Bromate-induced Changes in p21 DNA Methylation and Histone Acetylation in Renal Cells

Ramya T. Kolli,*,†,1 Travis C. Glenn,†,‡ Bradley T. Brown,§ Sukhneeraj P. Kaur,* Lillie M. Barnett,*† Lawrence H. Lash,¶ and Brian S. Cummings*,†,2

*Department of Pharmaceutical and Biomedical Sciences; †Interdisciplinary Toxicology Program; ‡Environmental Health Science; §College of Pharmacy, University of Georgia, Athens, Georgia 30602; and ¶Department of Pharmacology, Wayne State University, Detroit, Michigan 48201

1Present address: National Institute of Environmental Health Sciences, Building 101, 111 TW Alexander Drive, Durham, NC 27709.
2To whom correspondence should be addressed at 336 College of Pharmacy South, University of Georgia, Athens, GA 30602. Fax: (706) 542-5358. E-mail: briansc@uga.edu.

ABSTRACT

Bromate (BrO3−) is a water disinfection byproduct (DBP) previously shown to induce nephrotoxicity in vitro and in vivo. We recently showed that inhibitors of DNA methyltransferase 5-aza-2′-deoxycytidine (5-Aza) and histone deacetylase trichostatin A (TSA) increased BrO3− nephrotoxicity whereas altering the expression of the cyclin-dependent kinase inhibitor p21. Human embryonic kidney cells (HEK293) and normal rat kidney (NRK) cells were sub-chronically exposed to BrO3− or epigenetic inhibitors for 18 days, followed by 9 days of withdrawal. DNA methylation was studied using a modification of bisulfite amplicon sequencing called targeted gene bisulfite sequencing. Basal promoter methylation in the human p21 promoter region was substantially lower than that of the rat DNA. Furthermore, 5-Aza decreased DNA methylation in HEK293 cells at the sis-inducible element at 3 distinct CpG sites located at 691, 855, and 895 bp upstream of transcription start site (TSS). 5-Aza also decreased methylation at the rat p21 promoter about 250 bp upstream of the p21 TSS. In contrast, sub-chronic BrO3− exposure failed to alter methylation in human or rat renal cells. BrO3− exposure altered histone acetylation in NRK cells at the p21 TSS, but not in HEK293 cells. Interestingly, changes in DNA methylation induced by 5-Aza persisted after its removal; however, TSA- and BrO3−-induced histone hyperacetylation returned to basal levels after 3 days of withdrawal. These data demonstrate novel sites within the p21 gene that are epigenetically regulated and further show that significant differences exist in the epigenetic landscape between rat and human p21, especially with regards to toxicant-induced changes in histone acetylation.

Key words: kidney; nephrotoxicity; epigenetics; p21; chromatin immunoprecipitation; bromate; DNA methylation; next-generation sequencing.
kinase (Zhang et al., 2010, 2011). We have also demonstrated that BrO₃⁻ increases the expression of the cyclin-dependent kinase inhibitor p21, both in vitro and in vivo (Kolissetty, Bull, et al., 2013; Zhang et al., 2010).

p21 regulates cell cycle progression at the G₁ and S phase (Gartel and Radhakrishnan, 2005). p21 activation has been shown to be protective against various nephrotoxins such as cisplatin (di Pietro et al., 2012; Jiang and Dong, 2008; Nowak et al., 2003; Price et al., 2009). Several studies have shown that p21 can be activated by the tumor suppressor p53, and that such activation correlates with cell death (El-Deiry et al., 1994). However, our recent studies demonstrate that exposure of both cells and animals to lower, more environmentally relevant doses of BrO₃⁻ resulted in p21 activation independently of p53 (Kolissetty, Bull, et al., 2013; Kolissetty, Delker, et al., 2013; Scholpa et al., 2014). Hence, this study was focused on understanding the alternative mechanism mediating how BrO₃⁻ activates p21 independently of p53.

As aforementioned, we previously demonstrated, using immunoblot analysis, that sub-chronic exposure of renal cells to doses of BrO₃⁻ as low as 0.01 ppm (MCL) increased p21 protein expression (Scholpa et al., 2014). To our knowledge these were some of the lowest doses ever shown for BrO₃⁻ to induce molecular changes. This same study also showed that BrO₃⁻ exposure altered methylation of the coding region of p21 in correlation with increases in its expression. Although p21 is known to be regulated by epigenetic mechanisms (Bott et al., 2005; Yoon et al., 2012), including DNA methylation and histone modifications, almost all of these data have focused on epigenetic changes of p21 in cancer cells (Bott et al., 2005; Moreira et al., 2009; Teramen et al., 2011; Ying et al., 2004). In contrast, a gap-in-knowledge exists with regard to the epigenetic regulation of p21 by environmental oxidants like BrO₃⁻ or any other DBPs.

Furthermore, evidence supporting a role for epigenetics in the toxicity of BrO₃⁻ is the fact that both the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) and the DNA methyltransferase (DNMT) inhibitor 5-aza-2’-deoxycytidine (5-Aza) increased BrO₃⁻-induced cytotoxicity when compared with BrO₃⁻ or TSA and 5-Aza alone (Scholpa et al., 2014). The increase in toxicity correlated with decreased p21. Although changes in p21 expression correlated with changes in p21 DNA methylation, the specific CpG sites altered were not identified. We addressed this limitation in this study using a modification of the bisulfite amplicon sequencing (BSAS) methylation analysis approach called targeted gene bisulfite sequencing (TGBS).

Next-generation sequencing methods are more frequently used to assess methylation of whole-genomes, reduced representation of genomes, and target capture of many loci. Although these methods create a significant amount of data they can be somewhat complex, expensive and are not yet as amicable to studies testing multiple exposure and doses of toxicants. Thus, simple, flexible, and low-cost methods are needed to leverage NGS for sequencing single-locus amplicons from large numbers of samples. We developed TGBS as a 2-stage PCR approach for rapid analysis of DNA methylation of targeted loci. TGBS differs from most of the current next-generation bisulfite sequencing approaches, including reduced representation bisulfite sequencing and BSAS, which typically perform whole-genome or target-gene fragmentation during library preparation (Kruger et al., 2012; Masser et al., 2013; Meissner et al., 2005). These approaches have limitations in terms of inconsistent fragment lengths, complicated sequence mapping and methylation analysis. TGBS does not rely on fragmentation and because each amplicon is its own read, the library preparation and data analysis are comparatively simplified. We recently demonstrated the ability of TGBS to assess p21 DNA methylation in renal cells in response to 5-Aza (Scholpa et al., 2016).

In addition to DNA methylation, histone acetylation/decetylation is also believed to play a key role in p21 regulation in cancer cells (Shin et al., 2000; Sowa et al., 1997). For example, treatment of human colon cancer cells with HDAC inhibitors TSA or sodium butyrate increased the transcription of p21 by inducing the acetylation of histones H4 and H3 (Fang et al., 2004). We recently showed that TSA increases p21 protein expression in renal cells (Scholpa et al., 2014) by increasing the acetylation of lysine 9 and 14 on histone H3 (H3K9/14 Ac) (Scholpa et al., 2016). However, the effects of BrO₃⁻ on H3K9/14 Ac levels at the p21 promoter region are not known. We also showed the DNMT inhibitor 5-Aza increased p21 expression in rat and human renal cells (Scholpa et al., 2014), but similar to our studies with TSA, the exact site targeted by 5-Aza are not known. In this study, we address this gap-in-knowledge using TGBS and show that substantial differences exist in the basal methylation of the promoter regions of rat and human p21. We then determined the effect of BrO₃⁻ on this methylation. We also identified changes in H3K9/14 Ac levels after sub-chronic exposure of HEK293 and normal rat kidney (NRK) cells to low doses of BrO₃⁻ using chromatin immunoprecipitation (ChIP). Data derived from these studies are critical for understanding the role of epigenetics in the toxicity of BrO₃⁻ and in understanding the molecular mechanism by which nephrotoxins and epigenetics regulate p21, a critical nephroprotective gene.

MATERIALS AND METHODS

Normal rat kidney cells, human embryonic kidney cells (HEK293), and penicillin and streptomycin were purchased from American Type Culture Collection (Manassas, Virginia). Human proximal tubule (hPT) cells were derived from whole, de-identified human kidneys that were obtained through the International Institute for the Advancement of Medicine (Edison, New Jersey). All tissue was scored by a pathologist as normal (ie, derived from noncancerous, nondiseased tissue). Potassium bromate (KBrO₃), 5-Aza, TSA, trypsin EDTA, glycine, and 37 wt.% formaldehyde were purchased from Sigma-Aldrich (St. Louis, Missouri), Dulbecco’s modified Eagle medium (DMEM) was purchased from HyClone technologies (Logan, Utah), 5-Aza was dissolved in dimethyl sulfoxide (DMSO) from Fisher Scientific (Pittsburg, Pennsylvania). DNasey blood and tissue extraction kit was purchased from Qiagen (Valencia, California). The EZ-DNA methylation lightning kit was purchased from Zymo research (Irvine, California). The nucleoSpin gel and PCR clean-up kit was purchased from Macherey-Nagel (Düren, Germany). The EpiQuik acetyl-histone H3 ChIP kit was purchased from Epigentek (Farmingdale, New York). The PCR master mix was purchased from Promega (Madison, Wisconsin) for ChIP-PCR. The MiSeq reagent v3 kit was purchased from Illumina, Inc. (San Diego, California), the Kapa HiFi PCR kit from Kapa Biosystems (Wilmington, Massachusetts), and the Maxima hot-start taq DNA polymerase for bisulfite-PCR and the Sera-Mag magnetic speedbeads were purchased from Thermo Scientific (Waltham, Massachusetts).

Cell Culture and Sub-chronic Treatment

5-Aza and TSA were used as positive controls. 5-Aza is a DNA methyltransferase inhibitor used in many studies for its demethylating properties (Bott et al., 2005; Broday et al., 1999; Christman, 2002; Shin et al., 2000). TSA is a HDAC inhibitor and

Vol. 168, No. 2, 2018
used widely to study anticancer and anti-inflammatory mechanisms (Adcock, 2007; Drummond et al., 2005; Vanhaecke et al., 2004). HEK293 cells (3 × 10⁶) were seeded in T-175 tissue culture flasks and NRK cells (1.5 × 10⁶) were seeded in T-75 flasks and grown at 37°C in a 5% CO₂ incubator. Cells were treated with 0–100 ppm bromate (BrO₃⁻), 40 μM 5-Aza, 100 nM TSA, or 0.05% DMSO (vehicle control for TSA and 5-Aza) for 72 h at log phase (after 24 h of seeding). A portion of the cells was used for reseeding and the rest for sample collection for targeted gene bisulfite sequencing and chromatin immunoprecipitation. This was called passage 1 (P1) and the regimen continued until 18 days (P6). Cells were allowed to recover by discontinuing the exposures for the next 3 passages (Days 21–27 or P7–P9) to obtain withdrawal samples.

Figure 1. Sub-chronic dosing regimen for BrO₃⁻ and epigenetic inhibitors. Cells were exposed to various concentrations of BrO₃⁻ at log phase (after 24 h of seeding) for 72 h. A portion of the cells was used for reseeding and the rest for sample collection for targeted gene bisulfite sequencing and chromatin immunoprecipitation. This was called passage 1 (P1) and the regimen continued until 18 days (P6). Cells were allowed to recover by discontinuing the exposures for the next 3 passages (Days 21–27 or P7–P9) to obtain withdrawal samples.

The DNA methylation of p21 promoter and coding regions in HEK293, NRK, and hPT cells was analyzed using TGBS, which is a modification of BSAS. Cells (5 × 10⁶) 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 2 successive steps to obtain a maximum yield, using 120 μl followed by 40 μl of elution 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.

Bisulfite converted DNA (350 ng) was used to amplify different regions of the p21 promoter. The locus-specific primers
were designed using Methprimer (Li and Dahiya, 2002) and were synthesized by Integrated DNA Technologies, Inc (IDT, Coralville, Iowa). Partial TruSeqHT sequences corresponding to part of the Illumina Read1 (R1) and Illumina Read2 (R2) sequencing primer-binding sites (BadDNA, 2017; Faircloth and Glenn, 2012; Glenn et al., 2016) were added 5’0 to the locus-specific primers during primer synthesis. The locus-specific primers and the partial TruSeqHT sequences are listed in Table 1. Fusion primers were synthesized by IDT, where R1 was fused to forward primers and R2 was fused to reverse primers. For example, the primer pair for human p21 TSS (hp21-TSS) was: forward: “iTru R1:ACACTCTTTCCCTACACGACGCTCTTCCGATCT ATAGTGTTGTGTTTTTTTGGAGAGTG” and reverse: “iTru R2:GTGACTGGAGTTCCAGACGCTCTTCCGATCTGCTCTTCCGATCT AACTCTACACCTCAAATACTCAACTACCTCAACTAAC.

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) and was termed hp21-SIE1. The third site was the 208 bp fragment of the rat p21 promoter region near the TSS termed as rp21-TSS, and the 4th locus was the 326 bp rat p21 coding region approximately 9 kb downstream of the TSS, termed rp21-coding. The schematic of these regions are shown in Figure 2A.

The 25 ml 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 s, 61.8°C (hp21-TSS or hp21-SIE1), or 54.2°C (rp21-TSS or rp21-coding) for 45 s followed by, 72°C for 45 s 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

Table 1. TruSeqHT Fusion Stubs and Locus-specific Primers for TGBS

<table>
<thead>
<tr>
<th>Locus</th>
<th>Forward Primer (5’—3’)</th>
<th>Reverse Primer (5’—3’)</th>
<th>Tm,°C</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TruSeqHT fusion</td>
<td>iTru R1:ACACTCTTTCCCTACACGACGCTCTTCCGATCT</td>
<td>iTru R2:GTGACTGGAGTTCCAGACGCTCTTCCGATCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hp21-TSSb</td>
<td>ATAGTGTTGTGTTTTTTTGGAGAGTG</td>
<td>ACAACTCTACACCTCAACCTCAACTA</td>
<td>61.8</td>
<td>350</td>
</tr>
<tr>
<td>hp21-SIE1c</td>
<td>TTTTTTGGAGTTTTTTTGGAGAGTG</td>
<td>AAACAAAATATTTTTCTCATCTCC</td>
<td>61.8</td>
<td>355</td>
</tr>
<tr>
<td>rp21-codingd</td>
<td>TTTTTTGGAGTTTTTTTGGAGAGTG</td>
<td>ACCCTACACAAACCTCAACTA</td>
<td>54.2</td>
<td>326</td>
</tr>
<tr>
<td>rp21-TSSE</td>
<td>TTTTTTGGAGTTTTTTTGGAGAGTG</td>
<td>ACGAAATATACCTCTCCTCAAA</td>
<td>54.2</td>
<td>208</td>
</tr>
</tbody>
</table>

The iTru R1 or iTru5 fusion sequence was synthesized on the 5’ end of each of the 4 forward primers and the iTru R2 or iTru7 fusion sequence was synthesized on the 5’end of each of the 4 reverse primers.

aAnnealing temperature.
bHuman p21 transcription start site.
cHuman p21 sis-inducible element-1.
dRat p21 coding region.
eRat p21 transcription start site.

Figure 2. Schematic of p21 gene organization highlighting the loci of interest for (A) DNA methylation analysis: 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). (B) Histone acetylation analysis: the human p21 promoter region (hp21-ChIP) about 1000 bp upstream of the transcription start site and rat p21 promoter region (rp21-ChIP) at the TSS.
the 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 and Bioinformatics Core at the University of Georgia. All sequences obtained were verified for locus-specificity using the Basic Local Alignment Search Tool (Altschul et al., 1990).

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 8 nucleotide indexes, as represented in Supplementary Figure 1. The 15 µl limited cycle reaction contained 1X Kapa buffer, 0.3 mM of each dNTP, 0.3 µM of each primer, 15 ng template DNA, and 0.3 U of HiFi HotStart DNA polymerase (Kapa Biosciences). The reaction conditions were: 98°C for 5 min, 11 cycles of 98°C for 15 s, 60°C for 30 s, and 72°C for 30 s 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. The pool of sequences was prepared for Illumina MiSeq platform using MiSeq 600 cycle v3 kit as described by Glenn et al. (2016).

**Sequence Analysis**

Read quality and trimming. The paired end 250–350 bp reads obtained from Illumina MiSeq were demultiplexed using Illumina software bcl2fastq (Bcl2fastq2, 2013). The sequence reads in fastq format were trimmed for better alignment using Babraham Bioinformatics’ free software Trim Galore (Trim Galore, 2012) or Geneious (Geneious, 2017; Kearse et al., 2012). However, because the sequencing templates included mostly the uniformly sized gel extracted 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 (Krueger and Andrews, 2011). Methylation analysis using Bismark was carried out in 3 steps:

- **Genome preparation:** A reference genome was prepared where a genome sequence from the National Center for Biotechnology Information (NCBI) for the target locus (p21 promoter or coding region) was downloaded as a fasta file. The reference genome was prepared using the following command: “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 2 folders within the genome folder REF, 1 with C -> T genome index and another with G -> A for the reverse reads.

- **Read alignment:** The second step was running bowtie2 within Bismark using the command: “bismark –bowtie2/home/user/ DNA/bowtie2-2.3.0/bismark, v0.17.0/REF/SampleSeq_R1.fastq.gz,” where read alignment for sequences in the folder named SampleSeq_R1 with a single-end approach was performed. 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 and yielded a run statistics report. Output files included a bam file and report.txt file (Supplementary 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 –s –comprehensive SampleSeq_R1.fastq_bismark_b2t.bam.” This generated output files that included M-bias.txt file, M-bias.R1.png file, CpG (Supplementary Datafiles 2–4), CHH and CHG context b2t.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 (Supplementary Datafile 2). The targeted bisulfite sequencing with short products allowed for manual extraction of methylation values for comparison across samples. 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.

**VirtualBox With Ready-to-run Bismark Package**

VirtualBox is an open source software that runs on various operating systems and supports various guest operating systems (VirtualBox, 2017). The path to download a ready-to-run VirtualBox package containing all the tools and installations required for DNA methylation analysis of a given fastq sequence file is indicated below. The package includes a working Bismark, which is a Linux software, in a VirtualBox on Windows host system. This can be found at “http://toxicology.uga.edu/resources/dna_methylation_analysis/” for the VirtualMachine named “TGBS” that can be accessed with the username “user” and password “TGBSKoli.” The instructions are detailed in the Supplementary File.

**Chromatin Immunoprecipitation**

The effect of BrO₃⁻ on p21 histone acetylation was investigated using Epigentek’s EpiQuik acetyl-histone H3 ChIP kit based on the manufacturer’s protocol with conditions optimized for HEK293 and NRK cells (Supplementary File). The primer sequences for ChIP-PCR are described in Table 2 and schematically represented in Figure 2B. The p21 promoter regions selected previously showed TSA-induced hyperacetylation of lysine 9 and 14 on histone H3 (H3K9/14 Ac) in human gastric carcinoma

### Table 2. Primer Sequences for ChIP

<table>
<thead>
<tr>
<th>Locus</th>
<th>Forward Primer (5′→3′)</th>
<th>Reverse Primer (5′→3′)</th>
<th>Tₚ * (°C)</th>
<th>Product Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rp21-ChIP</td>
<td>GTTCCAGCCCTGGAACCGAAG</td>
<td>GTACCAAACACCCTTCACCTGGTAC</td>
<td>59</td>
<td>227</td>
<td>Yuan et al. (2013)</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>CACGGCAAGTCTCAAGGGCACGTCG</td>
<td>GTGGAAGACGCTAGATATTGACGAC</td>
<td>54.2</td>
<td>150</td>
<td>—</td>
</tr>
<tr>
<td>rGAPDH</td>
<td>GGGGCCCTTCTGGAATTTGG</td>
<td>CTGGCAGGCAAGGATTACC</td>
<td>54.2</td>
<td>116</td>
<td>Mitani et al. (2005)</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>AAGGTCCGAGTCACACGAT</td>
<td>TGGAGATGTGTATGGGATT</td>
<td>54.2</td>
<td>221</td>
<td>—</td>
</tr>
</tbody>
</table>

*a* Annealing temperature

*b* Rat p21 region analyzed using chromatin immunoprecipitation.

*c* Human p21 region analyzed using chromatin immunoprecipitation.

**VirtualBox**

VirtualBox is an open source software that runs on various operating systems and supports various guest operating systems (VirtualBox, 2017). The path to download a ready-to-run VirtualBox package containing all the tools and installations required for DNA methylation analysis of a given fastq sequence file is indicated below. The package includes a working Bismark, which is a Linux software, in a VirtualBox on Windows host system. This can be found at “http://toxicology.uga.edu/resources/dna_methylation_analysis/” for the VirtualMachine named “TGBS” that can be accessed with the username “user” and password “TGBSKoli.” The instructions are detailed in the Supplementary File.

**Chromatin Immunoprecipitation**

The effect of BrO₃⁻ on p21 histone acetylation was investigated using Epigentek’s EpiQuik acetyl-histone H3 ChIP kit based on the manufacturer’s protocol with conditions optimized for HEK293 and NRK cells (Supplementary File). The primer sequences for ChIP-PCR are described in Table 2 and schematically represented in Figure 2B. The p21 promoter regions selected previously showed TSA-induced hyperacetylation of lysine 9 and 14 on histone H3 (H3K9/14 Ac) in human gastric carcinoma.
cell lines (Mitani et al., 2005) and rat mesangial cells (Yuan et al., 2013). The human p21 promoter analyzed was approximately 1000 bp upstream of the TSS (hp21-ChIP) and the rat p21 promoter region at the TSS (rp21-ChIP).

Statistics
Samples isolated from a distinct cell passage represented 1 experiment (n = 1). Data are represented as mean ± SEM (standard error of the mean) from at least 3 separate experiments (n = 3). For cell number and histone acetylation data, an unpaired Student’s t test was used to compare individual dose to the controls (each dose to the control). For the methylation data containing multiple exposures and multiple CpG sites, 2-way analysis of variance was used followed by Bonferroni post hoc test. Statistical analysis was performed using Graphpad PRISM considering p < .05 indicative of a statistically significant difference between the mean values.

RESULTS
Differences in Basal DNA Methylation of the p21 Promoter Region Between Human and Rat Kidney Cells
Prior to determining the effect of BrO₃⁻ on the methylation of p21 we determined differences in basal DNA methylation between human and rat p21 promoters isolated from HEK293 and NRK cells. The sites analyzed included a 350 bp upstream fragment of the human p21 promoter adjacent to the TSS (hp21-TSS) and a 250 bp upstream fragment of the rat p21 promoter near the TSS (rp21-TSS). The basal methylation of promoter CpG sites in the rat p21 TSS was substantially higher than that measured in the human p21 TSS (Figs. 3A and 3B). The average total DNA methylation at CpG sites in the rat p21 TSS was 16.4%, as compared with 0.8% in the human p21 TSS (Figure 3C). In contrast, the average methylation of CpG site in the SIE-1 promoter site in human p21 was almost 60%. The methylation of CpG sites in the rat p21 coding region (rp21-coding) was also assessed as a control (Figure 3C). We have previously used methylation-specific PCR to show that this site was highly methylated (Scholpa et al., 2014). As expected, the average methylation at this site was almost 100%.

Regional Differences in Basal DNA Methylation of p21 Between HEK293 Cells and Freshly Isolated Human Proximal Tubule Cells
Having established differences in the basal methylation between the rat and human p21 promoter regions we expanded our analysis to differences in CpG methylation between renal cells from the same species. The regions analyzed were the human p21 TSS and the SIE-1 site of human p21 promoter (hp21-SIE1) as shown in Figure 2A. We investigated the differences in basal level methylation of these regions between HEK293 cells and freshly isolated hPT. The average methylation of human p21 TSS in hPT cells was 1.4% and was not significantly different from that in HEK293 cells (Figure 4A). In contrast, the average methylation of all 3 CpG sites of the human p21 SIE1 site at 691 and 855 bp upstream of the human p21 TSS in hPT cells were significantly lower than that measured in HEK293 cells (Figure 4B).

Effect of BrO₃⁻ on Cell Number and Morphology
Having established the differences in methylation between rat and human p21, we next investigated the effects of sub-chronic exposure of renal cells to environmentally relevant concentrations of BrO₃⁻ on p21 DNA methylation and histone acetylation. Our previous study (Scholpa et al., 2014) demonstrated concentrations of BrO₃⁻-induced p21 expression as early as 3 days of exposure at all doses tested including 0.01 ppm. In this sub-chronic study, we hypothesized that these changes in p21 expression are a result of epigenetic mechanisms. To test the general premise of stability of these epigenetic changes, we
introduced an extra 9 days of withdrawal studies where the cells did not receive doses of BrO$_3^-$ or epigenetic inhibitors.

In agreement with our previous study (Scholpa et al., 2014), treatment of HEK293 cells with doses of BrO$_3^-$ below 100 ppm did not significantly alter cell morphology or number after 18 days of treatment (Figs. 5A–F and 5J). We also did not observe significant alterations in cell number or morphology of the cells after 9 days of withdrawal (Figs. 6A–F and 6J). Similar results were seen with NRK cells (Supplementary Figs. 2 and 3). In our acute study, cells treated with 100 ppm BrO$_3^-$ showed initial signs of cell rounding, detachment and small decreases in cell number compared with the control cells after 3 days (data not shown). We could not study the sub-chronic effect of 100 ppm BrO$_3^-$ past 6 days (P2) in HEK293 cells and past 9 days (P3) in NRK cells due to further toxicity that correlated with an immense decrease in cell number. These data agree with our previous studies that the lower doses of BrO$_3^-$ used in this study do not induce detectable levels of cell death (Scholpa et al., 2014).

Exposure of HEK293 cells to 40 μM 5-Aza altered cell morphology and cell number after 3 days, a trend that continued through the 18 days of exposure (Figs. 5I and 5J). This morphology and number recovered after 9 days of withdrawal but were still significantly different from the DMSO withdrawal cells (Figs. 6I and 6J). Exposure of cells to 100 nM TSA did not alter cell morphology or number after 18 days of sub-chronic treatment. Similar results were observed in NRK cells with BrO$_3^-$ and the epigenetic inhibitors (Supplementary Figure 2).

**Effect of BrO$_3^-$** on p21 Promoter Methylation

Our previous studies (Scholpa et al., 2014) demonstrated changes in p21 methylation in the coding regions that correlated with sub-chronic exposure to environmentally relevant concentrations of BrO$_3^-$ at concentrations that did not induce detectable cell death. The regions analyzed were the same 350 bp fragment of the human p21 TSS comprising 24 CpG sites, a 335 bp fragment including the human p21 SIE1 site (Figs. 7G and 7H). Decreased methylation at these sites in rat p21 TSS after 9 days of exposure and BrO$_3^-$ again did not alter methylation at these same sites in rat p21 coding region at any concentration or exposure. The decrease in DNA methylation induced by 5-Aza was greater after 18 days of exposure at all 3 CpG sites, as compared with 9 days (Figs. 7E and 7F). Similar decrease was observed in NRK cells. 5-Aza decreased DNA methylation at the rat p21 TSS after 9 days of exposure and BrO$_3^-$ again did not alter methylation at these sites in rat p21 coding region after withdrawal (Supplementary Figs. 7C and 7D). These data suggest that the effect of 5-Aza on DNA methylation at these sites had some permanence.

**Effect of BrO$_3^-$** on p21 Promoter Histone Acetylation

In the absence of changes in DNA methylation we investigated the hypothesis that changes in p21 expression induced by BrO$_3^-$ were mediated by histone acetylation. This hypothesis is supported by our previous studies showing that treatment of HEK293 and NRK cells with the HDAC inhibitor TSA upregulated p21 protein expression (Scholpa et al., 2014). We also...
demonstrated that TSA-induced p21 expression correlated with the increased acetylation of lysine 9 and 14 on histone H3 (H3K9/14 Ac) in the p21 promoter region (Scholpa et al., 2016). However, we do not know the effect of short- and long-term exposure of BrO₃⁻ on these cells, nor do we know the persistence of these changes.

Acute exposure (3 days or P1) of renal cells to 0.001–10 ppm BrO₃⁻ and 100 nM TSA did not affect the H3K9/14 Ac levels of the human p21 promoter approximately 1000 bp upstream of the TSS (hp21-ChIP) but did alter acetylation in the rat p21 promoter at the TSS (rp21-ChIP) at concentrations of 0.001 and 1 ppm BrO₃⁻ in a concentration-independent manner (Figure 8A). The sub-chronic effects of BrO₃⁻ and the positive control TSA were assessed at 9 days (P3), 18 days (P6), and 27 days (P9).

Treatment of NRK cells with 0.001–10 ppm BrO₃⁻ or 100 nM TSA significantly increased H3K9/14 Ac levels of rat p21 promoter after 9 days (P3) (Figure 8B). Similar increases were observed after 18 days (P6) of treatment with 0.01–10 ppm BrO₃⁻ (Figure 8C). Withdrawal of BrO₃⁻ for 9 (P9) days resulted in a return of H3K9/14 acetylation to control levels (Figure 8D). This same reversion was seen in cells exposed to TSA. To understand the time-dependence on this reversion, we assessed the recovery of the acetylation mark after 3 days of withdrawal (P7), which was long enough to result in a decrease in acetylation comparable with controls (Supplementary Figure 8). In contrast to NRK cells, H3K9/14 acetylation in HEK293 cells at the promoter site was not altered by BrO₃⁻ or TSA at any concentration and time point measured (Supplementary Figure 9).
DISCUSSION

One of the primary goals of this study was to determine the ability of the DBP BrO$_3^-$ to induce epigenetic changes in the nephroprotective gene p21. Understanding the molecular effect of BrO$_3^-$, especially at the epigenetic level, in renal cells is important as this chemical is a probable human carcinogen (IARC, 1999), and is reported to induce renal cancer in both rats and mice (Kurokawa et al., 1990; Wolf et al., 1998). Although the ability of DBPs, including BrO$_3^-$, to induce epigenetic changes has been studied previously, such studies use high non-environmentally relevant doses as well as acute exposure protocols. Data in this study demonstrate that BrO$_3^-$ can induce changes in histone acetylation at doses as low as the MCL. These data also suggest that changes in p21 expression in both rat and human cells are not mediated by changes in methylation at the CpG sites in the promoter region of p21. It is possible that changes in DNA methylation may be occurring at sites distal to the TSS.

Data from these studies also identify 3 hitherto unidentified CpG sites in human p21 promoter that are targeted by DNA methyltransferase. As previously reported, 5-Aza, an inhibitor of DNA methyltransferases, induced p21 expression in human renal cells but the exact CpG sites involved were not identified (Scholpa et al., 2014). This study showed that 5-Aza significantly decreased percent methylation at the human p21 SIE1 site in HEK293 cells, and that this decrease was enhanced after 18 days of sub-chronic exposure. Furthermore, and more importantly, decreased methylation persisted for 9 days after the withdrawal...
Figure 7. Acute and sub-chronic effects of BrO3 on the percent DNA methylation of CpGs in the SIE-1 site of the human p21 promoter. HEK293 cells were treated with water (vehicle control for BrO3), 0.001–100 ppm BrO3, dimethyl sulfoxide (DMSO) (vehicle control for 5-Aza) or 40 μM 5-Aza (positive control) for 3 days or P1 (A and B), 9 days or P3 (C and D), 18 days or P6 (E and F). The cells were allowed to recover by discontinuing the exposures for 9 days or P9 (G and H). The first row in the heat map represents the position of the cytosine in the CpG dinucleotide context relative to the transcription start site. Data are represented as the average percent DNA methylation of 3 separate passages (n = 3) as determined by targeted gene bisulfite sequencing analysis. The effects of 5-Aza are emphasized in the bar graphs for the respective exposure times. Data are represented as the mean ± SEM of 3 different passages (n = 3). *p < .05 compared with DMSO.
of 5-Aza, which involved 3 passages of the cells. This supports the conclusion that changes in methylation at the CpG sites identified are true epigenetic changes. These CpG sites encompass an SIE-1 site. This site is recognized by members of the signal transducer and activator of transcription family. The binding of STAT1 protein to SIE-1 has also been shown to upregulate \( p21 \) expression (Chin et al., 1996). Our data suggest that the SIE-1 is an important region for epigenetic regulation of \( p21 \) expression. However, our data also suggest that BrO\(_3^-\)/CO\(_2\) itself does not target this region.

It is important to point out that even though BrO\(_3^-\)/CO\(_2\) failed to alter DNA methylation within the promoter region of either HEK293 or NRK \( p21 \) does not suggest that DNA methylation is not involved in the regulation of \( p21 \) by BrO\(_3^-\)/CO\(_2\). Rather, it suggests that changes in DNA methylation at these specific sites are not involved. It is quite possible that changes in methylation at sites distant from the promoter region mediate \( p21 \) expression. Our choice for the sites analyzed with TGBS is obvious as these sites are within the promoter region. Future studies are needed to address the above hypothesis.

In contrast to methylation, BrO\(_3^-\) clearly altered histone acetylation. These data agree with that from Fang et al. (2004) who previously reported that histone acetylation regulates \( p21 \) expression in many cancer cell lines. Shin et al. (2000) also showed that \( p21 \) promoter region was not methylated in gastric cancer cells but was regulated by histone deacetylase 1 (Shin et al., 2000). Various studies, including ours (Scholpa et al., 2014), reported that inhibition of HDAC increases \( p21 \) expression, including that induced by azelaic bishydroxamic acid (Burgess et al., 2001), n-butylrate (Dagtas et al., 2009), suberoylanilide hydroxamic acid (Gui et al., 2004; Richon et al., 2000), and statins (Lin et al., 2008). The novelty in this study is that this is one of the first report to demonstrate that BrO\(_3^-\) exposure increases H3K9/14 acetylation. Furthermore, this study is the first to identify the location of this acetylation. Of further interest is that changes in acetylation occurred at doses as low as the EPA limit (0.01 ppm). The fact that changes in acetylation occurred in the absence of cell death, and that these changes were not persistent supports the hypothesis that induction of \( p21 \) after exposure to BrO\(_3^-\) is a protective rather than a toxic response. Further strengthening our previous observation that the role of p53 is key to the difference in the protective effect and the toxic effect of \( p21 \). At environmentally relevant doses \( p21 \) is activated independently of p53, at higher acutely toxic doses p53 is activated (Scholpa et al., 2014). Another interesting finding from these studies is that BrO\(_3^-\) did not alter H3K9/14 acetylation in HEK293 cells. Data on the effect of both TSA and BrO\(_3^-\) on acetylation of H3K9/14 further support the hypothesis that epigenetic regulation of \( p21 \) is species-dependent.

It should be noted that histones are modified by marks like lysine methylation, arginine methylation and citrullination, serine, tyrosine or threonine phosphorylation, and that we have only assessed lysine acetylation on histone H3. It is possible

**Figure 8.** Sub-chronic effects of BrO\(_3^-\)/CO\(_2\) on acetylation of lysine 9 and 14 on histone H3 (H3K9/14 Ac) of the rat p21 promoter region. Normal rat kidney cells to were exposed to 0.001–100 ppm BrO\(_3^-\) for 3 days or P1 (A), 9 days or P3 (B), and 18 days or P6 (C). The cells were allowed to recover by discontinuing exposures for 9 days or P9 (D). Data are represented as the relative fold increase in H3K9/14 Ac levels as normalized to GAPDH as determined using the chromatin immunoprecipitation assay. Data are represented as the mean ± SEM of 3 different passages (\( n=3 \)). * \( p < 0.05 \) compared with 0 ppm BrO\(_3^-\)/CO\(_2\) and # \( p < 0.05 \) compared with DMSO.
that the lack of histone acetylation in human p21 in response to BrO₃⁻ may be a result of acetylation at different histones within the promoter region, or a different modification at a different location in the noncoding region. Nevertheless, such data would still support the premise that epigenetic regulation of p21 is species-dependent.

A major finding from these studies is that the basal DNA methylation of the promoter region of rat and human p21 are substantially different. This can be highlighted by the fact that the overall methylation of CpG sites in human p21 was about 10-fold lower than that measured in the similar region in rat p21. Furthermore, the promoter region of human p21 also contains a SIE-1 site not present in rat. These data demonstrate that the epigenetic landscape of the promoter region of rat and human p21 are not the same. The implication for this finding is that studies assessing the ability of nephrotoxicants to regulate p21 in rodent renal cells may not be comparable with human renal cells. As such, care should be taken in extrapolating epigenetic data, at least for BrO₃⁻ and p21, between species. Furthermore, this suggests that studies in human cells may be more relevant for such extrapolation and that changes in rat epigenetics in p21 may not be as relevant. It’s possible that epigenetic changes in other rat genes may mirror that of humans. Thus, we are unable to speculate globally if epigenetic effects should be studied for bromate.

Another interesting finding from these data is that the degree of basal DNA methylation of the promoter region of human p21 varied between cell types. This is demonstrated by the fact that the level of DNA methylation at the CpG sites –855 and –691 were significantly lower in hPT cells compared with HEK293 cells. These data suggest that basal DNA methylation may not only differ between species, but also different between similar cell types from the same species. Further studies are needed to confirm this hypothesis. It should be noted that hPT cells used in this study were freshly isolated from whole tissue. That is not the case for HEK293 cells, which are immortalized and grown in media and are embryonic in origin. Thus, it is possible that the culture conditions may mediate changes in basal DNA methylation in the p21 promoter region. It is reasonable to expect differences in basal DNA methylation between cell lines and freshly isolated cells. At the same time, it is known that the methylohe of embryonic and adult cells are different irrespective of tissue type (Varley et al., 2013). The toxicological manifestation of these differences is yet to be investigated. However, a limitation of this study is that epigenetic comparisons were made between human embryonic cell line and rat adult cell line. Future studies are needed to address this gap in knowledge. Regardless, these data suggest that epigenetic studies on the same gene may not translate from cell line to cell line, even within the same species.

In conclusion, our data demonstrate that the expression of rat p21 is regulated by histone acetylation and not DNA methylation in the regions analyzed, after sub-chronic exposure of rat renal cells to BrO₃⁻. In contrast, it appears that while sites for epigenetic regulation do exist within the promoter regions of human and rat p21, these sites exist at different regions of the respective promoters. These data also demonstrate that DNA methylation within the promoter sites does not appear to mediate changes in expression of either rat or human p21 in response to BrO₃⁻. Finally, these data demonstrate the species-specific differences in the epigenetic regulation of p21 and suggest an uncertainty in extrapolating rat epigenetic data for assessing the risk of toxicants in humans.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

ACKNOWLEDGMENTS

We thank everyone involved in the generation of data. We thank Troy J. Kieran and Patrick R. Watson (T.C.G. laboratory) for their help in sample pooling and MiSeq runs.

FUNDING

This project was supported in part with funds from the National Institutes of Health (NIH); National Institute of Biomedical Imaging and Bioengineering (NIBIB) (EB016010 to B.S.C.), as well as Interdisciplinary Toxicology Program Stipend Support and US-EPA’s Student Services Authority Support to R.T.K.

REFERENCES


