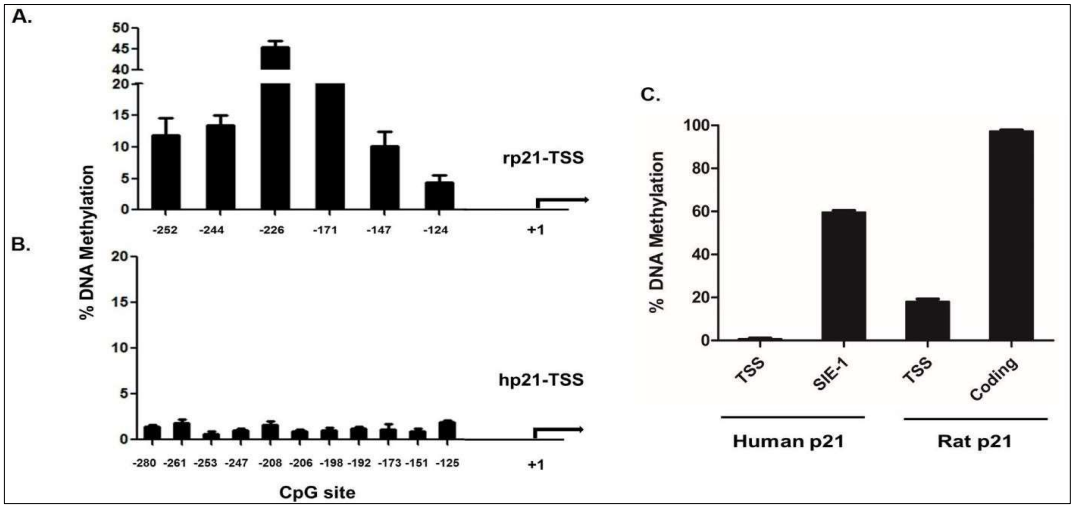

Figure 3: Differential methylation analysis

Comparison of methylation between rat (**A**) and human (**B**) p21 transcription start sites. DNA methylation data are represented as percent methylation of each CpG site in the analyzed fragments of the human and rat p21 promoter regions near the respective transcription start sites (rp21-TSS and hp21-TSS). **(C)** Comparison of methylation in different regions of the rat and human p21 gene.

Regional differences in basal DNA methylation of p21

A differential methylation analysis was performed on p21 DNA isolated from untreated cells. The regions analyzed are as shown in Figure 1. Across the two species, we observed differential methylation across these regions with an average of 0.8% at the hp21-TSS, 57.9% at the transcription factor binding site (SIE-1), 16.1% at the rat-TSS and 95.8% at the rat p21-coding region (Figure 3).

Effect of 5-Aza

We used 5-Aza as a positive control to verify the ability of TGBS to detect changes in DNA methylation. The basal level of methylation in the CpG sites spanning 350 bp adjacent to the human p21 transcription start site (hp21-TSS) showed a low level of total percent methylation (0.9%) in the presence of DMSO, which was similar to the 5-Aza treated cells (Figure 4A). Total percent methylation in this context is the sum of the average methylation of all the CpG sites in the region analyzed. In contrast, treatment of cells with 5-Aza caused about a 35% decrease in total methylation of the SIE-1, as compared to DMSO treated cells (Figure 4B). This correlated to increases in the protein expression of p21 as shown in our previously published.

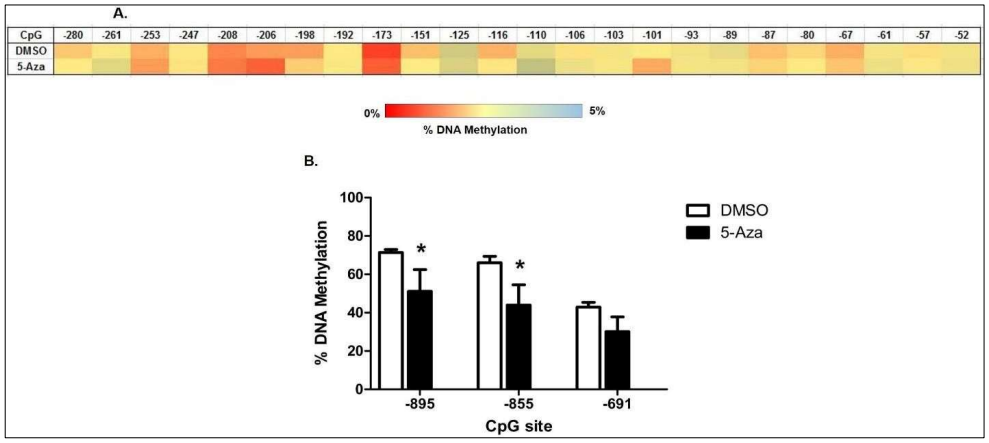

Figure 4: Effect of 5-Aza on DNA methylation of the promoter region of human p21

A) Heat-map of the site-specific percent DNA methylation changes as determined by TGBS in the human p21 promoter region at the transcription start site (hp21-TSS) after 3 days of exposure to DMSO (vehicle control) or 40 µM 5-Aza (positive control). The first row represents the position of the cytosine in the CpG dinucleotide context relative to the TSS. Heat map intensity is showed in the sidebar with deep red indicating percent methylation value towards zero and pale blue indicating relatively higher methylation of 5%. **B)** Effect of 5-Aza on DNA methylation of cytosine residues in the SIE-1 site in human p21 promoter region (hp21-SIE1). Data are represented as the mean ± SEM of three independent experiments (n=3). *P<0.05 compared with 0 ppm BrO_3^-.

Difference in basal DNA methylation of the p21 promoter region between HEK293 cells and freshly isolated human proximal tubule cells

As we observed decreases in the percent DNA methylation at the hp21-SIE1 site after treatment with the demethylating agent 5-Aza, we wanted to investigate the differences in basal level methylation between HEK293 cells and freshly isolated human proximal tubule (hPT) cells at the TSS and sites. The average methylation of hp21-TSS in hPT cells was 1.4% and not significantly different from that in HEK293 cells (Figure 5A). In contrast, the average methylation of all three CpG sties in hPT cells were lower than that measured in HEK293 cells at the hp21-SIE1 site (Figure 5B).

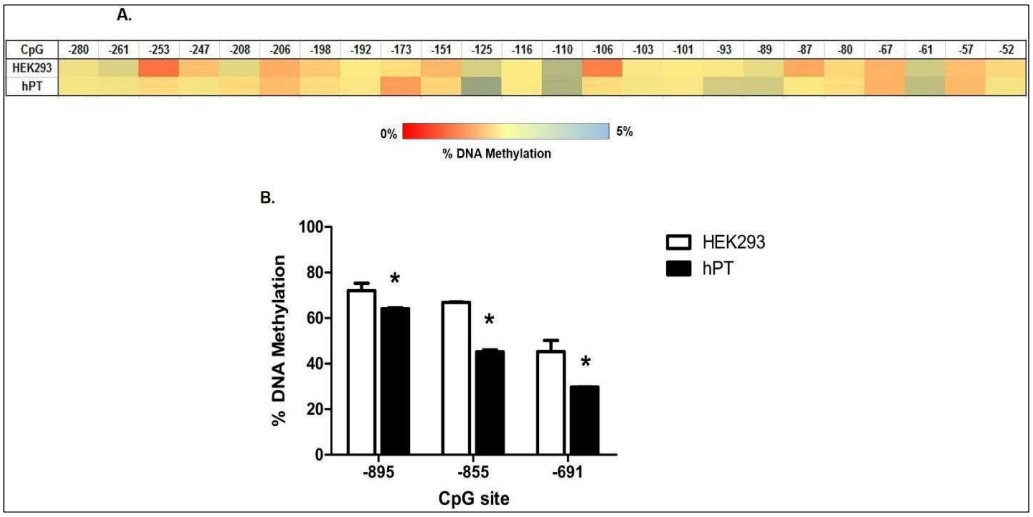

Figure 5: Comparison of basal DNA methylation of the p21 promoter region between HEK293 cells and freshly isolated human proximal tubule (hPT) cells.

A) Heat-map of the site-specific percent DNA methylation changes as determined by TGBS in the human p21 promoter region at the transcription start site (hp21-TSS). Heat map intensity is showed in the sidebar with deep red indicating percent methylation value towards zero and pale blue indicating relatively higher methylation of 5%. **B)** Comparison of methylation of human p21 promoter at the transcription factor binding site SIE-1 between HEK293 and hPT cells. Data are represented as the mean ± SEM of three different passages of HEK293 cells and three different pools of the hPT isolated cells (n=3).

*P<0.05 compared with HEK293.

Effects of nephrotoxicants on HEK293 and NRK cells

The above studies assessed the effect of acutely toxic concentrations of BrO_3^-. Our recent studies demonstrated changes in p21 DNA methylation in the coding region in the presence of low environmentally relevant concentrations of BrO_3^- after sub-chronic exposures. These previous studies did not determine changes in methylation in the promoter region of p21 or differences between rat and human p21 methylation. We addressed this gap-in-knowledge by exposing both NRK and HEK293 cells to BrO_3^- at concentrations we previously demonstrated not to induce cell death. In agreement with this recent study treatment of HEK293 cells with doses of BrO_3^- below 100 ppm did not significantly alter cell morphology or number after 72 hrs of treatment (Figure 6A-F, L). Cells treated with 100 ppm BrO_3^- showed initial signs of cell rounding, detachment and small decreases in cell number compared to the control cells (Figure 6 G and L). In contrast, cisplatin (1 µM), used a positive control, significantly altered cell morphology and cell number

(Figure 6 K-L). Exposure of cells to 5-Aza, as well as to the histone deacetylase inhibitor trichostatin A (TSA), also did not alter cell morphology or number compared to the vehicle control DMSO (Figure 6 H-J, L). Similar results were observed in NRK cells, with the exception that concentrations of 5-Aza of 40 µM did slightly decrease the cell number (Figure 7 A-L).

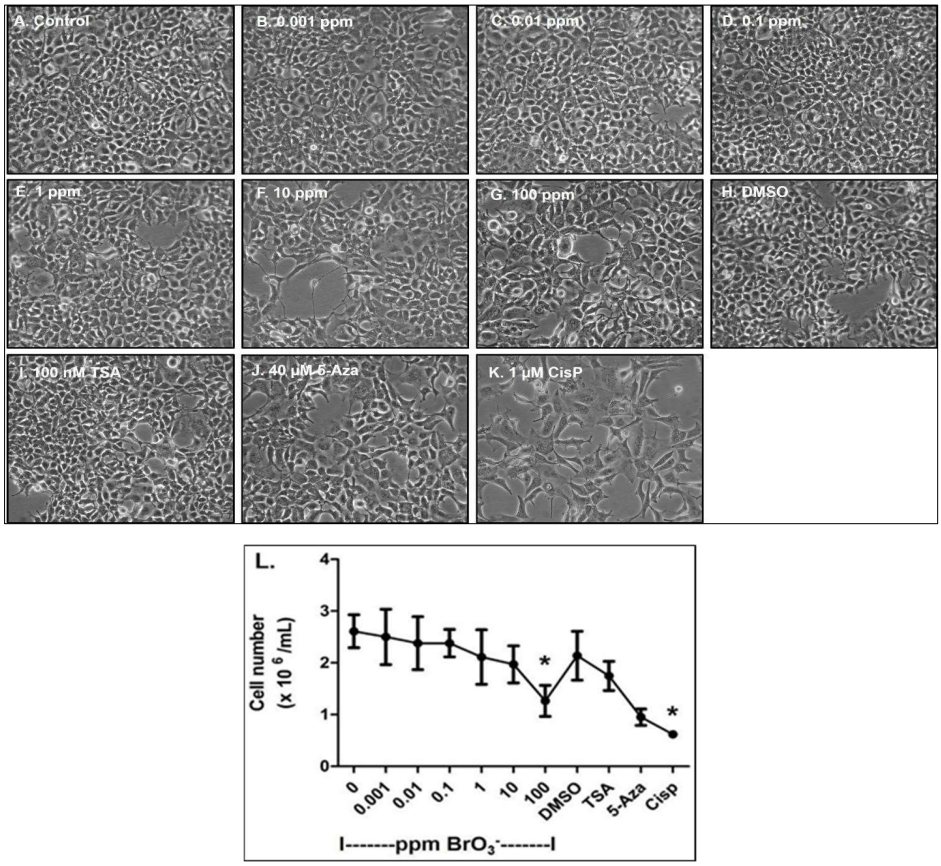

Figure 6: Effect of nephrotoxicants and epigenetic inhibitors on HEK293 cell morphology and number

HEK293 cells were exposed to 0-100 ppm BrO_3^- (A-G), vehicle control DMSO (H), 40 µM 5-Aza (I), 100 nM TSA (J) or 1 µM cisplatin (K) for 72 hrs. The cell number data in L are represented as mean ± SEM of three separate passages (n=3).

*P<0.05 compared with 0 ppm BrO_3^-.

Effects of nephrotoxicants on DNA methylation

Treatment of HEK293 cells with environmentally relevant concentrations of BrO_3^- for 72 hrs did not significantly alter the DNA methylation in the hp21-S1E1 site at any position assessed, which include the CpG cytosines at -691, -855 and -895 bp upstream of the TSS (Figure 8). In contrast, 5-Aza significantly decreased DNA methylation, as compared to its DMSO control, at the CpG sites -895 and -855. We also used TGBS to assess changes in methylation in the CpG sites located in the

human p21 promoter region adjacent to the transcription start site (hp21-TSS) (Appendix Figure 2) and did not detect any changes in methylation induced by BrO_3^- or 5-Aza. It should be noted that we have shown that these same concentrations and exposure periods of BrO_3^- and 5-Aza do increase the protein expression of p21.

Differential methylation data are represented as percent DNA methylation of the transcription start site (TSS), sis-inducible element (SIE-1) and gene coding regions of human and rat p21. Next-generation sequencing data was analyzed using Bismark bisulfite mapper. Data are represented as the mean ± standard error of the mean (SEM) of three independent experiments (n=3).

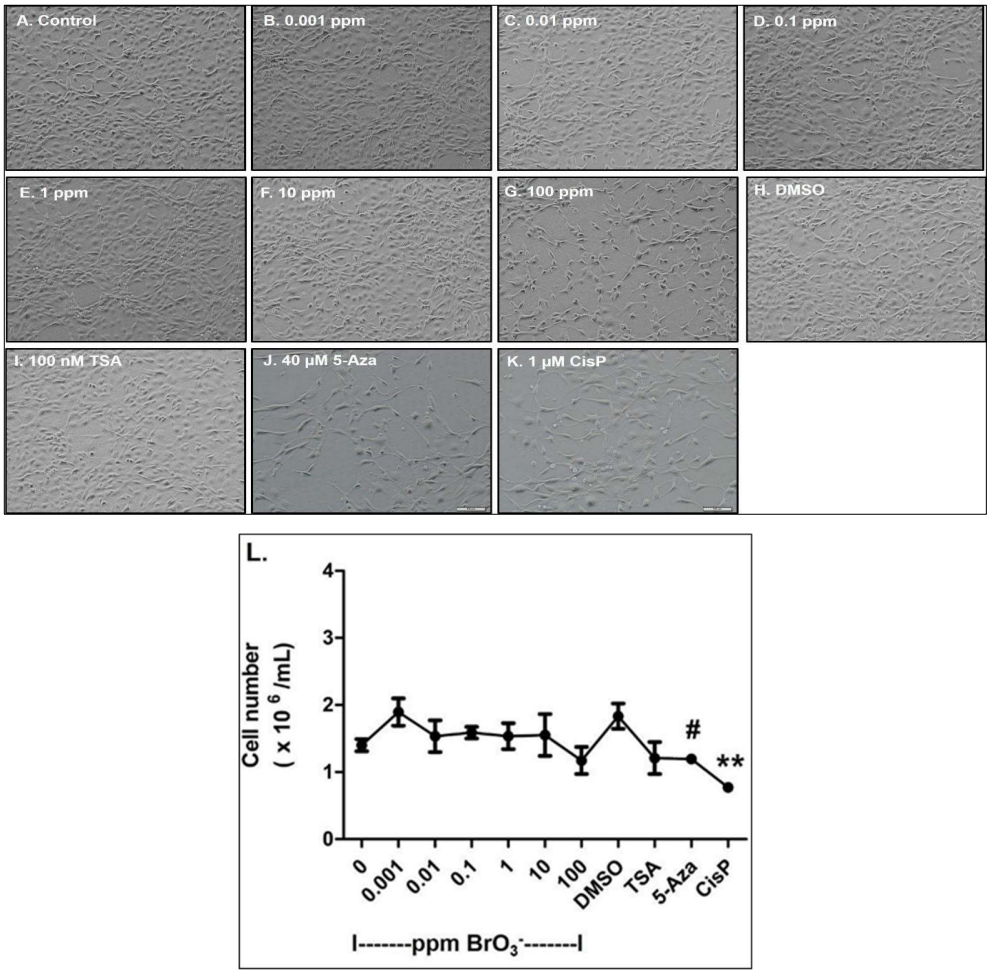

Figure 7: Effect of nephrotoxicants and epigenetic inhibitors on NRK cell morphology and number.

NRK cells were exposed to 0-100 ppm BrO_3^- (A-G), vehicle control DMSO (H), 40 µM 5-Aza (I), 100 nM TSA (J) and 1 µM cisplatin (K) for 72 hrs. The cell number data in **L** are represented as mean ± SEM of three separate passages (n=3). *P<0.05 compared with 0 ppm BrO_3^- and $^{\#}$P<0.05 compared with DMSO.

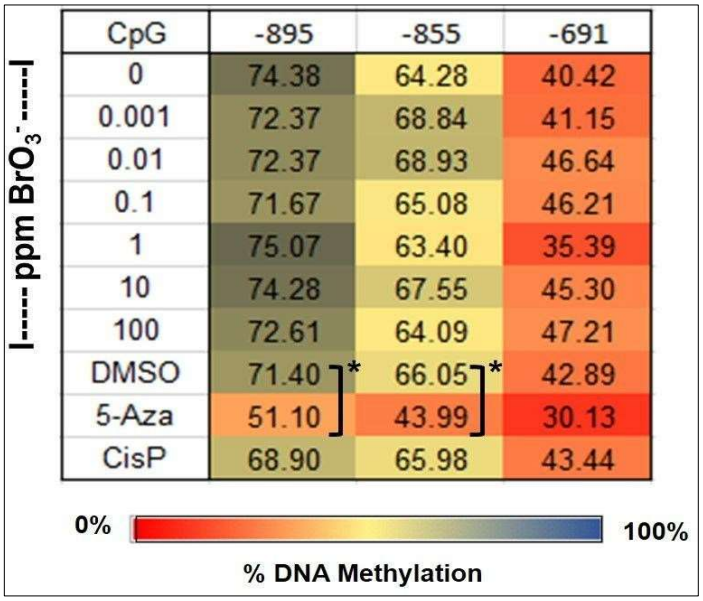

Figure 8: Effects of the nephrotoxicants $BrO3^-$ and cisplatin on the percent DNA methylation of cytosines in the SIE-1 site in human p21 promoter.

HEK293 cells were treated with water (vehicle control for $BrO3^-$ and cisplatin), 0.001 to 100 ppm $BrO3^-$, 1 µM cisplatin, DMSO (vehicle control for 5-Aza) or 40 µM 5-Aza (positive control) for 72 hrs. The first row represents the position of the cytosine in the CpG dinucleotide context relative to the TSS. Heat map intensity is showed in the sidebar with deep red indicating percent methylation value towards zero and deep blue indicating towards 100%. Data are presented as the average percent DNA methylation of three separate passages (n=3).

*P<0.05 compared with compared with DMSO.

DISCUSSION

This study explored whether nephrotoxicants induce renal protective p21 expression through DNA methylation changes in its promoter region and compared the epigenetic regulation of human and rat p21. Targeted gene bisulfite sequencing (TGBS) with Illumina MiSeq and Bismark was used to analyze CpG methylation patterns across species and cell types. Results showed that the DNA methyltransferase inhibitor 5-Aza altered methylation at the SIE-1 site in the human p21 promoter, but nephrotoxicants such as cisplatin and bromate did not significantly affect methylation, even though they increased p21 protein expression. This suggests that p21 induction by these agents occurs through mechanisms independent of promoter methylation. Importantly, the study found lower basal methylation in the human p21 promoter compared to rat, highlighting species-specific epigenetic differences and cautioning against direct extrapolation of rat data to humans. While TGBS proved valuable for cost-effective, site-specific methylation analysis, its scope was limited

to ~1,000 bp upstream of the transcription start site, excluding more distant regulatory regions. Overall, the findings demonstrate that nephrotoxicant-induced p21 expression is not mediated by CpG methylation at the analyzed sites, while underscoring the utility of TGBS and the significance of interspecies epigenetic differences in protective gene regulation.

CONCLUSION

This study demonstrates that nephrotoxicant-induced p21 expression is not mediated by CpG methylation changes in the analyzed promoter regions, as only 5-Aza treatment altered methylation at the human SIE-1 site, while cisplatin and bromate did not. Importantly, basal promoter methylation differed significantly between human and rat p21, underscoring species-specific epigenetic regulation and caution in extrapolating data across models. Targeted gene bisulfite sequencing (TGBS) proved to be a robust, cost-effective method for site-specific DNA methylation analysis, offering advantages over conventional approaches. These findings highlight the need for further exploration of distal regulatory regions and emphasize the importance of epigenetic context in understanding renal protective mechanisms of p21.

REFERENCES

1. Allan, L. A., Duhig, T., Read, M., & Fried, M. (2000). The p21(WAF1/CIP1) promoter is methylated in Rat-1 cells: stable restoration of p53-dependent p21(WAF1/CIP1) expression after transfection of a genomic clone containing the p21(WAF1/CIP1) gene. *Mol Cell Biol, 20*(4), 1291-1298.
2. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol, 215*(3), 403-410. doi:10.1016/S0022-2836(05)80360-2
3. Alyea, R. A., Gollapudi, B. B., & Rasoulpour, R. J. (2014). Are we ready to consider transgenerational epigenetic effects in human health risk assessment? Environ Mol Mutagen, 55(3), 292-298. doi:10.1002/em.21831
4. Analyzer, B. BiQ Analyzer - a software tool for DNA methylation analysis.
5. Anway, M. D., Cupp, A. S., Uzumcu, M., & Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science, 308(5727), 1466-1469. doi:10.1126/science.1108190
6. Archer, S. Y., & Hodin, R. A. (1999). Histone acetylation and cancer. Curr Opin Genet Dev, 9(2), 171-174.
7. Avissar-Whiting, M., Veiga, K. R., Uhl, K. M., Maccani, M. A., Gagne, L. A., Moen, E. L., & Marsit, C. J. (2010). Bisphenol A exposure leads to specific microRNA alterations in placental cells. Reprod Toxicol, 29(4), 401-406. doi:10.1016/j.reprotox.2010.04.004

8. Bannister, A. J., & Kouzarides, T. (2011). Regulation of chromatin by histone modifications. Cell Res, 21(3), 381-395. doi:10.1038/cr.2011.22
9. Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C., & Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature, 410(6824), 120-124. doi:10.1038/35065138.
10. Baylin, S. B., & Ohm, J. E. (2006). Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? Nat Rev Cancer, 6(2), 107-116. doi:10.1038/nrc1799
11. Baynes, R. E. (2012). Quantitative risk assessment methods for cancer and noncancer effects. Prog Mol Biol Transl Sci, 112, 259-283. doi:10.1016/B978-0-12-415813-9.00009-X bcl2fastq2. (2013). bcl2fastq2 Conversion Software v2.19.1.
12. Belinsky, S. A. (2005). Silencing of genes by promoter hypermethylation: key event in rodent and human lung cancer. Carcinogenesis, 26(9), 1481-1487. doi:10.1093/carcin/bgi020
13. Berger, J., & Currie, P. D. (2012). Zebrafish models flex their muscles to shed light on muscular dystrophies. Dis Model Mech, 5(6), 726-732. doi:10.1242/dmm.010082
14. Beyersmann, D. (2002). Effects of carcinogenic metals on gene expression. Toxicol Lett, 127(1-3), 63-68.
15. Bhasin, M., Reinherz, E. L., & Reche, P. A. (2006). Recognition and classification of histones using support vector machine. J Comput Biol, 13(1), 102-112. doi:10.1089/cmb.2006.13.102
16. Bismark. (2017). Babraham Bioinformatics - Bismark Bisulfite Read Mapper and Methylation Caller.
17. Blomen, V. A., & Boonstra, J. (2011). Stable transmission of reversible modifications: maintenance of epigenetic information through the cell cycle. Cell Mol Life Sci, 68(1), 27-44. doi:10.1007/s00018-010-0505-5
18. Bock, C., Reither, S., Mikeska, T., Paulsen, M., Walter, J., & Lengauer, T. (2005). BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. Bioinformatics, 21(21), 4067-4068. doi:10.1093/bioinformatics/bti652
19. Bott, S. R., Arya, M., Kirby, R. S., & Williamson, M. (2005). p21WAF1/CIP1 gene is inactivated in metastatic prostatic cancer cell lines by promoter methylation. Prostate Cancer Prostatic Dis, 8(4), 321-326. doi:10.1038/sj.pcan.4500822
20. Broday, L., Lee, Y. W., & Costa, M. (1999). 5-azacytidine induces transgene silencing by DNA methylation in Chinese hamster cells. Mol Cell Biol, 19(4), 3198-3204.

21. Bull, R. J., & Cottruvo, J. A. (2006). Research strategy for developing key information on bromate's mode of action. Toxicology, 221(2-3), 135-144.
22. Burgess, A. J., Pavey, S., Warrener, R., Hunter, L. J., Piva, T. J., Musgrove, E. A., . . . Gabrielli, B. G. (2001). Up-regulation of p21(WAF1/CIP1) by histone deacetylase inhibitors reduces their cytotoxicity. Mol Pharmacol, 60(4), 828-837.
23. Cantor, K. P. (1997). Drinking water and cancer. Cancer Causes Control, 8(3), 292-308.
24. Carthew, R. W., & Sontheimer, E. J. (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell, 136(4), 642-655. doi:10.1016/j.cell.2009.01.035
25. Carvan, M. J., 3rd, Kalluvila, T. A., Klingler, R. H., Larson, J. K., Pickens, M., Mora-Zamorano, F. X., Skinner, M. K. (2017). Mercury-induced epigenetic transgenerational inheritance of abnormal neurobehavior is correlated with sperm epimutations in zebrafish. *PLoS ONE, 12*(5), e0176155. doi:10.1371/journal.pone.0176155
26. Chanda, S., Dasgupta, U. B., Guhamazumder, D., Gupta, M., Chaudhuri, U., Lahiri, S., Chatterjee, D. (2006). DNA hypermethylation of promoter of gene p53 and p16 in arsenic-exposed people with and without malignancy. *Toxicol Sci, 89*(2), 431-437. doi:10.1093/toxsci/kfj030