

## The Value of DNA Methylation Analysis in Basic, Initial Toxicity Assessments

Rebecca E. Watson,\* James M. McKim,† Gary L. Cockerell,† and Jay I. Goodman\*,<sup>1</sup>

\*Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan 48824, and †Pharmacia Corporation, Investigative Toxicology, Kalamazoo, Michigan 49048

Received December 5, 2003; accepted January 27, 2004

DNA methylation is an epigenetic mechanism regulating patterns of gene expression. Our goal was to see if the assessment of DNA methylation might be a useful tool, when used in conjunction with initial, basic *in vitro* tests, to provide a more informative preliminary appraisal of the toxic potential of chemicals to prioritize them for further evaluation. We sought to give better indications of a compound's toxic potential and its possible mechanism of action at an earlier time and, thereby, contribute to a rational approach of an overall reduction in testing by making improved early decisions. Global and GC-rich patterns of DNA methylation were evaluated along with more traditional cytolethality measurements, e.g., cytolethality and genotoxicity assessments, on rat hepatoma (H4IIE) cells. The relative toxic potential of model compounds camptothecin, 5-fluorouracil, rotenone, and staurosporine was estimated by employing DNA methylation assessments combined with our cytolethality data plus genotoxicity information gleaned from the literature. The overall contribution of the methylation assessment was threefold; it (1) strengthened a ranking based on genotoxicity; (2) provided an indication that a compound might be more potentially problematic than what cytolethality and genotoxicity assessments alone would indicate; and (3) suggested that compounds, particularly nongenotoxins, that are more potent regarding their ability to alter methylation, especially at noncytolethal concentrations, may be more potentially toxic. Altered methylation per se is not proof of toxicity; this needs to be viewed as a component of an evaluation.

There is an increasing need for more informative preliminary tests to predict the toxic potential of chemicals to prioritize them for further evaluation. This is pertinent for the screening of environmental compounds as well as for the development of medicines and consumer products. For practical purposes, when faced with large numbers of small amounts of compounds, initial evaluations are based on the results of *in*

*vitro* studies. Clearly, the initial assessments should be predictive of *in vivo* toxic effects and amenable to dose- (concentration-) response analysis. In light of the recent change in the paradigm of drug discovery leading to the development of very limited quantities of numerous potential lead compounds using combinatorial chemistry, the need for enhanced *in vitro* approaches for basic, initial assessment of toxicity is particularly acute in the pharmaceutical industry (Furka, 2002). In this context, it is important to note that a high percentage of potential new medicines currently fail due to toxicity, often during preclinical or clinical trials, resulting in a significant waste of time and resources (Cockerell *et al.*, 2002).

Typically, initial assessments of toxicity include measurements of cytolethality and genotoxicity (including mutagenicity). Knowledge concerning the mutagenic potential of a compound is an important component of a basic, initial safety assessment (Ames *et al.*, 1979; Rueff *et al.*, 1996). However, different mutagenicity assays performed on the same compound can produce markedly disparate results (Choi *et al.*, 1996). Structure-activity relationships often provide a basis for selection of potential drug candidates in the pharmaceutical industry, and this approach has also been used to try to identify compounds acting at sites known to elicit a toxic response (Woo *et al.*, 1995). Toxicogenomics holds out the potential to develop into a useful screening tool for identification of the toxic potential of chemicals (Tennant, 2002). However, a substantial effort, including data analysis, is necessary to evaluate this approach more thoroughly before it can be employed on a routine basis.

We propose that DNA methylation analysis might be a useful tool when used in conjunction with initial, basic *in vitro* tests, e.g., cytolethality and genotoxicity assessments. This can provide increased knowledge of a chemical's toxic potential and contribute to an enhanced ability to prioritize compounds for further evaluation. Methylation of DNA cytosine residues is an epigenetic mechanism that regulates gene expression as well as tissue-specific, developmental, immunological, and neurological processes (Robertson and Jones, 2000). Both hypo- and hypermethylation may lead to deleterious effects. In general, increases in methylation at promoter regions lead to

<sup>1</sup> To whom correspondence should be addressed at Department of Pharmacology and Toxicology, Michigan State University, B440 Life Sciences Building, East Lansing, MI 48824. Fax: (517) 353-8915. E-mail: goodman3@msu.edu.

transcriptional silencing by directly hindering the binding of transcription factors or by recruiting proteins that bind methylated cytosines, e.g., chromatin deacetylase (Attwood *et al.*, 2002). Conversely, hypomethylation may lead to the increased expression of certain oncogenes and/or the loss of genomic stability by the expression of transposable elements that are normally silenced by methylation (Carnell and Goodman, 2003; Counts and Goodman, 1995). Alterations to normal patterns of methylation have been shown to play a role in cancer (Counts and Goodman, 1995) as well as in developmental, neurological, and immunological disorders (reviewed in Watson and Goodman, 2002a). Thus, altered methylation can lead to aberrant transcription of genes and, therefore, might form a basis for a variety of toxic outcomes. However, there is a possible positive side in that compounds that are found to affect methylation might be useful in cancer therapy; a currently employed anticancer drug, azacytosine, acts by decreasing DNA methylation.

We performed DNA methylation analysis in conjunction with more traditional cytolethality assessments on rat hepatoma (H4IIE) cells treated with the known demethylating agent azacytidine and the model compounds camptothecin, 5-fluorouracil (5-FU), rotenone, and staurosporine. Our goal was to see if assessments of DNA methylation might assist in improving basic, initial toxicological screens.

In our view, the appropriate initial approach should be a general one, involving an evaluation of global methylation status and an assessment of methylation in GC-rich regions of the genome, rather than an attempt at gene-specific evaluations. We do not want to simply add new tests. On the contrary, our aim was to determine if the assessment of DNA methylation, with an emphasis on dose-response relationships, could provide a useful added dimension to basic, initial toxicity assessment of a compound's toxic potential and an earlier indication of its possible mechanism of action. This could aid in selecting and prioritizing those compounds that should be considered for further evaluation and contribute to an overall reduction in testing by making improved early decisions.

## MATERIALS AND METHODS

**Cell culture and DNA purification.** H4IIE rat hepatoma cells (between passages 7–9) were grown in 96- and 6-well plates for *in vitro* toxicity analysis and for methylation analysis, respectively. We have conducted experiments to ascertain that results from these *in vitro* toxicity assessments do not vary between 96- and 6-well plates (data not shown). Cells to be used for methylation analysis were dosed with concentrations of compounds deemed to be cytolethal and noncytolethal based on a battery of *in vitro* cytolethality assessments. After a 72 h incubation, cells were washed twice with PBS, trypsinized, centrifuged, and frozen at  $-80^{\circ}\text{C}$  until use. DNA was extracted using Trizol reagent (Sigma-Aldrich, St. Louis, MO) and stored at  $4^{\circ}\text{C}$  until use.

**Proof of principle compound: 5-aza-2'-deoxycytidine.** Our initial studies focused on our proof-of-principle compound 5-aza-2'-deoxycytidine (dAzaC, purchased from Sigma-Aldrich), a cytosine analog known to cause demethylation by incorporating into DNA and irreversibly binding DNA methyltrans-

ferase, thus inhibiting methylation of newly replicated DNA (Lu and Randerath, 1984).

**Model compounds.** Following the initial studies with dAzaC, four model compounds with varying modes of action and different toxic effects were selected. None of these compounds were known to have any effect on DNA methylation. Camptothecin is an S-phase-specific anticancer agent that inhibits the activity of DNA topoisomerase I, leading to replication fork arrest as well as single- and double-strand DNA breaks (Morris and Geller, 1996). 5-FU is a pyrimidine analog that is metabolized to 5-fluorodeoxyuridine monophosphate, a compound that competes with deoxyuridine monophosphate for thymidylate synthetase. Normally, thymidylate synthetase catalyzes the conversion of deoxyuridine monophosphate to thymidine monophosphate, a precursor of thymidine triphosphate and a necessary component of DNA (Parker and Cheng, 1990). Thus, the overall effect of 5-FU is to inhibit replication. Rotenone inhibits complex I of the mitochondrial oxidative phosphorylation chain, stopping the supply of electrons to quinol cytochrome c oxidoreductase. This decreases adenosine triphosphate (ATP) production and the release of cytochrome c from the mitochondria; the increased permeability of the mitochondrial membrane leads to caspase-mediated apoptosis (Pei *et al.*, 2003). Staurosporine is a nonspecific inhibitor of protein kinases that promotes apoptosis through both caspase-dependent and -independent mechanisms (Belmokhtar *et al.*, 2001). Staurosporine also inhibits the catalytic activity of topoisomerase II by blocking the transfer of phosphodiester bonds from DNA to the active tyrosine site (Lassota *et al.*, 1996). All compounds described were purchased from Sigma-Aldrich.

**In vitro toxicity assessments.** *In vitro* toxicity assessments for each compound included measurements of ATP, cell number, glutathione-S-transferase (GST), and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) as part of the Tox Cluster battery of assays described elsewhere (McKim *et al.*, 2001).

**ATP assay.** ATP serves as the principal immediate donor of free energy and is present in all metabolically active cells (Crouch *et al.*, 1993). Levels of ATP decline rapidly when cells are injured, and this can be easily measured using an ATP bioluminescence assay in which a luciferin ATP substrate was added and interacted with ATP and oxygen to form oxyluciferin, AMP, PP<sub>i</sub>, CO<sub>2</sub>, and light (Crouch *et al.*, 1993). The ATPLite-M bioluminescence assay kit (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA) was used to measure the amount of ATP in the H4IIE cells. The amount of ATP is extrapolated from the amount of light emitted as measured by a spectrophotometer (Packard, Palo Alto, CA). Results are expressed as percentages of control values.

**Cellular proliferation assay.** Measurements of cellular proliferation provide a general measure of toxicity. Cell number was assessed using the CyQUANT cell proliferation assay kit from Molecular Probes (Eugene, OR), a highly sensitive, fluorescence-based microplate assay for determining the number of cultured cells (Jones *et al.*, 2001). Cells were rinsed with PBS to remove dead cells no longer adhering to the plate, lysed, and the DNA was stained using the CyQUANT fluorescent dye. Fluorescence was measured using a Packard Spectracount fluorescence reader. Using a standard curve generated from fluorescence readings of known amounts of H4IIE cells, the cell numbers in our samples were extrapolated.

**GST assay.** GST leakage is linked to a loss of membrane integrity and necrosis in hepatocytes, and, thus, the amount of GST is related to cell viability (Giannini *et al.*, 2000). To measure GST release into the serum, we used the rat  $\alpha$  GST enzyme immunoassay (Biotrin International, Dublin, Ireland). After 72 h, serum from the cells was removed, diluted 1:4 with media, and 100  $\mu\text{l}$ /well of the diluted serum were placed into 96-well plates coated with IgG antibody. The cells were incubated for 1 h at room temperature using a rotary mixer. Plates were then washed six times using the Biotrin wash buffer. After removing all the fluid from the plate, 100  $\mu\text{l}$ /well of the Biotrin conjugate were added. This conjugate binds to the IgG-bound GST. Plates were incubated with the conjugate for 1 h at room temperature using a rotary mixer and then washed six times using Biotrin wash buffer. After removing all the fluid from the plate,

100  $\mu$ l of Biotrin TMB substrate were added to each well. The plates were incubated for 15 min at room temperature using a rotary mixer. Following incubation, 50  $\mu$ l stop solution were added to each well and plates were read using a Packard Spectracount spectrophotometer. The percentages of damaged GST-releasing cells and nondamaged cells (not releasing GST above basal values) were determined using a standard curve generated from standards containing known percentages of control and 50  $\mu$ M digitonin-treated cells. Digitonin damages cells and elicits GST release. GST results are presented as the percentages of control cells not releasing GST above basal values.

**MTT assay.** MTT analysis provides a general measurement of mitochondrial dehydrogenase activity and cell viability (Rodriguez and Acosta, 1997). The MTT assay is based on the reduction of the soluble yellow MTT tetrazolium salt to a blue MTT formazan product by mitochondrial dehydrogenases (Mosman, 1983). Each well of H4IIE cells within 96-well plates was incubated with 100  $\mu$ l of a 0.5 mg/ml MTT solution for 3 h. Following the MTT incubation, the media were removed by aspiration and 200  $\mu$ l isopropanol were added to each well to dissolve and solubilize the intracellular MTT formazan product. After a 20 min incubation with isopropanol (with shaking) in the dark, the optical density of each well was assessed at 570 and 850 nm using a Packard Spectracount spectrophotometer. Results are expressed as percentages of control values. The MTT assay was purchased from Sigma-Aldrich.

**Rationale by which cytolethal and noncytolethal concentrations of compounds were selected.** Based on dose-response analysis, the threshold concentration was estimated to be the first concentration below which there was no statistically significant change compared to measurements in untreated control cells and above which there was a significant change in at least two of the parameters. A concentration equal to 10–25% of this value was used as the noncytolethal concentration. The cytolethal concentration was selected as the first concentration at which the percentages of control values for at least two of the assays were between 25 and 40%. Thus, noncytolethal and cytolethal concentrations were chosen in a uniform manner for each model compound. Also, these parameters were used to select noncytolethal concentrations of dAzaC.

**Global DNA methylation analysis: SssI methylase assay.** SssI methylase uses S-adenosyl methionine (SAM) as a methyl group donor to methylate the 5' position of cytosine at unmethylated CpG sites in DNA. Thus, the level of global DNA methylation can be determined by the amount of tritiated methyl groups from [<sup>3</sup>H-CH<sub>3</sub>] S-adenosyl-L-methionine incorporated into DNA, since there is an inverse relationship between incorporation of radioactivity and the original degree of methylation (Balaghi and Wagner, 1993). DNA (1 mg) was incubated with 2  $\mu$ Ci [<sup>3</sup>H-CH<sub>3</sub>] S-adenosyl-L-methionine (New England Nuclear, Boston, MA) and 3 units of SssI methylase (New England Biolabs, Beverly, MA) for 1 h at 30°C. Results are presented as counts per minute per microgram (cpm/ $\mu$ g) DNA. Five replicates were performed per sample. Graphical presentation was performed using Excel (Microsoft Corp., Redmond, WA). Statistical analysis was performed with Excel using two-tailed *t*-tests to compare the average cpm/ $\mu$ g DNA measurements of treatment groups and controls. A *p* value of < 0.05 was considered statistically significant.

**Methylation analysis of GC-rich regions: restriction digests.** For each DNA sample, three restriction digests were performed as follows: *RsaI* alone, *RsaI* and *MspI*, and *RsaI* and *HpaII*. *RsaI* is a methylation-insensitive enzyme used to cut the DNA into smaller fragments. Both *MspI* and *HpaII* are methylation-sensitive enzymes that cut between cytosine residues at 5'-CCGG-3' sites. *MspI* will not cut if the external cytosine is methylated, and *HpaII* will not cut if the internal cytosine is methylated. Both *MspI* and *HpaII* will cut if the site is unmethylated (Mann and Smith, 1977). All enzymes used were from Roche (Indianapolis, IN). Restriction digests were performed with 1  $\mu$ g DNA and 5.0 units *RsaI* in Roche buffer L. After a 1 h incubation (with shaking) in a water bath at 37°C, two 2.5-unit aliquots of *MspI* or *HpaII* were added, 2 h apart. The total incubation time was 18 h. The enzymes were inactivated by a 10 min incubation at 65°C, and the digests were stored at 4°C until amplified by PCR.

**Arbitrarily primed (AP)-[<sup>33</sup>P] PCR.** PCR was performed on restriction digests using a single primer (5'-AACCTCACCTAACCCCGG-3') that arbitrarily binds within GC-rich regions of DNA (Gonzalzo *et al.*, 1997). Reactions were composed of 5  $\mu$ l of the restriction digest (containing 1  $\mu$ g digested DNA), 0.4  $\mu$ M of each primer, 1.25 units of Taq polymerase (Gibco BRL, Rockville, MD), 1.5 mM MgCl<sub>2</sub>, 60 mM Tris, 15 mM ammonium sulfate, 1.65  $\mu$ Ci  $\alpha$ -[<sup>33</sup>P]-dATP (New England Nuclear, Boston, MA), and glass-distilled water to volume. Samples were heated for 5 min at 94°C before the addition of dNTPs to minimize the possibility of primer-dimer formation. Cycling conditions included a single denature cycle for 2 min at 94°C, followed by five cycles under the following conditions: 30 s at 94°C, 1 min at 40°C, 1.5 min at 72°C; then, 30 cycles at 94°C for 30 s, 55°C for 15 s, and 72°C for 1 min, a time delay cycle for 5 min at 72°C, and a soak cycle at 4°C. PCR products (5  $\mu$ l of each) were separated on a 6% polyacrylamide sequencing gel at 45 watts for 2¼–2½ h. The gel was soaked for 10 min in a fixing solution with 5% acetic acid and 5% methanol, rinsed for 10 min in glass-distilled water, dried, and placed into a cassette with a storage phosphorimage screen to visualize labeled PCR products. Compared with larger DNA fragments on the upper halves of gels, smaller fragments on the lower halves of gels sometimes required a longer exposure to clearly discern bands. Thus, a short exposure of 3 d followed by a longer exposure of 8 d was often performed on a gel. Phosphorimages were analyzed using Quantity One software (Bio-Rad, Hercules, CA).

## RESULTS

Firstly, we wanted to determine how the existing *in vitro* toxicity analysis compared with methylation analysis using dAzaC, a drug known to alter DNA methylation. Studies were performed using H4IIE cells treated with 10 and 100  $\mu$ M dAzaC; both concentrations were found to be noncytolethal based on *in vitro* toxicity analysis. These concentrations of dAzaC decreased global methylation levels in a dose-dependent manner (Fig. 1). Furthermore, arbitrarily primed PCR results showed that, in the treated samples, there were five GC-rich regions in which there was a greater amount of methylation at the external cytosines at 5'-CCGG-3' sites and two

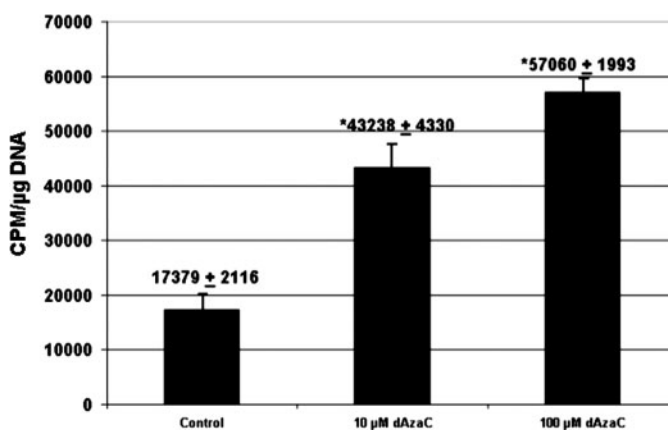


FIG. 1. Global methylation levels in and H4IIE cells treated with 10 and 100  $\mu$ M 5-aza-2'-deoxycytidine (dAzaC) for 72 h. Control H4IIE cells were untreated and grown for 72 h. Each bar shown represents the mean CPM/ $\mu$ g value from five replicates performed on each of four DNA samples, each from a separate well. \*Statistically significant difference (*p* < 0.05) compared with control group.

GC-rich regions in which there was a greater amount of methylation at the internal cytosines at 5'-CCGG-3' sites, compared with untreated controls (Fig. 2).

Cytolethal and noncytolethal concentrations were chosen for the model compounds based on *in vitro* toxicity data, as shown in Figure 3. Global methylation status in cells treated with both cytolethal and noncytolethal concentrations of these compounds is shown in Figure 4. Global methylation levels of cells

treated with both cytolethal and noncytolethal concentrations of camptothecin were not significantly different from the untreated controls. However, treatment with a cytolethal dose of 5-FU and a noncytolethal dose of staurosporine led to statistically significant ( $p < 0.05$ ) decreases in the global level of methylation. A cytolethal concentration of staurosporine led to a reduction in global methylation levels, but this was not statistically significant ( $p = 0.08$ ). Also, at both cytolethal and

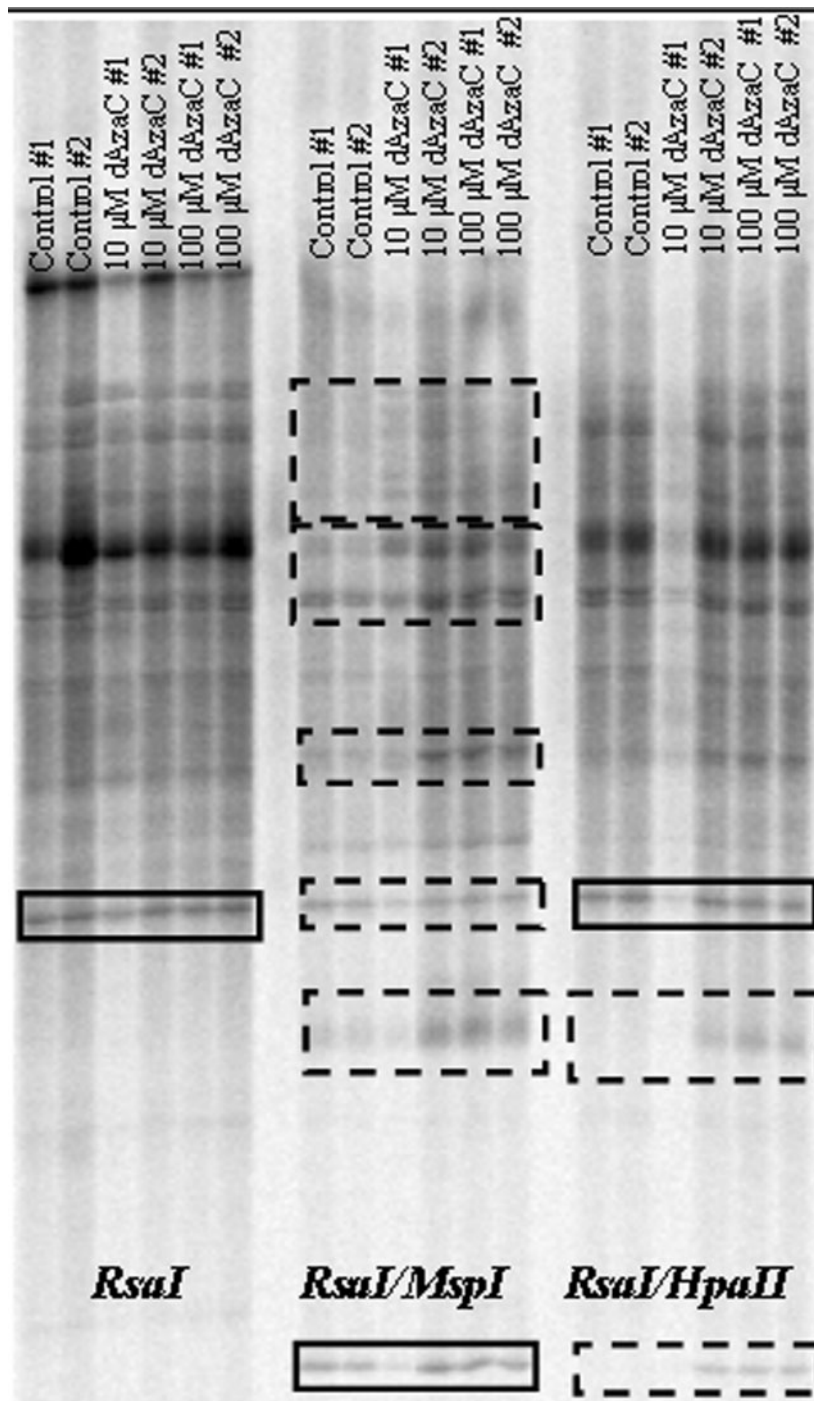
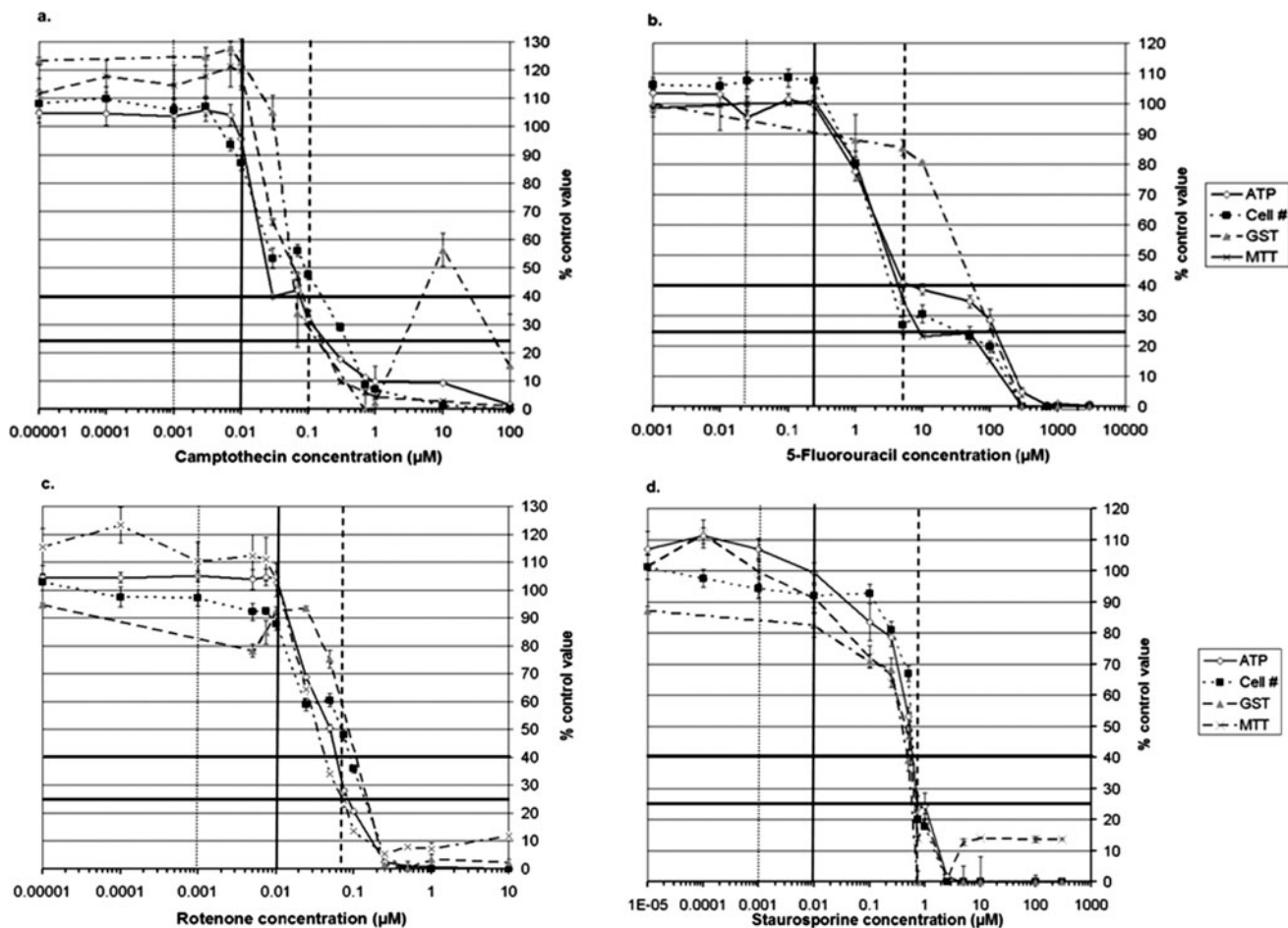


FIG. 2. Methylation status of GC-rich regions in H4IIE cells treated with 10 and 100  $\mu\text{M}$  5-aza-2'deoxyctidine (dAzaC) for 72 h. Results shown indicate GC-rich methylation patterns from two separate wells each (1 and 2 as labeled at the top of the gel), of untreated control cells, and of cells treated with 10 or 100  $\mu\text{M}$  of dAzaC. These results are representative of DNA from four separate wells of samples from each group. Dashed boxes indicate regions in which bands are seen more distinctly in the treated samples compared with controls. Solid boxes indicate reference rows of bands that are reasonably constant and highlighted to show that lane-to-lane loading was relatively consistent.



Compound	Non-cytotoxic (NCL) concentration	Cytotoxic (CL) concentration
Camptothecin	0.001 $\mu$ M	0.1 $\mu$ M
5-Fluorouracil	0.25 $\mu$ M	5 $\mu$ M
Rotenone	0.001 $\mu$ M	0.07 $\mu$ M
Staurosporine	0.001 $\mu$ M	0.75 $\mu$ M

**FIG. 3.** *In vitro* toxicity assessments of (A) camptothecin, (B) 5-FU, (C) rotenone, and (D) staurosporine. *In vitro* assays included measurements of ATP, cell number, GST, and MTT. Percentages of control values for a range of concentrations are indicated. Thick horizontal bars indicate the 25–40% control range used to select cytotoxic concentrations for each compound. The thick vertical line indicates the threshold dose below which at least three parameters do not vary significantly from control values and above which at least two parameters are statistically below the percentages of control values of the preceding concentration. The dotted vertical line indicates the noncytotoxic concentration, chosen to be 10–25% of the threshold. The dashed vertical line indicates the cytotoxic concentration. Data were analyzed and cytotoxic and noncytotoxic concentrations were selected as indicated in a uniform manner for each model compound. Two replicates of the full set of toxicological parameters were performed for each compound and the results were highly reproducible. (E) A summary of threshold, cytotoxic, and noncytotoxic concentrations chosen for each compound is shown.

noncytotoxic concentrations, rotenone seemed to increase global levels of DNA methylation, though this was not statistically significant ( $p = 0.12$  and  $0.09$ , respectively).

GC-specific methylation status was assessed for cytotoxic doses of all the model compounds (Fig. 5). A cytotoxic concentration of camptothecin (Fig. 5A) or rotenone (Fig. 5C) resulted in no detectable alterations in the GC-rich methylation

patterns of H4IIE cells compared with controls. Treatment with a cytotoxic concentration of 5-FU (Fig. 5B) induced hypermethylation at the internal cytosine of the 5'-CCGG-3' site in six GC-rich regions, hypomethylation at the internal cytosine in three GC-rich regions, and hypomethylation at the external cytosine in two GC-rich regions. Treatment with a cytotoxic concentration of staurosporine (Fig. 5D) resulted in hyper-

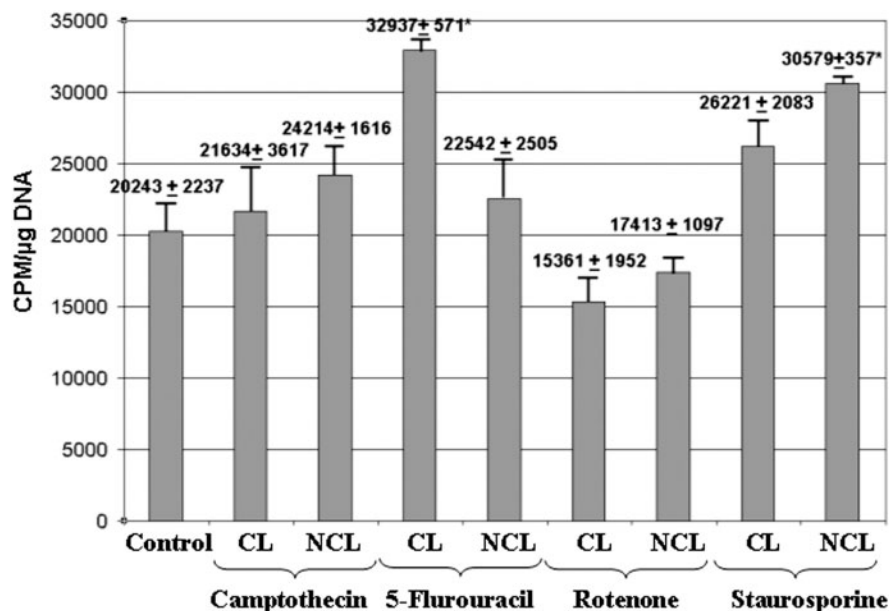


FIG. 4. Global methylation levels in H4IIE cells treated for 72 h with camptothecin, 5-FU, rotenone, and staurosporine. Global methylation status in untreated control cells and cells treated with cytolethal (CL) and noncytolethal (NCL) concentrations of camptothecin, 5-FU, rotenone, and staurosporine is presented. Each bar shown represents the mean CPM/ $\mu$ l value from five replicates performed on each of four DNA samples, each from a separate well. \*Statistically significant difference ( $p < 0.05$ ) compared with controls.

methylation at the external cytosine of the 5'-CCGG-3' site in five GC-rich regions. Since treatment with cytolethal concentrations of 5-FU and staurosporine led to alterations in GC-rich methylation, the GC-rich methylation status of cells treated with noncytolethal concentrations of these com-

pounds was assessed (Fig. 6). The noncytolethal concentration of 5-FU did not lead to any GC-specific methylation alterations (Fig. 6A), but the noncytolethal concentration of staurosporine led to increases and decreases in methylation at the internal cytosine of the 5'-CCGG-3' site in five and

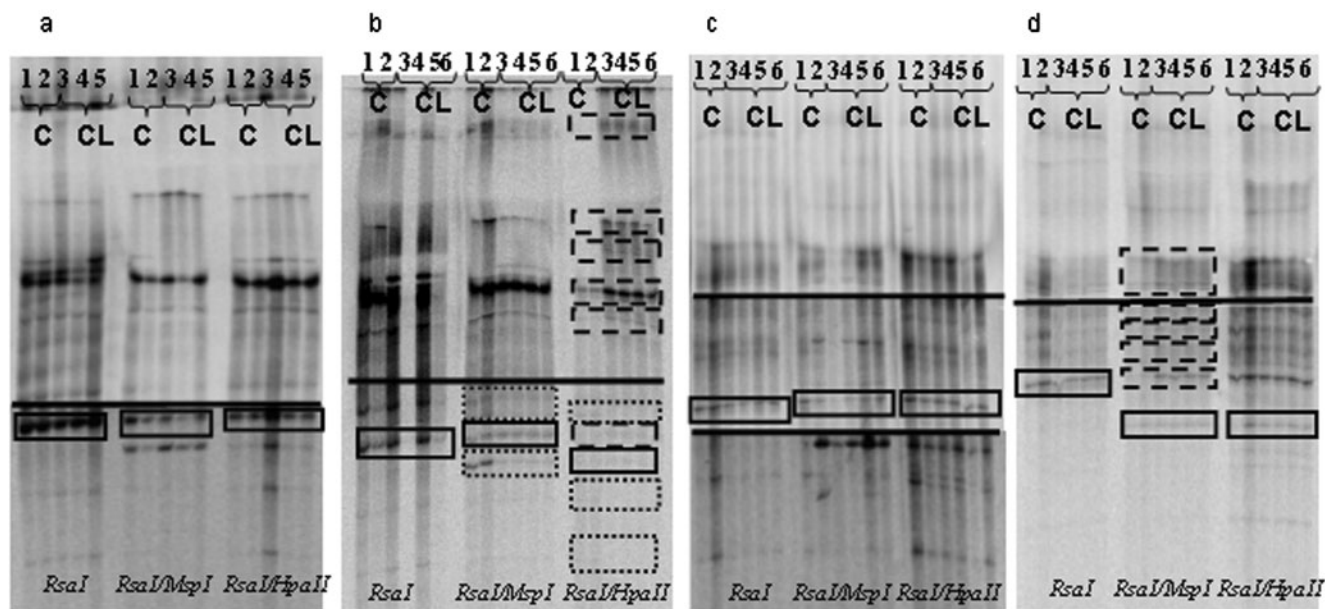


FIG. 5. GC-rich methylation in H4IIE cells treated with cytolethal concentrations of (A) camptothecin, (B) 5-FU, (C) rotenone, and (D) staurosporine. C, control, untreated cells; CL, cells treated with cytolethal concentrations of each compound. Numbers underneath the brackets at the top of the gel indicate individual samples from separate wells within 6-well plates, such that  $n = 2$  for control cells and  $n = 3$  or 4 for cells treated with a cytolethal dose of each compound. For each sample, *RsaI*, *RsaI/MspI*, and *RsaI/HpaII* digests were performed. Dashed boxes indicate rows of bands seen more prominently in treated cells compared with controls and dotted boxes indicate rows of bands seen less prominently in treated cells compared with controls. Solid boxes indicate reference rows (R) of bands that are reasonably constant and highlighted to show that lane-to-lane loading was relatively consistent. The top part of the images is from a 1- to 2-day exposure, the middle part (in B) is from a 3-day exposure, and the bottom part is from a 7-day exposure.

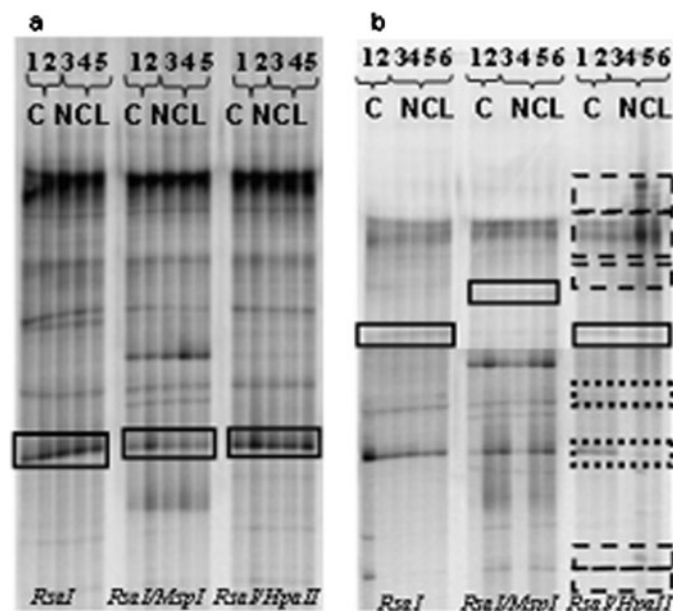


FIG. 6. GC-rich methylation in H4IIE cells treated with noncytotoxic concentrations of (A) 5-FU and (B) staurosporine. C, control, untreated cells; NCL, cells treated with noncytotoxic concentrations of 5-FU and staurosporine. Numbers underneath the brackets at the top of the gel indicate individual samples from separate wells within 6-well plates, such that  $n = 2$  for control cells and  $n = 3$  or 4 for cells treated with a cytotoxic dose of each compound. For each sample, *RsaI*, *RsaI/MspI*, and *RsaI/HpaII* digests were performed. Dashed boxes indicate rows of bands seen more prominently in treated cells compared with controls and dotted boxes indicate rows of bands seen less prominently in treated cells compared with controls. Solid boxes indicate reference rows (R) of bands that are reasonably constant and highlighted to illustrate that lane-to-lane loading was relatively consistent. The images shown are from 4-day exposures.

two regions, respectively (Fig. 6B). A summary of the results of global and GC-rich DNA methylation analysis is presented in Table 1.

## DISCUSSION

Based on the results of our investigation, we believe that the inclusion of an assessment of methylation status, with an emphasis on dose-response relationships, as a component of initial, preliminary toxicity testing can help in the prioritization of compounds at early screening stages and contribute to a better understanding of possible mechanisms underlying toxicity. For instance, if two compounds exhibit similar results from cytotoxicity and genotoxicity assays but one compound affects methylation at low doses and the other does not, this could provide a basis for considering the latter to be less potentially toxic. More effective prioritization is expected to result in an overall decrease in time, cost, and testing.

We performed methylation analysis by examining both global and GC-rich methylation in H4IIE cell DNA. These approaches assess different and, importantly, complementary aspects of genome-wide methylation. A focus on gene-specific methylation would not be appropriate during initial toxicity testing, in part because one would not know which gene(s) to examine and compound-specific changes could be anticipated. Global and GC-rich methylation patterns might be regulated by different methyltransferases and may be affected as a result of the administration of a specific compound. For example, our data show that dAzaC, a demethylating agent that irreversibly binds and, thus, inactivates methyltransferases, decreased global methylation as expected but also increased methylation at GC-rich sites. Other studies have reported that dAzaC treat-

TABLE 1  
Summary of Global and GC-Rich DNA Methylation Analysis

Compound	Effect on global DNA methylation status	Effect on GC-rich DNA methylation	
		Hypermethylated sites	Hypomethylated sites
DNA methylation analysis at cytotoxic concentrations			
Camptothecin	No change	0	0
5-FU	Decreased ( $\downarrow$ ) <sup>a</sup> (hypomethylation)	6	5
Rotenone	No change	0	0
Staurosporine	Decreased ( $\downarrow$ ) <sup>b</sup> (hypomethylation)	6	0
DNA methylation analysis at noncytotoxic concentrations			
dAzaC	Decreased ( $\downarrow$ ) <sup>a</sup> (hypomethylation)	8	0
Camptothecin	No change	Not analyzed <sup>c</sup>	Not analyzed <sup>c</sup>
5-FU	No change	0	0
Rotenone	No change	Not analyzed <sup>c</sup>	Not analyzed <sup>c</sup>
Staurosporine	Decreased ( $\downarrow$ ) <sup>b</sup> (hypomethylation)	5	2

Note. These data are shown in Figures 1–6.

<sup>a</sup>Statistically significant in two-tailed *t*-test,  $p < 0.05$ .

<sup>b</sup>There is a trend toward a decrease in global DNA methylation, though not statistically significant, in two-tailed *t*-test,  $p = 0.08$ .

<sup>c</sup>GC-rich DNA methylation status was not analyzed for camptothecin and rotenone at noncytotoxic concentrations because no such changes were observed at cytotoxic concentrations.

TABLE 2  
Estimated Toxic Potential Rankings of Model Compounds Based on Methylation Analysis at Cytolethal and Noncytolethal Concentrations

Compound <sup>a,b</sup>	Effect on global DNA methylation status	Effect on GC-rich DNA methylation		Estimated toxic potential ranking <sup>c</sup>
		Hypermethylated sites	Hypomethylated sites	
Methylation analysis at cytolethal concentrations				
B	Decreased (↓) <sup>d</sup> (hypomethylation)	6	5	1
D	Decreased (↓) <sup>e</sup> (hypomethylation)	6	0	1
A, C	No change	0	0	2
Methylation analysis at noncytolethal concentrations				
D	Decreased (↓) <sup>d</sup> (hypomethylation)	5	2	1
A, B, C	No change	Not analyzed	Not analyzed	2

*Note.* For each compound, the threshold value was estimated to be the concentration below which there was no statistically significant change in cytolethality compared with untreated control cells and above which there was a significant change in at least two cytolethality parameters. A concentration used that was equal to 10–25% of the threshold value was employed. The cytolethal concentration used was selected as the lowest concentration at which the percentages of control values for at least two cytolethality parameters were between 25 and 40%.

<sup>a</sup>The model compounds presented in Table 1 are now indicated by letter to de-emphasize the knowledge of their mechanisms of action and to focus on the use of methylation data to derive an estimated toxic potential ranking.

<sup>b</sup>A, camptothecin; B, 5-FU; C, rotenone; and D, staurosporine.

<sup>c</sup>Estimated relative potential to elicit a toxic response, such that a higher number represents a potentially safer compound and a lower number represents one more likely to be problematic.

<sup>d</sup>Statistically significant in two-tailed *t*-test,  $p < 0.05$ .

<sup>e</sup>There is a trend toward a decrease in global DNA methylation that is close to reaching statistical significance in a two-tailed *t*-test,  $p = 0.08$ .

ment reduces global levels of methylation while increasing methylation at select regions (Broday *et al.*, 1999; Grassi *et al.*, 2003). The basis for the latter effect is not known, but this finding indicates that dAzaC might affect methylation at GC-rich regions in a manner secondary to its known mode of action and provides an additional example of the importance of examining both global and GC-specific methylation patterns.

In support of this, it should be noted that DNMT1 and DNMT3b methyltransferases have a greater *in vivo* binding avidity to dAzaC-containing DNA compared with DNMT2 and 3a (Liu *et al.*, 2003). Perhaps in response to decreasing availability of DNMT1 and 3b, amounts of DNMT2 and 3a are upregulated as a result of azacytidine treatment and the GC-rich regions, shown to be hypermethylated as a result of dAzaC treatment, are methylated by these methyltransferases. Even if methylation is affected by a secondary or tertiary mechanism, this finding nonetheless provides more insight into a compound's actions than solely relying on cytotoxicity assessments. Furthermore, mice given phenobarbital exhibited global hypomethylation in liver DNA (Counts *et al.*, 1996) with increased hypermethylation in GC-rich regions (Watson and Goodman, 2002b). Thus, it is informative to look at both global and GC-specific changes.

There are multiple ways in which DNA methylation may be altered, including perturbing the 1-carbon choline/folate/methionine metabolic pathway required for the synthesis of SAM, the proximate methyl group donor for DNA (Zeisel and Blusztajn, 1994). For example, arsenic is methylated by SAM, and administration of arsenic is thought to hypomethylate

DNA by decreasing the availability of SAM (Okoji *et al.*, 2002). Maintenance of normal DNA methylation may be viewed as a basic homeostatic mechanism. Therefore, detec-

TABLE 3  
Toxic Potential Ranking of the Model Compounds Based on the Concentration Needed to Elicit a Cytolethal Response

Compound <sup>a,b</sup>	Cytolethal concentration (μM)	Estimated toxic potential rank <sup>c</sup>
A, C <sup>d</sup>	0.10, 0.07	1
D	0.75	2
B	5.0	3

*Note.* For each compound, the threshold value was estimated to be the concentration below which there was no statistically significant change in cytolethality compared with untreated control cells and above which there was a significant change in at least two cytolethality parameters. A concentration equal to 10–25% of the threshold value was used as the noncytolethal concentration. The cytolethal concentration was selected as the first concentration at which the percentages of control values for at least two cytolethality parameters were between 25 and 40%.

<sup>a</sup>The model compounds presented in Table 1 are now indicated by letter to de-emphasize the knowledge of their mechanisms of action and to focus on the use of methylation data to derive an estimated toxic potential ranking.

<sup>b</sup>A, camptothecin; B, 5-FU; C, rotenone; and D, staurosporine.

<sup>c</sup>Estimated relative potential to elicit a toxic response, such that a higher number represents a potentially safer compound and a lower number represents one more likely to be problematic.

<sup>d</sup>These compounds were assigned the same rank because the cytolethal concentrations were nearly equivalent.

TABLE 4  
Summary of *In Vitro* Genotoxicity Data for the Model Compounds

Compound <sup>a,b</sup>	<i>In vitro</i> genotoxicity data			
	Ames test	Sister chromatid exchange test	Chromosomal aberration test	Mouse lymphoma test
A	Not available	Positive	Positive	Positive
B	Negative	Positive	Positive	Positive
C	Negative	Negative	Negative	Positive
D	Not available	Not available	Not available	Not available

Note. For the purposes of this table, the search for genotoxicity data was limited to results of the four common tests presented. These data were obtained from references cited in TOXLINE, National Institutes of Health, and National Toxicology Program databases (<http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?TOXLINE>, <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?Multi>, and <http://ntp-server.niehs.nih.gov>, respectively).

<sup>a</sup>The model compounds presented in Table 1 are now indicated by letter to de-emphasize the knowledge of their mechanisms of action and to focus on the use of methylation data to derive an estimated toxic potential ranking.

<sup>b</sup>A, camptothecin; B, 5-FU; C, rotenone; and D, staurosporine.

tion of alterations of this might enhance early basic toxicity screening by providing a broader picture of a compound's potentially toxic effects.

Typically, basic, initial toxicity assessments involve *in vitro* studies aimed at ascertaining the compound-of-interest's cytolethal and genotoxic effects. To show the potential importance of the evaluation of DNA methylation for compound prioritization, we estimated the relative toxic potentials of the model compounds used in this study by employing DNA methylation assessments combined with the cytolethality data presented plus genotoxicity information gleaned from the literature. For the latter, we searched for data concerning four basic *in vitro* tests: the Ames test, the sister chromatid exchange, chromosome aberrations, and the mouse lymphoma assay. Our model compounds are indicated by letters (to de-emphasize the fact that we have knowledge of their mechanisms of action) as follows: A represents camptothecin, B represents 5-FU, C represents rotenone, and D represents staurosporine. Using methylation analysis alone, the compounds were ranked according to estimated toxic potentials at cytolethal and noncytolethal concentrations, such that a higher number indicates a safer compound compared to one ranked with a lower number (Table 2). At cytolethal concentrations, compounds B and D affected multiple changes in DNA methylation while A and C did not. Thus, B and D would be ranked as more toxic than A and C (Table 2). However, at noncytolethal concentrations, only compound D affected DNA methylation. We viewed methylation alterations at noncytolethal concentrations as being more significant than changes that occurred only at cytolethal concentrations. Therefore, D is ranked more toxic than A, B, and C (Table 2). Thus, if all four compounds were non-genotoxic, methylation data plus information concerning cytolethality could be very helpful to an initial prioritization of potential toxicity.

Compounds were also ranked based on the concentrations at which a cytolethal effect was obtained (Table 3). This is a

crude method of ranking, likely to be important only if a compound is toxic at very low (pM) concentrations or if there is an extreme range of potencies of the compounds-of-interest. In this case, the cytolethal concentrations of the model compounds are within a 100-fold range. The genotoxicity data are presented in Table 4. We then used the genotoxicity data with and without the cytolethality data (Table 3) to rank the compounds based on estimated toxic potential. Genotoxicity data alone indicated that, since A and B are genotoxic and C is not, A and B would be more likely to pose a toxic response than C (Table 5). Cytolethality data indicated that a lower dose of A

TABLE 5  
Estimated Toxic Potential Rankings Based on *In Vitro* Genotoxicity Data (Table 4) Without and Using Cytolethality Data (Table 3)

Compound <sup>a,b</sup>	Estimated toxic potential rank <sup>c</sup>
Toxic potential ranking using <i>in vitro</i> genotoxicity data	
A, B	1
C	2
D	<sup>d</sup>
Toxic potential ranking using <i>in vitro</i> genotoxicity data combined with cytolethality data	
A	1
B	2
C	3
D	<sup>d</sup>

<sup>a</sup>The model compounds presented in Table 1 are now indicated by letter to de-emphasize the knowledge of their mechanisms of action and to focus on the use of methylation data to derive an estimated toxic potential ranking.

<sup>b</sup>A, camptothecin; B, 5-FU; C, rotenone; and D, staurosporine.

<sup>c</sup>Estimated relative potential to elicit a toxic response, such that a higher number represents a potentially safer compound and a lower number represents one more likely to be problematic.

<sup>d</sup>Due to a lack of *in vitro* genotoxicity data, D could not be ranked.

was needed to elicit a cytolethal effect than B, thus permitting these two compounds to be separated in ranking (Table 5). Finally, methylation data at noncytolethal concentrations were combined with genotoxicity and cytolethality data to rank the compounds (Table 6). Though compound C was most cytolethal, it ranked least potentially toxic when genotoxicity and methylation data were also considered (Table 6). If data were available indicating that compound D was equal to or more genotoxic than A and B, then D would be considered the most potentially toxic of the group (Fig. 6B).

The overall contribution of DNA methylation assessments in this initial, toxic-potential evaluation exercise was threefold: (1) it both supported and strengthened the ranking based on genotoxicity, indicating that compound C was less potentially toxic than compounds A and B in this example; (2) it provided an indication that compound D was more potentially problematic than indicated by cytolethality and genotoxicity assessments; and (3) for nongenotoxic compounds, it showed that those that were more potent with regard to their ability to alter methylation, particularly at noncytolethal concentrations (Table 2), may be more potentially toxic.

It is very important to recognize that an alteration in DNA methylation alone is not necessarily indicative of toxicity; certain changes in methylation might be representative of nor-

mal biological processes, and methylation is a reversible mechanism (Ramchandani *et al.*, 1999). Also, one must consider that human cells are more capable of maintaining normal methylation status compared to rodent cells (reviewed in Goodman and Watson, 2002). For these reasons, methylation analysis needs to be viewed as a component of an overall toxicity assessment.

This study serves as a promising first step in assessing the utility of methylation analysis in early stages of toxicity testing. A next step should involve *in vitro* and *in vivo* dose-response comparisons linked to histopathology and take reversibility into consideration (e.g., are the *in vitro* data predictive of *in vivo* toxicity and is methylation status altered in target organs?). Future experiments should examine the effects of different compounds within particular chemical classes. Currently, gene array approaches to assess methylation status are too cumbersome as far as cost and data analysis issues to be used on a routine, initial basis. Attempts should be made to adapt methylation analysis to high-throughput approaches (e.g., separation of random primed PCR products by capillary electrophoresis rather than the use of polyacrylamide gels).

#### ACKNOWLEDGMENTS

Research support from Pharmacia Corporation, including stipend support for R.E.W., is gratefully acknowledged.

#### REFERENCES

- Ames, B., Haroun, L., Andrews, A. W., Thibault, L. H., and Lijinsky, W. (1979). The reliability of the Ames assay for the prediction of chemical carcinogenicity. *Mutat. Res.* **62**, 393–399.
- Attwood, J. T., Yung, R. L., and Richardson, B. C. (2002). DNA methylation and the regulation of gene transcription. *Cell. Mol. Life Sci.* **59**, 241–257.
- Balaghi, M., and Wagner, C. (1993). DNA methylation in folate deficiency: Use of CpG methylase. *Biochem. Biophys. Res. Commun.* **193**, 1184–1190.
- Belmokhtar, C. A., Hillion, J., and Segal-Bendridjian, E. (2001). Staurosporine induces apoptosis through both caspase-dependent and caspase-independent mechanisms. *Oncogene* **20**, 3354–3362.
- Brodsky, L., Lee, Y.-W., and Costa, M. (1999). 5-Azacytidine induces transgene silencing by DNA methylation in Chinese hamster cells. *Mol. Cell. Biol.* **19**, 3198–3204.
- Carnell, A. N., and Goodman, J. I. (2003). The long (LINEs) and the short (SINEs) of it: Altered methylation as a precursor to toxicity. *Toxicol. Sci.* **75**, 229–235.
- Choi, M. J., Lee, J. W., and Lee, B. M. (1996). Comparative assessment of DNA adduct formation, Salmonella mutagenicity, and chromosome aberration assays as short-term tests for DNA damage. *J. Toxicol. Environ. Health* **49**, 271–284.
- Cockerell, G. L., McKim, J. M., and Vonderfecht, S. L. (2002). Strategic importance of research support through pathology. *Toxicol. Pathol.* **30**, 4–7.
- Counts, J. L., and Goodman, J. I. (1995). Alterations in DNA methylation may play a variety of roles in carcinogenesis. *Cell* **83**, 13–15.
- Counts, J. L., Sarmiento, J. I., Harbison, M. L., Downing, J. C., McClain, R. M., and Goodman, J. I. (1996). Cell proliferation and global methylation status changes in mouse liver after phenobarbital and/or choline-devoid, methionine-deficient diet administration. *Carcinogenesis* **17**, 1251–1257.

TABLE 6

Estimated Toxic Potential Rankings Based on Methylation Analysis at Noncytolethal Concentrations (Table 2) in Combination with Cytolethality (Table 3) and *In Vitro* Genotoxicity Data (Table 4)

Compound <sup>a,b</sup>	Estimated toxic potential rank <sup>c</sup>
Estimated toxic potential ranking assuming compound D <sup>a,b</sup> is less genotoxic than A and B	
A	1
B	2
D	1 or 2 <sup>d</sup>
C	3
Estimated toxic potential ranking assuming compound D <sup>a,b</sup> is as or more genotoxic than A and B	
D	1
A	2
B	3
C	4

<sup>a</sup>The model compounds presented in Table 1 are now indicated by letter to de-emphasize the knowledge of their mechanisms of action and to focus on the use of methylation data to derive an estimated toxic potential ranking.

<sup>b</sup>A, camptothecin; B, 5-FU; C, rotenone; and D, staurosporine.

<sup>c</sup>Estimated relative potential to elicit a toxic response, such that a higher number represents a potentially safer compound and a lower number represents one more likely to be problematic.

<sup>d</sup>The ranking of D relative to A and B was not determined because we cannot compare the relative toxic potentials of a compound that elicited changes in methylation (D) with those that elicit positive results in genotoxicity assays (A and B).

- Crouch, S. P. M., Kozlowski, R., Slater, K. J., and Fletcher, J. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J. Immunol. Methods* **160**, 81–88.
- Furka, A. (2002). Combinatorial chemistry: 20 years on... *Drug Disc. Today* **7**, 1–4.
- Giannini, E., Risso, D., Ceppa, P., Botta, F., Chiarbonello, B., Fasoli, A., Malfatti, F., Romagnoli, P., Lantieri, P. B., and Testa, R. (2000). Utility of  $\alpha$ -glutathione S-transferase assessment in chronic hepatitis C patients with near normal alanine aminotransferase levels. *Clin. Biochem.* **33**, 297–301.
- Gonzalzo, M. L., Liang, G., Spruck, C. H., III, Zingg, J.-M., Rideout, W. M., III, and Jones, P. A. (1997). Identification and characterization of differentially methylated regions of genomic DNA by methylation-sensitive arbitrarily primed PCR. *Cancer. Res.* **57**, 594–599.
- Goodman, J. I., and Watson, R. E. (2002). Altered DNA methylation: A secondary mechanism involved in carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **42**, 501–525.
- Grassi, G., Maccaroni, P., Meyer, R., Kaiser, H., D'Ambrosio, E., Pascale, E., Grassi, M., Kuhn, A., DiNardo, P., Kandolf, R., et al. (2003). Inhibitors of DNA methylation and histone deacetylation activate cytomegalovirus promoter-controlled reporter gene expression in human glioblastoma cell line U87. *Carcinogenesis* **24**, 1625–1635.
- Jones, L. J., Gray, M., Yue, S. T., Haugland, R. P., and Singer, V. L. (2001). Sensitive determination of cell number using the CyQUANT cell proliferation assay. *J. Immunol. Methods* **254**, 85–98.
- Lassota, P., Singh, G., and Kramer, R. (1996). Mechanism of topoisomerase II inhibition by staurosporine and other protein kinase inhibitors. *J. Biol. Chem.* **271**, 26418–26423.
- Liu, K., Wang, Y. F., Cantemir, C., and Muller, M. T. (2003). Endogenous assays of DNA methyltransferases: Evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells *in vivo*. *Mol. Cell. Biol.* **23**, 2709–2719.
- Lu, L. J., and Randerath, K. (1984). Long term instability and molecular mechanism of 5-azacytidine-induced DNA hypomethylation in normal and neoplastic tissues *in vivo*. *Mol. Pharmacol.* **26**, 594–603.
- Mann, M. B., and Smith, H. O. (1977). Specificity of *HpaII* and *HaeIII* DNA methylases. *Nucleic Acids Res.* **4**, 4238–4243.
- McKim, J. M., Wilga, P. C., Petrella, D. K., Pregoner, J. F., Patel, R. K., and Cockerell, G. L. (2001). A new approach to *in vitro* toxicity screening based on multi-endpoint analysis provides information of mechanism and predicts *in vivo* toxicity. *Toxicol. Sci. Suppl.* **60**, 306.
- Morris, E. J., and Geller, H. M. (1996). Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-I: Evidence for cell cycle-independent toxicity. *J. Cell Biol.* **134**, 757–770.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytolethality assays. *J. Immunol. Methods* **65**, 55–63.
- Okoji, R. S., Yu, R. C., Maronpot, R. R., and Froines, J. R. (2002). Sodium arsenite administration via drinking water increases genome-wide and Haras DNA hypomethylation in methyl-deficient C57BL/6J mice. *Carcinogenesis* **23**, 777–785.
- Parker, W. B., and Cheng, Y. C. (1990). Metabolism and mechanism of action of 5-fluorouracil. *Pharmacol. Ther.* **48**, 381–395.
- Pei, W., Liou, A. K., and Chen, J. (2003). Two caspase-mediated apoptotic pathways induced by rotenone toxicity in cortical neuronal cells. *FASEB J.* **17**, 520–522.
- Ramchandani, S., Bhattachary, S. K., Cervoni, N., and Szyf, M. (1999). DNA methylation is a reversible biological signal. *Proc. Natl. Acad. Sci. USA* **96**, 6107–6112.
- Robertson, K. D., and Jones, P. A. (2000). DNA methylation: Past, present, and future directions. *Carcinogenesis* **21**, 461–467.
- Rodriguez, R. J., and Acosta, D., Jr. (1997). *N*-Deacetyl ketoconazole-induced hepatotoxicity in a primary culture system of rat hepatocytes. *Toxicology* **117**, 121–131.
- Rueff, J., Chiappella, C., Chipman, J. K., Darroudi, F., Silva, I. D., Duverger-van Bogaert, M., Fonti, E., Glatt, H. R., Isern, P., Laires, A., et al. (1996). Development and validation of alternative metabolic systems for mutagenicity testing in short-term assays. *Mutat. Res.* **353**, 151–176.
- Tennant, R. W. (2002). The national center for toxicogenomics: Using new technologies to inform mechanistic toxicology. *Environ. Health Perspect.* **110**, A8–A10.
- Watson, R. E., and Goodman, J. I. (2002a). Epigenetics and DNA methylation come of age in toxicology. *Toxicol. Sci.* **67**, 11–16.
- Watson, R. E., and Goodman, J. I. (2002b). Effects of phenobarbital on DNA methylation in GC-rich regions of hepatic DNA from mice that exhibit different levels of susceptibility to liver tumorigenesis. *Toxicol. Sci.* **68**, 51–58.
- Woo, Y. T., Lai, D. Y., Argus, M. F., and Arcos, J. C. (1995). Development of structure-activity relationship rules for predicting carcinogenic potential of chemicals. *Toxicol. Lett.* **79**, 219–228.
- Zeisel, S. H., and Blusztajn, J. K. (1994). Choline and human nutrition. *Ann. Rev. Nutr.* **14**, 269–296.