

Genotoxicity profiles of common alkyl halides and esters with alkylating activity

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Abstract

Drug synthesis and/or formulation can generate genotoxic impurities. For instance, strong acid/alcohol interactions during the process of drug salt formation produce alkylating agents such as alkyl halides and alkyl esters of alkyl sulfonic acids. The genotoxicity of a few classic alkylating agents such as methyl and ethyl methanesulfonate have been previously well characterized, whereas the majority of compounds from this class have only been tested in the Salmonella reversion assay. Therefore, the goal of this study was to investigate clastogenicity and DEL recombination profiles of 22 halogenated alkanes and alkylesters of sulfuric and alkane-, aryl-sulfonic acids using a battery of cellular and molecular assays. The in-vitro micronucleus assay in CHO cells was used to measure clastogenicity and the deletion recombination (DEL) assay in *S. cerevisiae* provided a measure of DNA deletions. We also examined the compounds' reactivity towards 4-(*p*-nitrobenzyl)pyridine (NBP), a surrogate molecule for biological ring nitrogens. Methylating agents were most potent in all three assays and the alkyl chlorides evaluated in our study were negative in all three assays. Also, a strong correlation was found between the MN, DEL and NBP assays. In summary, this study contributes to a better understanding of the genotoxic properties of common alkyl halides and alkyl esters with alkylating activity and might provide guidance for managing risk of genotoxic process-related impurities of drug substances and products.

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1. Introduction

Alkylating agents are potentially carcinogenic compounds that, due to their electrophilic nature, can introduce lesions at nucleophilic centers of DNA. Industrial syntheses generate these compounds as products or byproducts and human exposure can occur through either

direct contact with the product or through environmental contamination. Pharmaceutical companies often formulate therapeutic compounds as salts for better stability and absorption. Drug salt formation involves strong acid/base interactions in the presence of alcohol solvents, which can form by-products such as alkyl halides, as well as aryl and alkyl esters of strong acids [1,2]. The synthesis and formulation of drugs can, thus, form potentially hazardous DNA alkylating agents that become incorporated into the final product.

Alkylating compounds can vary greatly in their reactivity towards DNA. One property of an alkylating compound, which can determine DNA reactivity, is

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whether the nucleophilic attack occurs via an SN1 or SN2 mechanism. Compounds that react with DNA bases via an SN2 mechanism will primarily lead to N-alkylation because they are selective for highly nucleophilic sites [3], whereas a compound able to react via an SN1 mechanism will lead to high levels of O-alkylation [4]. *O*⁶-methylguanine is a highly promutagenic lesion leading to G:C to A:T transition mutations but this lesion is not clastogenic, whereas N-methylation is clastogenic and not mutagenic [5]. Furthermore, efficiency of DNA repair mechanisms and exposure dose can also influence the observed genotoxicity profile of an alkylating agent [6]. The fact that alkylating agents can lead to various DNA lesions and thus different genotoxic endpoints, greatly complicates the evaluation of these compounds' DNA-damaging activity and carcinogenic potential.

The goal of this study is to examine the clastogenicity and DNA deletion profiles of 22 alkylating agents commonly encountered during the drug synthesis process (Table 1). A majority of the 22 compounds have been examined in the past using the Salmonella reversion assay but very little is known about their clastogenic activity. The chemical structure of most of these compounds suggests that they are "SN2" acting compounds and are expected to primarily cause nitrogen alkylation of DNA bases. These compounds include halogenated alkanes, alkyl esters of sulfuric acid, alkyl esters of alkane- and aryl-sulfonic acids. Three assays were chosen for this study. The 4-(*p*-nitrobenzyl)pyridine (NBP) reactivity assay provided a compound's chemoselectivity for ring nitrogens. NBP serves as a surrogate molecule for biological ring nitrogens and the fraction of NBP alkylated by the test compound provides a quantitative measure of reactivity [7]. It is expected that the magnitude of ring nitrogen reactivity would reflect a compound's SN2 reactivity and thus clastogenicity. The in-vitro micronucleus assay in CHO cells (MN) measured clastogenic response [8]. Micronuclei are membrane bound fragments of DNA that have been separated from the nuclei during cell division due to chromosome breakage [9]. The yeast deletion recombination (DEL) assay provided a measure of DNA deletions [10]. Deletions occur as a result of intrachromosomal homologous recombination between direct repeats separated by a 6 kb fragment. DNA strand breaks can be deleterious to the stability of the genome. The MN assay measures chromosomal damage such as DNA strand breaks and the DEL assay measures DNA deletions, which are a type of genomic rearrangement that can result from strand breaks. Genome rearrangements are involved in carcinogenesis [11] and therefore both assays measure an endpoint that is relevant in the process of carcinogenesis.

2. Materials and methods

2.1. Chemicals

2.1.1. Yeast DEL assay

Synthetic complete (SC) medium, SC medium lacking histidine (SC-HIS) and inoculation (-LEU) medium were prepared as previously described [12]. The diploid *Saccharomyces cerevisiae* strain RS112 was used to determine the frequency of DEL recombination: MATa/MAT α ura3-52/ura3-52 leu2-3,112/leu2- Δ 98 trp5-27/TRP5 arg4-3/ARG4 ade2-40/ade2-101 ilv1-92/ILV1 HIS3::pRS6/his3 Δ 200 LYS2/lys2-801.

2.1.1.1. The recombination assay. The DEL recombination substrate was constructed by integration of plasmid pRS6 containing an internal fragment of the *HIS3* gene at the genomic *HIS3* site of the yeast strain. This resulted in two copies of the *his3* gene, one with a terminal deletion at the 3' end and the other with a terminal deletion at the 5' end [10]. Homologous recombination events between the two copies result in deletions (DEL) of the intervening sequence and restoration of the functional *HIS3* allele. Thus, cells that have undergone DEL recombination events can be detected as colonies growing on agar medium lacking histidine.

The yeast DEL assay was performed as previously described [12]. In brief, single colonies of RS112 strain were grown overnight and subcultured in the presence of test compound for 17 h at 30 °C under constant shaking. Cells were plated onto SC medium to determine the number of survivors and onto SC-HIS medium to score for DEL events.

The frequency of DEL recombination is expressed as a fraction of DEL recombination events per 10,000 viable cells.

2.1.2. Micronucleus assay

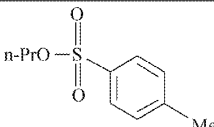
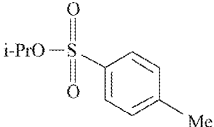
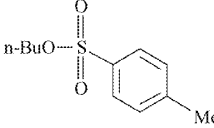
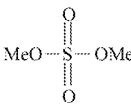
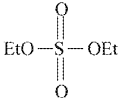
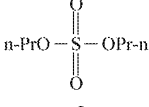
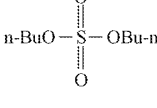
2.1.2.1. Chemicals. McCoy's 5A medium, Dulbecco's phosphate-buffered saline (1 $\times$), fetal bovine serum (FBS), antibiotics solution (5000 U/mL penicillin and 5000 μ g/mL streptomycin), trypsin (0.025%), L-glutamine—200 mM (100 $\times$) liquid, and Versene (0.2 g/L EDTA in D-PBS) were purchased from Invitrogen Corporation (Frederick, MD). Cytochalasin B (CYB) and mitomycin-C were purchased from Molecular Toxicology Incorporated (Boone, NC). Potassium chloride (KCl) was purchased from Sigma-Aldrich. Methanol and glacial acetic acid were purchased from J.T. Baker (Phillipsburg, NJ). Acridine orange was purchased from Molecular Probes Inc. (Eugene, OR). Dimethylsulfoxide (DMSO) was purchased from Pierce Endogen (Rockford, IL). The CHO-WBL cell line was obtained from the laboratory of Dr. Sheila Galloway (Merck, West Point PA).

2.1.2.2. Cell culture. CHO-WBL cells were grown in McCoy's 5A medium containing 5–10% FBS, 2% penicillin–streptomycin and 2 mM L-glutamine and maintained in a humidified incubator at 37 °C, 5% CO₂. Approximately 24 h prior to treatment, the cultures were

Table 1
List of compounds

Compound name	Structure	CAS#	Source
Alkyl halides			
<i>n</i> -Propyl chloride (<i>n</i> PC)	$\text{H}_3\text{C}-\text{CH}_2-\text{CH}_2-\text{Cl}$	540-54-5	Fluka
<i>i</i> -Propyl chloride (<i>i</i> PC)	$\begin{array}{c} \text{Cl} \\ \\ \text{H}_3\text{C}-\text{CH}-\text{CH}_3 \end{array}$	75-29-6	Aldrich
<i>n</i> -Butyl chloride (<i>n</i> BC)	$\text{H}_3\text{C}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{Cl}$	109-69-3	Fluka
Ethyl bromide (EB)	$\text{Br}-\text{CH}_2-\text{CH}_3$	74-96-4	Fluka
<i>n</i> -Propyl bromide (<i>n</i> PB)	$\text{Br}-\text{CH}_2-\text{CH}_2-\text{CH}_3$	106-94-5	Aldrich
<i>i</i> -Propyl bromide (<i>i</i> PB)	$\begin{array}{c} \text{Br} \\ \\ \text{H}_3\text{C}-\text{CH}-\text{CH}_3 \end{array}$	75-26-3	Aldrich
<i>n</i> -Butyl bromide (<i>n</i> BB)	$\text{Br}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_3$	109-65-9	Aldrich
Esters of alkyl sulfonic acids			
Methyl methanesulfonate (MMS)	$\begin{array}{c} \text{O} \\ \\ \text{MeO}-\text{S}-\text{Me} \\ \\ \text{O} \end{array}$	66-27-3	Aldrich
Ethyl methanesulfonate (EMS)	$\begin{array}{c} \text{O} \\ \\ \text{EtO}-\text{S}-\text{Me} \\ \\ \text{O} \end{array}$	62-50-0	Aldrich
<i>n</i> -Propyl methanesulfonate (<i>n</i> PMS)	$\begin{array}{c} \text{O} \\ \\ \text{n-PrO}-\text{S}-\text{Me} \\ \\ \text{O} \end{array}$	1912-31-8	
Esters of Aryl-sulfonic acids			
Methyl benzenesulfonate (MBS)	$\begin{array}{c} \text{O} \\ \\ \text{Ph}-\text{S}-\text{OMe} \\ \\ \text{O} \end{array}$	80-18-2	Acros Organics
Ethyl benzenesulfonate (EBS)	$\begin{array}{c} \text{O} \\ \\ \text{Ph}-\text{S}-\text{OEt} \\ \\ \text{O} \end{array}$	515-46-8	TCI America
<i>n</i> -Butyl benzenesulfonate (<i>n</i> BBS)	$\begin{array}{c} \text{O} \\ \\ \text{Ph}-\text{S}-\text{OBu-n} \\ \\ \text{O} \end{array}$	80-44-4	TCI America
Methyl toluenesulfonate (MTS)	$\begin{array}{c} \text{O} \\ \\ \text{MeO}-\text{S}-\text{C}_6\text{H}_4-\text{Me} \\ \\ \text{O} \end{array}$	80-48-8	Fluka
Ethyl toluenesulfonate (ETS)	$\begin{array}{c} \text{O} \\ \\ \text{EtO}-\text{S}-\text{C}_6\text{H}_4-\text{Me} \\ \\ \text{O} \end{array}$	80-40-0	Fluka

Table 1 (Continued)

Compound name	Structure	CAS#	Source
<i>n</i> -Propyl toluenesulfonate (<i>n</i> PTS)		599-91-7	TCI America
<i>i</i> -Propyl toluenesulfonate (<i>i</i> PTS)		2307-69-9	
<i>n</i> -Butyl toluenesulfonate (<i>n</i> BTS)		778-28-9	TCI America
Esters of sulfuric acid			
Dimethyl sulfate (DMS)		77-78-1	Fluka
Diethyl sulfate (DES)		64-67-5	Aldrich
Dipropyl sulfate (DPS)		598-05-0	TCI America
Dibutyl sulfate (DBS)		625-22-9	TCI America

Structures were obtained from the SciFinder database.

trypsinized and the cells were seeded in eight-well chambered slides at a density of $1.0\text{--}1.4 \times 10^4$ cells/well.

2.1.2.3. Compound preparation and cell treatment. Tested compounds were prepared as 100× stock concentrations in DMSO just prior to use. A dosing volume of 5 μL of stock concentrations diluted with 500 μL of dosing medium ensured a final DMSO concentration of ≤1%. Cells were treated with fresh treatment medium supplemented with 5% FBS and 3 μg/mL CYB along with various concentrations tested compounds, negative or positive control. CHO-WBL cells were exposed for 24 continuous hours in a humidified incubator at 37 °C, 5% CO₂. All compounds as well as the negative and positive controls were dosed in duplicate. Mitomycin-C (0.05 μg/mL) was used as the positive control and DMSO was used as the negative control. One set of negative and positive controls were run for each experimental day.

2.1.2.4. Culture termination and slide reading. The cultures were harvested using KCl (0.075 M) and 3:1 methanol to glacial acetic acid approximately 24 h post initial exposure. Analysis was performed using epi-fluorescence microscopy under high magnification (40–100×) after staining with acridine orange (31.25 μg/mL). Initially, for any given concentration or positive or negative control culture, 500 consecutive cells were scored for the presence of 1, 2 or ≥3 nuclei per well to determine %nuclear division [(total #cells with ≥2 nuclei/total #cells analyzed) × 100]. At least 500–1000 binucleated cells were scored for the presence of micronuclei in order to determine %micronucleated cells [(#micronucleated binucleate cells/total #binucleates scored) × 100]. In addition, the relative nuclear division [(%ND per culture/mean %ND negative controls) × 100] was calculated at each of the concentrations as compared to concurrent negative control data. Micronucleus scoring was consistent with the estab-

lished criteria in which micronuclei were distinct and free in the cytoplasm, $\leq 1/3$ the size of the main nucleus with similar staining intensity and within binucleated cells only with intact cytoplasmic and nuclear membranes. For each of the test articles evaluated at least three concentrations per test were scored for the presence of micronuclei. To allow statistical evaluation of the data, at least three independent experiments were performed. Attempts were made to ensure that each compound was evaluated up to a concentration that elicited a $>30\%$ suppression in relative nuclear division representing signs of cellular toxicity or reached the limits of solubility.

2.2. 4-(*p*-Nitrobenzyl)pyridine (NBP) assay

The NBP assay was performed according to Epstein et al. [13]. Tested compounds were dissolved in acetone at 4.0 mM. The reaction mix was assembled by combining 1 mL of test compound solution, 2 mL dH₂O and 4 mL of a 2% (w/v) NBP (Fluka)/Ethylene Glycol (Sigma). Blank samples consisted of 1 mL acetone, 2 mL dH₂O, and 4 mL NBP. Mixtures were incubated in a 37 °C waterbath for, 30 min, 60 min, 120 min, 180 min and 240 min. A sample at the start of the experiment (0 min) was used for background assessment. At each time interval, 0.5 mL of test solution or blank was removed and cooled on ice for approximately 1 min and 420 μ L of a 50% (v/v) solution of triethylamine (Sigma) and acetone (JT Baker) was added and thoroughly vortexed. Subsequently, the NBP turnover was quantified spectrophotometrically via measuring absorbance at 560 nm using Spectramax Plus (Molecular Devices, Sunnyvale, CA). Two replicate samples per time point were measured in each of three independent experiments. The mean of the replicates at each time interval was graphed and the corresponding linear trend line equation was calculated. The rate of the NBP turnover, derived from the equations, was then expressed as OD/h. A total of three independent experiments were performed with each tested compound and the average of the three tests was reported.

2.3. Statistical analysis

Statistical significance in the DEL and MN assays was determined using one-way ANOVA analysis followed by Dunnett multiple comparison test (comparing each exposure concentration to the zero control). Correlation analysis was determined using Pearson test. Significance in the NBP assay was determined using the student's *t*-test. All statistical analysis was carried out using GraphPad Prism statistical software.

3. Results

Table 2 summarizes the data obtained for the three assays in this study. The MN assay in CHO cells, the DEL assay in yeast cells and the NBP chemical reactivity assay. The percent relative division (RD%) in CHO cells

and percent relative survival (RS%) in yeast cells provide a measure of cellular toxicity.

3.1. Alkyl halides

All three alkyl chlorides tested in this study were negative in the MN and DEL assays and were not reactive towards the nucleophile NBP. Alkyl bromides exhibited no clastogenic activity in the MN assay. However, ethyl bromide and *n*- and *i*-propyl bromides were positive for inducing deletions in the DEL assay. *n*-Butyl bromide did not elicit a response in either genotoxicity assay but it weakly reacted with NBP. All six alkyl halides led to toxicity in the yeast cells, but induced very little toxicity in the CHO cells. Therefore, it is not clear whether the negative results in the CHO cells are due to lack of genotoxic response or simply lack of availability of the compounds.

3.2. Esters of alkyl sulfonic acids

Methyl-, ethyl-, and *n*-propyl methanesulfonate produced a positive response in the MN and DEL assays. Ethyl methanesulfonate, however, tested negative for NBP reactivity because it did not cause a reproducibly significant increase in NBP alkylation. Since EMS causes 11-fold higher NBP alkylation than the control, the negative response is most likely due to a high degree of experimental variation rather than a true lack of reactivity.

3.3. Esters of aryl-sulfonic acids

All three benzenesulfonates produced a positive response in the MN and DEL assays and reacted with NBP. Methyl-, ethyl-, and *n*-butyl-toluenesulfonate tested positive in all three assays and were reactive with NBP. *n*-Propyl toluenesulfonate was positive in the DEL assay, negative in the MN assay and weakly reactive towards NBP. *i*-Propyl toluenesulfonate was positive in the MN assay and was not reactive with NBP but was not studied in the DEL assay due to the limited availability of the compound. Methyl benzenesulfonate and methyl toluenesulfonate were the most reactive towards NBP in the aryl-sulfonic group. Both compounds alkylated NBP approximately 700-fold over the control. The other esters in this group all led to a 50-fold or lower increase in NBP alkylation.

3.4. Esters of sulfuric acid

Dimethyl sulfate, diethyl sulfate, dipropyl sulfate and dibutyl sulfate were positive in the MN and DEL assays.

Table 2
Results of the micronucleus assay (MN), DEL assay and NBP test system^a

Compound	Micronucleus (CHO cells)				DEL (<i>S. Cervisiae</i>)				NBP alkylation $\Delta 560/1$ h
	Concentration ($\mu\text{g/mL}$)	%MN ^b	Ave. fold increase	RD% ^c	Concentration ($\mu\text{g/mL}$)	His+/10 ⁴ viable cells ^b	Ave. fold increase	RS% ^c	Fold increase ^d
<i>n</i> -Propyl chloride	0	1.07 $\pm$ 0.07	1.0	100.0	0	1.46 $\pm$ 0.33	1.0	100.0	
	3211	1.03 $\pm$ 0.07	1.0	99.9	4450	1.92 $\pm$ 0.38	1.3	52.4	
	3784	1.13 $\pm$ 0.03	1.1	99.7	6675	1.84 $\pm$ 0.35	1.3	42.9	
	4460	1.37 $\pm$ 0.12	1.3	91.9	8900	2.02 $\pm$ 0.41	1.4	39.6	(0.0049 $\pm$ 0.0020)
Assay result			Negative				Negative		2.11 Negative
<i>i</i> -Propyl chloride	0	1.13 $\pm$ 0.07	1.0	100.0	0	1.41 $\pm$ 0.44	1.0	100.0	
	3673	1.43 $\pm$ 0.33	1.3	104.0	6450	1.77 $\pm$ 0.37	1.3	42.4	
	4286	1.30 $\pm$ 0.12	1.1	103.1	8600	1.56 $\pm$ 0.13	1.1	63.7	
	5000	1.97 $\pm$ 0.42	1.7	96.8	12900	2.25 $\pm$ 1.63	1.6	40.0	(0.0028 $\pm$ 0.0022)
Assay result			Negative				Negative		1.20 Negative
<i>n</i> -Butyl chloride	0	1.20 $\pm$ 0.11	1.0	100.0	0	1.85 $\pm$ 0.54	1.0	100.0	
	3154	1.17 $\pm$ 0.23	1.0	94.8	890	1.89 $\pm$ 0.45	1.0	80.0	
	3544	1.60 $\pm$ 0.15	1.3	88.8	2225	2.05 $\pm$ 0.27	1.1	68.9	
	4430	1.90 $\pm$ 0.06*	1.6	90.7	4450	1.94 $\pm$ 0.07	1.0	46.6	
					8900	3.30 $\pm$ 0.60	1.8	5.0	(0.0031 $\pm$ 0.0013)
Assay result			Negative*				Negative		1.37 Negative
Ethyl bromide	0	1.20 $\pm$ 0.00	1.0	100.0	0	1.10 $\pm$ 0.30	1.0	100.0	
	5000	1.40 $\pm$ 0.15	1.2	91.5	3650	7.18 $\pm$ 1.7	6.5	51.4	
	6257	1.07 $\pm$ 0.09	0.9	98.5	7300	14.33 $\pm$ 3.54**	13.1	25.6	
	7300	1.03 $\pm$ 0.09	0.9	93.5	14600	14.50 $\pm$ 2.59**	13.2	1.8	
					21900	19.20 $\pm$ 4.50**	17.5	0.4	(0.0244 $\pm$ 0.0175)
Assay result			Negative				Positive**		10.59 Negative
<i>n</i> -Propyl bromide	0	1.23 $\pm$ 0.15	1.0	100.0	0	1.07 $\pm$ 0.15	1.0	100.0	
	3687	1.07 $\pm$ 0.20	0.9	92.0	1350	2.21 $\pm$ 0.21	2.1	64.3	
	4326	1.00 $\pm$ 0.10	0.8	92.7	3375	4.36 $\pm$ 0.67	4.1	43.2	
	5000	1.10 $\pm$ 0.26	0.9	98.8	6750	10.06 $\pm$ 1.33*	9.4	0.3	(0.0179 $\pm$ 0.0104)
Assay result			Negative				Positive**		7.80 Negative
<i>i</i> -Propyl bromide	0	1.20 $\pm$ 0.00	1.00	100.0	0	1.40 $\pm$ 0.43	1.0	100.0	
	3472	1.00 $\pm$ 0.10	0.83	99.4	3275	4.87 $\pm$ 0.32	3.5	44.2	
	4167	1.03 $\pm$ 0.07	0.83	91.3	6550	10.94 $\pm$ 2.87**	7.8	30.4	
	5000	1.13 $\pm$ 0.03	0.92	81.0	13100	11.75 $\pm$ 1.54**	8.4	14.0	
					19650	8.16 $\pm$ 0.38*	5.8	3.0	(0.0048 $\pm$ 0.0022)
Assay result			Negative				Positive**		2.10 Negative

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Table 2 (Continued)

Compound	Micronucleus (CHO cells)				DEL (<i>S. Cervisiae</i>)				NBP alkylation $\Delta 560/1$ h
	Concentration ($\mu\text{g/mL}$)	%MN ^b	Ave. fold increase	RD% ^c	Concentration ($\mu\text{g/mL}$)	His+/10 ⁴ viable cells ^b	Ave. fold increase	RS% ^c	Fold increase ^d
<i>n</i> -Butyl bromide	0	1.20 $\pm$ 0.12	1.0	100.0	0	1.72 $\pm$ 0.65	1.0	100.0	
	3472	0.90 $\pm$ 0.10	0.8	89.2	64	1.11 $\pm$ 0.23	0.6	105.4	
	4167	1.07 $\pm$ 0.15	0.9	84.1	128	1.81 $\pm$ 0.34	1.0	100.0	
	5000	1.17 $\pm$ 0.07	1.0	78.9	1280	2.31 $\pm$ 0.77	1.3	73.0	(0.0175 $\pm$ 0.0039*)
Assay result			Negative				Negative		7.62
Methyl methanesulfonate	0	1.27 $\pm$ 0.09	1.0	100.0	0	2.21 $\pm$ 0.64	1.0	100.0	
	39.4	10.77 $\pm$ 0.88**	8.5	86.1	13	4.94 $\pm$ 0.53	2.2	54.8	
	47.28	10.40 $\pm$ 0.85**	8.2	73.2	65	14.21 $\pm$ 0.40**	6.4	23.7	
	68.1	9.57 $\pm$ 0.98**	7.6	68.2	130	21.27 $\pm$ 3.47**	9.6	16.5	(0.9166 $\pm$ 0.2846*)
Assay result			Positive**				Positive**		398.54
Ethyl methanesulfonate	0	1.33 $\pm$ 0.03	1.0	100.0	0	1.34 $\pm$ 0.35	1.0	100.0	
	929	5.43 $\pm$ 0.38**	4.1	72.6	58.35	4.31 $\pm$ 0.57	3.2	84.9	
	1114	6.30 $\pm$ 0.26**	4.7	64.1	116.7	7.35 $\pm$ 0.90	5.5	71.6	
	1338	5.10 $\pm$ 0.75**	3.8	50.7	583.5	11.43 $\pm$ 0.27	8.5	47.1	
Assay result			Positive**				Positive**		18.5
					1167	20.22 $\pm$ 7.33*	15.1		(0.0261 $\pm$ 0.0113)
					2917.5	29.83 $\pm$ 5.37**	22.2		11.35
									Negative
<i>n</i> -Propyl methanesulfonate	0	1.27 $\pm$ 0.03	1.0	100.0	0	1.09 $\pm$ 0.03	1.0	100.0	
	941	10.00 $\pm$ 1.08**	7.9	79.1	130	3.69 $\pm$ 0.52	3.4	77.9	
	1471	11.13 $\pm$ 0.29**	8.8	61.6	650	9.07 $\pm$ 1.56*	8.3	47.4	
	1838	12.93 $\pm$ 1.04**	10.2	41.6	1300	23.97 $\pm$ 2.66**	22.1	13.5	(0.0157 $\pm$ 0.0004**)
Assay result			Positive**				Positive**		6.83
Methyl benzenesulfonate	0	1.23 $\pm$ 0.07	1.0	100.0	0	2.09 $\pm$ 0.55	1.0	100.0	
	20.9	6.00 $\pm$ 1.44	4.9	83.5	12.5	31.73 $\pm$ 8.07*	15.2	47.8	
	26.2	9.83 $\pm$ 3.26*	8.0	72.3	62.5	46.44 $\pm$ 7.85**	22.2	7.3	
	32.7	8.37 $\pm$ 0.99	6.8	60.4	125	48.43 $\pm$ 8.29**	23.2	3.2	(1.5691 $\pm$ 0.1135**)
Assay result			Positive*				Positive**		682.20
Ethyl benzenesulfonate	0	1.27 $\pm$ 0.03	1.0	100.0	0	1.63 $\pm$ 0.36	1.0	100.0	
	110	1.20 $\pm$ 0.00	0.9	96.3	121	7.46 $\pm$ 1.52	4.6	91.0	
	137	2.20 $\pm$ 0.40	1.7	76.8	1210	26.96 $\pm$ 11.08*	16.6	39.3	
	172	2.63 $\pm$ 0.26**	2.0	46.8	3025	20.43 $\pm$ 4.28	12.6	10.2	(0.1259 $\pm$ 0.0073**)
Assay result			Positive**				Positive*		54.74
<i>n</i> -Butyl benzenesulfonate	0	1.20 $\pm$ 0.06	1.0	100.0	0	1.27 $\pm$ 0.29	1.0	100.0	
	17.5	1.87 $\pm$ 0.12	1.6	87.7	2875	3.61 $\pm$ 0.68	2.8	67.4	
	21.9	1.83 $\pm$ 0.17	1.5	59.6	5750	4.55 $\pm$ 0.58*	3.6	49.2	
	27.4	2.60 $\pm$ 0.26**	2.2	71.1	11500	4.60 $\pm$ 0.51*	3.6	25.3	
Assay result			Positive**				Positive**		17.8
					17250	6.10 $\pm$ 0.68**	4.8		(0.0928 $\pm$ 0.0098**)
					23000	6.57 $\pm$ 1.26**	5.2		40.35
									Positive

Table 2 (Continued)

Compound	Micronucleus (CHO cells)				DEL (<i>S. Cervisiae</i>)				NBP alkylation $\Delta 560/1$ h
	Concentration ($\mu\text{g/mL}$)	%MN ^b	Ave. fold increase	RD% ^c	Concentration ($\mu\text{g/mL}$)	His+/10 ⁴ viable cells ^b	Ave. fold increase	RS% ^c	Fold increase ^d
Methyl toluenesulfonate	0	1.13 $\pm$ 0.03	1.0	100.0					
	86	2.07 $\pm$ 0.09	1.8	91.4					
	108	3.97 $\pm$ 0.29**	3.5	81.0					
	135	11.80 $\pm$ 0.53**	10.4	61.3					(1.6382 $\pm$ 0.1401**)
Assay result			Positive**				N/A		712.28 Positive
Ethyl toluenesulfonate	0	1.07 $\pm$ 0.07	1.0	100.0	0	1.96 $\pm$ 0.19	1.0	100.0	
	154	1.87 $\pm$ 0.09**	1.8	86.0	58.5	3.37 $\pm$ 0.21	1.7	149.0	
	165	2.07 $\pm$ 0.23**	1.9	66.1	117	6.07 $\pm$ 0.74	3.1	136.6	
	177	2.77 $\pm$ 0.09**	2.6	53.6	585	15.18 $\pm$ 3.62*	7.7	14.0	
					1170	17.97 $\pm$ 5.15**	9.2	9.7	(0.0918 $\pm$ 0.0138*)
Assay result			Positive**		2925	19.24 $\pm$ 2.78**	9.8	21.7	39.91 Positive
<i>n</i> -Propyl toluenesulfonate	0	1.23 $\pm$ 0.09	1.0	100.0	0	1.07 $\pm$ 0.42	1.0	100.0	
	30	1.17 $\pm$ 0.03	0.9	90.8	575	3.46 $\pm$ 0.33**	3.2	34.0	
	38	0.90 $\pm$ 0.00	0.7	76.4	2875	3.28 $\pm$ 0.07**	3.1	34.9	
	45	1.07 $\pm$ 0.15	0.9	57.4	5750	3.65 $\pm$ 0.09**	3.4	32.0	
					11500	3.59 $\pm$ 0.26**	3.3	14.9	(0.0556 $\pm$ 0.0066**)
Assay result			Negative				Positive**		24.19 Positive
<i>i</i> -Propyl toluenesulfonate	0	1.07 $\pm$ 0.14	1.0	100.0					
	655	3.83 $\pm$ 0.64*	3.5	89.6					
	819	3.37 $\pm$ 0.78	3.1	72.2					(0.0165 $\pm$ 0.0057)
	1024	4.57 $\pm$ 0.63**	4.2	49.1					7.18
Assay result			Positive*				N/A		Negative
<i>n</i> -Butyl toluenesulfonate	0	1.10 $\pm$ 0.06	1.0	100.0	0	1.56 $\pm$ 0.38	1.00	100.0	
	44	1.67 $\pm$ 0.15	1.5	80.3	1120	2.11 $\pm$ 0.29	1.36	66.7	
	48	2.90 $\pm$ 0.21**	2.6	68.6	2800	1.94 $\pm$ 0.28	1.24	94.3	
	52	2.43 $\pm$ 0.42*	2.2	54.9	11200	2.35 $\pm$ 0.73	1.51	44.6	
					16800	5.03 $\pm$ 0.47**	3.23	7.6	(0.0528 $\pm$ 0.0005**)
Assay result			Positive**				Positive**		22.97 Positive
Dimethyl sulfate	0	1.20 $\pm$ 0.06	1.0	100.0	0	0.94 $\pm$ 0.17	1.00	100.0	
	58.5	11.97 $\pm$ 1.99**	10.0	75.4	13.3	11.92 $\pm$ 0.42**	12.62	63.4	
	73.1	14.33 $\pm$ 0.48**	11.9	58.4	66.5	21.17 $\pm$ 0.66**	22.40	38.9	
	91.4	16.87 $\pm$ 0.27**	14.1	39.0	133	34.78 $\pm$ 0.66**	36.81	15.9	(6.7632 $\pm$ 0.3500**)
Assay result			Positive**				Positive**		2940.51 Positive
Diethyl sulfate	0	1.17 $\pm$ 0.15	1.0	100.0	0	1.76 $\pm$ 0.27	1.00	100.0	
	385	3.57 $\pm$ 0.38*	3.1	87.1	118	11.86 $\pm$ 1.15	6.73	71.3	
	482	5.33 $\pm$ 0.22**	4.6	71.7	590	19.12 $\pm$ 5.0**	10.85	48.3	
	602	7.20 $\pm$ 0.81**	6.2	49.3	1180	15.04 $\pm$ 1.69*	8.53	38.7	(0.7891 $\pm$ 0.0364**)
Assay result			Positive**				Positive*		343.10 Positive

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Table 2 (Continued)

Compound	Micronucleus (CHO cells)			DEL (<i>S. Cervisiae</i>)			NBP alkylation Δ560/1 h	
	Concentration (μg/mL)	%MN ^b	Ave. fold increase	RD% ^c	Concentration (μg/mL)	His+/10 ⁴ viable cells ^b	Ave. fold increase	RS% ^c
Dipropyl sulfate	0	1.23 ± 0.03	1.0	100.0	0	2.14 ± 0.50	1.00	100.0
	568	2.77 ± 0.07**	2.2	77.9	113	8.71 ± 1.54	4.08	114.7
	710	2.30 ± 0.20*	1.9	67.8	565	15.48 ± 1.72	7.25	79.0
	888	2.70 ± 0.35**	2.2	50.2	1130	24.71 ± 4.86**	11.56	43.9
Assay result					2825	36.63 ± 5.27**	17.14	20.0
			Positive**					(0.5097 ± 0.0132**)
								221.61
								Positive
Dibutyl sulfate	0	1.23 ± 0.03	1.0	100.0	0	1.87 ± 0.55	1.00	100.0
	114	1.60 ± 0.15	1.3	86.8	106	2.88 ± 0.37	1.54	117.0
	142	2.00 ± 0.26*	1.6	70.4	530	6.14 ± 1.26	3.28	108.0
	178	3.10 ± 0.21**	2.5	62.5	1060	9.84 ± 1.43*	5.26	53.3
Assay result					2650	10.42 ± 1.03*	5.57	62.5
			Positive**		5300	16.85 ± 3.57**	9.00	275.65
								(0.6340 ± 0.0776**)
								Positive

^a (*) *p*-value < 0.05, (**) *p*-value < 0.01. Asterisks for qualitative results (positive/negative) denote significance level based on ANOVA analysis and asterisks for individual exposure concentrations denote significance based on Dunnett multiple comparison test.

^b Results are presented as the mean ± standard error for at least three independent experiments.

^c %Relative division (%RD) for the MN assay and %relative survival (RS%) for the DEL assay are presented as the average of at least three independent experiments.

^d Results are presented as an average fold-increase over the control, which was 0.0023 ± 0.0015. The numbers in parentheses represent the mean ± standard error for the NBP assay for at least three independent experiments.

The three compounds were highly reactive towards NBP. Dimethyl sulfate was the most reactive of all 22 compounds in this study, leading to an approximately 3000-fold increase in NBP alkylation. The other esters in this group led to an approximately 300-fold increase in NBP alkylation.

3.5. Magnitudes of induction

Table 3 compares the genotoxic potency of the positive compounds within each assay. For the MN and DEL assays, a two-fold increase in genotoxic endpoint over the control is the minimum increase required for a positive response. We, therefore, extrapolated the concentration that leads to a two-fold induction from the dose–response curves of each compound in the MN and DEL assays. This provided a way to rank the compounds based on the minimum concentration required to elicit a positive response. In the NBP test system, the compounds are tested at identical concentrations and the compounds are ranked by fold increase over control. For the MN and DEL assay, the magnitude of positive responses is generally associated with cellular toxicity. To evaluate the association between magnitude of genotoxicity and magnitude of cellular toxicity we first extrapolated the concentration that leads to 70% relative division in the CHO cells and 50% relative survival in the yeast cells (data not shown). This allowed us to quantify the magnitude of toxicity of each compound in the two systems. We then tested the correlation between magnitude of toxicity and magnitude of genotoxicity. We found that in both the DEL assay (*R*² = 0.59, *P*-value = 0.0005) and in the MN assay (*R*² = 0.42, *P*-value = 0.012) there is a significant correlation between cellular toxicity and genotoxicity.

Fig. 1 graphically illustrates the potency of each compound in the three assays. The y-axis provides a reciprocal value of the concentrations presented in Table 3. This allows for a more intuitive presentation of magnitude since a small concentration means higher potency. Methylating agents are most potent in all three test systems. In the NBP reactivity assay, methylating agents are followed in potency by esters of sulfuric acid and esters of aryl-sulfonic acid. This implies that the leaving group determines potency of ring nitrogen alkylation. In the DEL assay, ethylating and propylating agents are next most potent followed by butylating agents and alkyl bromides. In the MN assay butylating agents are next most potent after methylating agents, followed by propylating and ethylating agents.

Table 3
Genotoxic potency ranking

Compound	2-fold %MN Induction		
	mM	µg/mL	Rank
(A) MN assay			
Methyl benzenesulfonate	0.03	5.5	1
Methyl methanesulfonate	0.05	5	2
Dimethyl sulfate	0.05	6	3
<i>n</i> -Butyl benzenesulfonate	0.12	26	4
<i>n</i> -Butyl toluenesulfonate	0.20	46	5
Methyl toluenesulfonate	0.47	88	6
Diethyl sulfate	0.52	80	7
Dibutyl sulfate	0.75	157	8
Ethyl toluenesulfonate	0.83	166	9
Ethyl benzenesulfonate	0.92	172	10
<i>n</i> -Propyl methanesulfonate	0.94	130	11
<i>i</i> -Propyl toluenesulfonate	1.21	260	12
Ethyl methanesulfonate	2.42	300	13
Dipropyl sulfate	2.47	450	14
Compound	2-fold DEL induction		
	mM	µg/mL	Rank
(B) DEL assay			
Dimethyl sulfate	0.01	1.15	1
Methyl benzenesulfonate	0.05	9	2
Methyl methanesulfonate	0.10	10.5	3
Diethyl sulfate	0.14	21	4
Ethyl benzenesulfonate	0.18	34	5
Dipropyl sulfate	0.20	36	6
Ethyl methanesulfonate	0.21	26	7
Ethyl toluenesulfonate	0.35	70	8
<i>n</i> -Propyl methanesulfonate	0.39	54	9
Dibutyl sulfate	1.05	220	10
<i>n</i> -Propyl toluenesulfonate	1.21	260	11
Ethyl bromide	6.06	660	12
<i>n</i> -Butyl benzenesulfonate	7.19	1540	13
<i>n</i> -Propyl bromide	10.24	1260	14
<i>i</i> -Propyl bromide	10.73	1320	15
<i>n</i> -Butyl toluenesulfonate	56.07	12800	16
Compound	NBP reactivity		Rank
	Fold increase		
(C) NBP alkylation Δ560/1 h			
Dimethyl sulfate	2940.5		1
Methyl toluenesulfonate	712.28		2
Methyl benzenesulfonate	682.2		3
Methyl methanesulfonate	398.54		4
Diethyl sulfate	343.1		5
Dibutyl sulfate	275.65		6
Dipropyl sulfate	221.61		7
Ethyl benzenesulfonate	54.74		8
<i>n</i> -Butyl benzenesulfonate	40.35		9
Ethyl toluenesulfonate	39.91		10
<i>n</i> -Propyl toluenesulfonate	24.19		11
<i>n</i> -Butyl toluenesulfonate	22.97		12
<i>n</i> -Butyl bromide	7.62		13
<i>n</i> -Propyl methanesulfonate	6.83		14

For the MN and DEL assays, concentrations leading to a two-fold induction in genotoxic endpoint were extrapolated from the dose–response curves for each compound. The concentrations were then used to rank the compounds by potency.

3.6. Comparison with other studies

Table 4 summarizes the qualitative assay results for this study and also presents available data from the literature for the Salmonella reverse mutation assay and animal carcinogenicity studies. The concordance between the DEL assay and the available data for the SAL assay is 94%, MN and SAL is 74% and NBP and SAL is 74%. Alkyl chlorides are negative in all three test systems. Alkyl bromides are negative in the MN assay when tested to limits of solubility, and show no suppression in relative division (Table 2). Alkyl bromides are negative in the NBP test system with the exception of *n*-butyl bromide, which is weakly reactive towards NBP. Ethyl bromide, *n*-propyl bromide and *i*-propyl bromide are positive in the DEL assay. Alkyl methanesulfonates are positive in all three test systems with the exception of EMS, which did not cause a statistically significant increase in NBP alkylation, probably due to high experimental variability. Alkyl esters of aryl-sulfonic acids are positive in all test systems with the exception of *n*-propyl toluenesulfonate, which is negative for MN induction and *i*-propyl toluenesulfonate, which is negative for NBP reactivity. Without exception, alkyl esters of sulfuric acid are positive in all test systems.

4. Discussion

In this study, we examined the genotoxicity profiles of 22 alkyl esters with alkylating activity that are often encountered as byproducts of drug synthesis and formulation [1]. We used the MN assay for clastogenicity, the DEL assay for DNA deletions and the NBP assay for DNA reactivity. The 20 compounds evaluated in the MN and DEL assays demonstrated 80% concordance between the two assays, of the 22 compounds evaluated in the MN and NBP assays, there was 82% concordance and of the 20 compounds examined in DEL and NBP, there was 75% concordance (Table 4). Of the 12 compounds positive in both MN and DEL assays 11 were also positive for NBP reactivity, the exception being EMS. The NBP result for EMS is very likely a false negative since it led to an 11-fold increase in alkylation and EMS has been previously shown to be reactive towards NBP [23]. The high degree of concordance between the clastogenicity assay, the DNA deletion assay and NBP reactivity further underlines the paradigm that ring nitrogen alkylation is an important factor leading to chromosome damage. Nonetheless, published results for the Salmonella reversion assay (Table 4) demonstrate that this set of alkylating compounds possess both mutagenic and clastogenic activity.

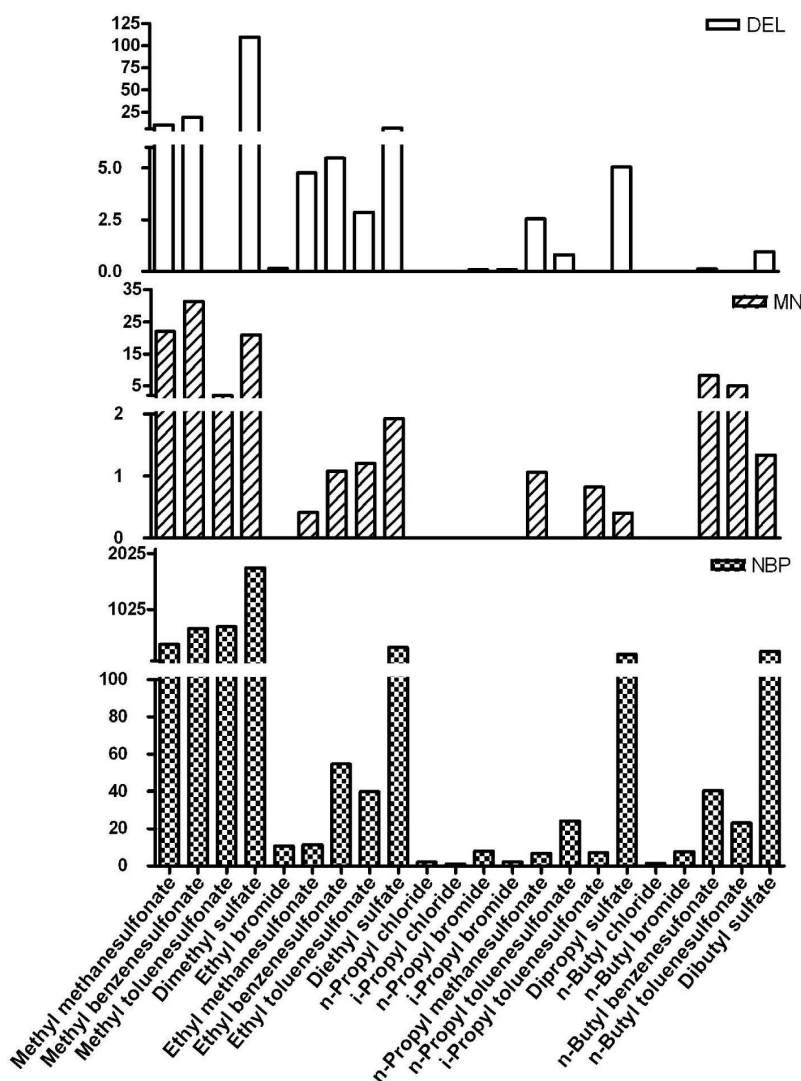


Fig. 1. Graphical representation of genotoxic potency. The values presented in this graph are reciprocals of the concentrations presented in Table 3 for the MN and DEL assays. For NBP, the graph shows actual fold-increase over control. Compounds are organized by alkyl group.

Alkylating agents can lead to a variety of DNA damage and regardless of the strong link between ring nitrogen alkylation and clastogenicity, other factors must influence a compound's genotoxic potential. This becomes evident when we compare the compounds' genotoxic potency in the three different assays (Table 3, Fig. 1). The size of the alkyl group appears to be an important factor in the two whole-cell systems, whereas the leaving group determines the magnitude of NBP reactivity. The importance of the alkyl group on geno-

toxicity has been shown previously [2]. The disparity in the potency pattern suggests that the magnitude of clastogenic potential of alkylating agents cannot be easily predicted by ring nitrogen reactivity alone. *n*-Propyl methanesulfonate (*n*PMS) is the least reactive towards NBP of all compounds that tested positive but it is a strong inducer of both micronuclei and DNA deletions (Table 3). Previous studies have shown that *n*PMS is not only weakly reactive toward NBP but also very weakly reactive toward trifluoroacetic acid (TFA) and

Table 4
Summary of genotoxicity data for 22 alkylating compounds

Compound	MN ^a	DEL ^a	NBP ^a	Salmonella reversion ^b	Animal carcinogenicity ^b
<i>n</i> -Propyl chloride	—	—	—	—[14]	
<i>i</i> -Propyl chloride	—	—	—		
<i>n</i> -Butyl chloride	—	—	—	—[15,16]	—[17]
Ethyl bromide	—	+	—	+ [16]	+ [18,19]
<i>n</i> -Propyl bromide	—	+	—	+ [16]	
<i>i</i> -Propyl bromide	—	+	—	+ [20]+S9	
<i>n</i> -Butyl bromide	—	—	+	+ [16]	
Methyl methanesulfonate	+	+	+	+ [21]	+ [22]
Ethyl methanesulfonate	+	+	—	+ [21]	+ [22]
<i>n</i> -Propyl methanesulfonate	+	+	+	+ [23]	
Methyl benzenesulfonate	+	+	+	+ [2]	
Ethyl benzenesulfonate	+	+	+	+ [2]	
<i>n</i> -Butyl benzenesulfonate	+	+	+	+ [2]	
Methyl toluenesulfonate	+		+	+ [2]	
Ethyl toluenesulfonate	+	+	+	+ [24]	
<i>n</i> -Propyl toluenesulfonate	—	+	+	+ [2]	
<i>i</i> -Propyl toluenesulfonate	+		—	+ [2]	
<i>n</i> -Butyl toluenesulfonate	+	+	+	+ [2]	
Dimethyl sulfate	+	+	+	+ [25]	+ [22]
Diethyl sulfate	+	+	+	+ [25]	+ [22]
Dipropyl sulfate	+	+	+		
Dibutyl sulfate	+	+	+		

Blanks in the table indicate that no data were available.
^a Results obtained in the current study.
^b Results from published literature.

H₂O, both measure oxygen reactivity [23]. This highlights that the magnitude of nitrogen or oxygen reactivity does not necessarily predict the magnitude of genotoxicity in a whole-cell system. Perhaps the distribution of alkyl DNA products predicts genotoxicity better than one specific lesion, as previously suggested [26]. Unfortunately, only four compounds (MMS, DMS, EMS and DES) in this study have been studied for their distribution of alkyl DNA products in double stranded DNA [27]. We have performed a correlation analysis for these four compounds between magnitudes of DEL and MN induction and the three major DNA alkylation products: N3 of adenine, N7 of guanine and O⁶ of guanine (data not shown). There was no statistically significant correlation between any parameter. The very small data set prohibits further analysis of the relationship between sites of DNA alkylation and magnitude of genotoxicity.

A compound's ability to traverse the cell membrane might also influence its magnitude of genotoxicity in a whole-cell system. However, if this were the case, we would expect that the size of the whole molecule would be a factor rather than the size of the alkyl group. We would expect the large benzenesulfonates and toluenesulfonates to be less able to permeate the cell than the much smaller propyl methanesulfonate; however, methyl benzenesulfonate is much more potent

than propyl methanesulfonate in both CHO and yeast cells.

Besides permeability, efficiency and mechanisms of DNA repair may play an important role in determining genotoxic outcome in a whole-cell system. Overall DNA repair capacity of a cell can influence formation of micronuclei [28]. The O⁶-methylguanine lesion, usually associated with point mutations, can lead to recombination and clastogenicity if it is not repaired by base excision repair enzymes before the cell undergoes DNA replication [29,30]. Bulky DNA lesions that lead to polymerase arrest can cause chromosome damage or point mutations depending on whether they are repaired by a low fidelity lesion by-pass polymerase or simply cause replication fork arrest [31]. The 3-methyladenine lesion is both clastogenic and recombinagenic [32]. This lesion is located in the minor groove of DNA where steric interactions might have an effect on alkylating activity. Such steric interactions would not factor into reactivity toward NBP or other chemical reactivity assays. Thus, the disparity in potency between the NBP assay, where the leaving group determines potency, and the whole-cell systems, where the alkyl group determines potency, can result from many confounding factors.

It is not surprising to find that a chemical assay might not be sensitive to all the nuances that affect a genotoxic

response in a whole cell. However, we also found differences between the two whole-cell systems. In CHO cells, the butylating agents were potent inducers of micronuclei and in the yeast DEL assay the butylating agents were the least potent. Differences in cell type must play some role in this distinction. Previously published data on a similar set of compounds shows that butyl benzene-sulfonate, a potent inducer of micronuclei in the current study, failed to induce micronuclei in a mouse lymphoma cell line [2]. Differences between cell lines can result from differences in absorption, as well as mechanisms and fidelity of DNA repair, as discussed above.

DNA repair mechanisms become particularly important when we consider that the MN assay and DEL assay measure different genotoxic endpoints. The MN assay measures chromosome breakage (clastogenicity) or chromosome loss (aneugenicity). Clastogenic activity leads to the inclusion of fragments of chromosomes in the micronuclei, whereas aneugenic activity would lead to the inclusion of an entire chromosome [33]. Alkylating agents can induce micronuclei via base excision and nucleotide excision repair intermediates [34,35]. DNA deletions, as measured by the yeast DEL assay, can occur as a result of an intrachromosomal rearrangement event. This rearrangement can proceed via several possible pathways and is highly inducible by double strand DNA breaks [36]. Alkylating agents induce DEL recombination during DNA replication [37]. This implies that in response to alkylating DNA damage DEL recombination occurs via a single strand break that is converted to a double strand break during DNA replication. This single strand break can result from base excision repair or polymerase arrest during replication [38,39]. Polymerase arresting lesions are known to cause DNA deletions such as the ones measured by the DEL assay [40]. Since double strand breaks, polymerase arrest and DNA excision repair intermediates lead to both DEL recombination and micronuclei, there is a great deal of overlap in the type of DNA damage measured by both assays. Therefore, it is not clear whether the difference in sensitivity to various alkylating agents is due to differences in cell type or differences in the endpoints measured.

A striking discrepancy between DEL and the other two assays in this study is in the results for the alkyl bromide compounds. A possible explanation is that these compounds are highly volatile and the DEL assay, unlike MN and NBP, is carried out in closed, screw-cap tubes thus making the compound more accessible to the yeast cells. Lack of accessibility to the CHO cells could also explain why the alkyl halides did not cause a significant reduction in percent relative division. This explanation is supported by studies done with the Salmonella rever-

sion assay adapted for volatile compounds, where ethyl, *n*-propyl and butyl bromides were positive and *n*-butyl chloride was negative [16]. Furthermore, ethyl bromide is classified as an animal carcinogen [18,19]. For this reason alkyl bromides may be of considerable concern in spite of the fact that they test negative in some test systems.

Some patterns observed in our study have been published previously. Methylating agents have previously exhibited highest reactivity towards NBP [7] and were more genotoxic in the Salmonella reverse mutation assay [23]. In studies with isolated bacterial DNA, DMS and MMS are much more reactive than DES and EMS [41]. Although the published literature provides extensive information about MMS, DMS, EMS and DES, much less is known about the other compounds in this study. It is, therefore, notable that methyl esters of aryl sulfonic acids are also much more potent than the ethyl esters.

One of the goals of genotoxicity testing is to predict carcinogenic potential. Consequently, it was important to compare known carcinogenicity data with the results collected in this study. However, animal carcinogenicity data are available for only six compounds in this study. A comparison reveals 100% concordance with the DEL assay, 100% concordance with the SAL assay, 83% concordance with the MN assay and 67% concordance with the NBP assay. A standardized TD50 value (a daily dose that induced tumors in half of the test animals) is available for only two compounds in this study. In the mouse, TD50 for methyl methanesulfonate is 31.8 mg/kg/day and for ethyl bromide the TD50 is 535 mg/kg/day [42]. This potency is consistent with the DEL assay results in this study where methyl methanesulfonate induces deletions at 0.1 mM and ethyl bromide induces deletions at 6.06 mM (Table 3B). A similar comparison with the other assays in this study was not possible because ethyl bromide was negative in the MN and NBP assays.

The high concordance between the DEL assay and other in vitro genotoxicity assays presented in this study highlights the DEL assay as a powerful tool in genotoxicity testing. The assay design makes it possible to study volatile compounds without modifying the basic protocol, i.e. evaluation of alkyl halides. The fact that the DEL assay correctly identified five animal carcinogens as positive and one non-carcinogen as negative further proves that DNA deletions, which are a type of genomic rearrangement, are an important measure of carcinogenic potential.

The findings of this study might also have implications for drug formulations. For instance, we report

that methylating agents are most potent clastogens and recombinagens, whereas alkyl chlorides are generally non-genotoxic. Thus, in a broader context of physical and chemical properties of a drug substance and analytical and purification methods, our data might facilitate selection of drug substance specific solvent–acid combinations while mitigating risk of exposure to genotoxic impurities.

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