Table 1
Summary of *in vitro* micronucleus tests.

Chemical	Solvent	Dose (μg/mL)	S9 mix	Cytotoxicity (relative cell survival) (%)	MN frequency (%)[b]	Control MN frequency (%)[b]
EMS	PBS	1000	–	101.0	10.15[c]	1.65[c]
MNNG	DMSO	2	–	98.5	13.25[c]	1.90[c]
PhIP	DMSO	12	+	79.5	16.00[c]	0.75[c]
B[*a*]P	DMSO	10	+	52.5	11.00[c]	1.25[c]
DMBA	DMSO	3	+	69.5	17.50[c]	0.75[c]
4-NQO	DMSO	0.5	–	62.1	5.90[c]	0.70[c]
Caffeine	DW	2000	–	92.7	4.35[c]	0.60[c]
Maltol	Saline	200	–	69.3	4.75[c]	0.55[c]
NaCl	MEM[a]	7500	–	85.2	4.00[c]	0.75[c]

[a] Culture medium (MEM supplemented with 10% CS).
[b] Mean of duplicate culture.
[c] $p < 0.001$ vs. controls by Fisher's exact test.

conducted to confirm reproducibility. The peak area was calculated using Masslynx version 4.0 (Waters) and normalized using the peak areas of dG and the internal standard (I.S.) as described by the following equation: Normalized peak area = (peak area of putative DNA adducts)/(dG area)/(I.S. area) × 10^7.

3. Results

3.1. Induction of micronucleated (MN) cells

The results from *in vitro* micronucleus tests with CHL/IU cells are summarized in Table 1. Since all test compounds are known to induce MN cells with various MOA in the presence or absence of S9-mix, the appropriate experimental conditions were determined in the present experiments. All test compounds induced significantly higher MN incidences (>4.0%) than the corresponding controls (solvents) at the concentrations giving higher than 50% cell survival. The incidence of MN cells in the negative control (solvent) ranged from 0.7 to 1.9%. The carcinogens, PhIP, B[*a*]P, and DMBA, significantly induced MN in the presence of S9-mix ($p < 0.001$), whereas other carcinogens, EMS, MNNG, and 4-NQO, and non-carcinogens, caffeine, maltol, and sodium chloride, induced MN in the absence of S9-mix ($p < 0.001$). These treatment conditions were used for the subsequent comprehensive DNA adductome analysis.

3.2. DNA adductome analysis

In the LC–MS/MS chromatograms of all samples derived from the cells treated with the 6 test carcinogens (groups A and B), putative DNA adduct peaks were detected. The detected peak molecular ion (*m/z*), retention times, normalized peak areas, and identified or presumed DNA adducts obtained from the chromatograms are summarized in Table 2. Among the test carcinogens, most adduct peaks were detected by both digestion methods; however, the PhIP-8-dG adduct was detected only by the nuclease P1 method, and the B[*a*]P and DMBA-induced DNA adducts were detected only by the MCN/SPD method. Non-carcinogens(group C) yielded no DNA adduct peaks, even under the conditions that showed positive results in the MN tests. The possible structures of some DNA adducts were estimated from their *m/z* according to the findings of previous reports (Fig. 2).

A representative chromatogram of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-treated samples is shown in Fig. S1. Two peaks at *m/z* 282 corresponding to the molecular ion of methylated dG were detected in the MNNG-treated samples. The first peak (retention time: 7.6 min) was identified as N^7-methyl-2′-deoxyguanosine (N^7-methyl-dG), and the second peak (retention time: 13.7 min) was identified as O^6-methyl-2′-deoxyguanosine (O^6-methyl-dG) by comparison with the chromatograms of each standard substance.

For ethylmethanesulfonate (EMS), two peaks at *m/z* 296 were detected (Fig. S2), and the molecular ion corresponded to ethylated dG. The first and second peaks were thought to be N^7-ethyl-2′-deoxyguanosine (N^7-ethyl-dG) and O^6-ethyl-2′deoxyguanosine (O^6-ethyl-dG), respectively, because the amount and polarity of N^7-ethyl-dG would be higher than those of O^6-ethyl-dG [6].

For 2-amino-6-phenyl-1-methylimidazo[4,5-b]pyrene (PhIP), the peaks at *m/z* 450 and 490 were detected (Figs. S3 and S4), and the *m/z* 490 corresponded to *N*-(deoxyguanosin-8-yl)-PhIP (PhIP-8-dG).

For benzo[*a*]pyrene (B[*a*]P), two peaks at *m/z* 570 were detected (Fig. S5). These peaks were considered to be 10-(deoxyguanosine-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (B[*a*]P-DE-N^2-dG).

For 7,12-dimethylbenz[*a*]anthracene (DMBA), 12 possible DNA adducts were detected (Figs. S6–S12).

For 4-nitroquinoline-1-oxide (4-NQO), several peaks were detected (Fig. S13–S16). The *m/z* 410 corresponded to 3-(deoxyadenosin-N^6-yl)-4-aminoquinoline 1-oxide (4-AQO-N^6-dA), and *m/z* 426 corresponded to 3-(deoxyguanosine-N^2-yl)-4-aminoquinoline 1-oxide (4-AQO-N^2-dG) and *N*-(deoxyguanosine-8-yl)-4-aminoquinoline 1-oxide (4-AQO-8-dG).

All adduct peaks with their *m/z*, retention times, and peak areas are illustrated in the adductome maps (Fig. 3).

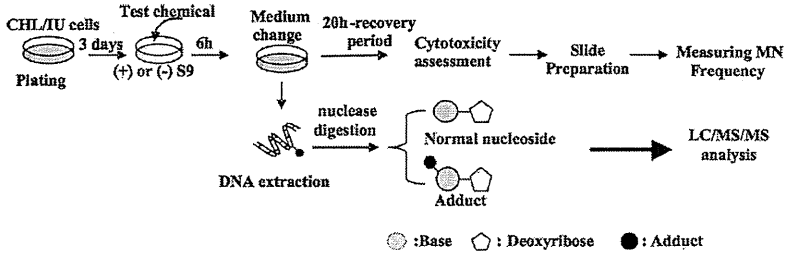

Fig. 1. Schematic outline of the *in vitro* MN test and adductome analysis.

Table 2
Summary of adductome analysis.

Group	Chemical	Peak no.	*m/z*	RT (min)	Normalized peak area		Identified or presumed adducts
					MCN/SPD method	NucleaseP1 method	
A	MNNG	1	282	7.6	2163	2240	N^7-methyl-dG*
		2		13.7	157	158	O^6-methyl-dG*
	EMS	1	296	9.6	2994	5816	N^7-ethyl-dG
		2		16.0	33	235	O^6-ethyl-dG
B	PhIP	1	450	19.1	N.D.	5	–
		2	490	19.6	N.D.	16	PhIP-dG
	B[*a*]P	1	570	22.1	2	N.D.	B[*a*]P-DE-N^2-dG
		2		22.5	6	N.D.	
	DMBA	1	558	24.6	3	N.D.	DMBA-DE-dA
		2	572	19.3	8	N.D.	–
		3		21.4	10	N.D.	–
		4	574	19.3	5	N.D.	DMBA-DE-dG
		5		22S.7	13	N.D.	
		6		23.5	25	N.D.	
		7	590	17.6	12	N.D.	–
		8		18.1	17	N.D.	
		9	596	23.5	5	N.D.	Sodium adducts of No.6
		10	606	18.7	14	N.D.	–
		11		20.7	3	N.D.	–
		12	612	19.3	4	N.D.	–
	4NQO	1	371	14.2	7	4	–
		2	410	12.3	155	112	4-AQO-N^6-dA
		3		17.3	N.D.	6	
		4	426	14.2	4	3	4-AQO-N^2-dG or 4-AQO-8-dG
		5		14.7	4	24	
		6	456	14.6	4	12	–
C	Caffeine Maltol NaCl	No specific peak was detected					

"N.D." means "not detected". "–" represents unknown adduct.
Adducts with and without asterisk show "identified" and "presumed" adducts, respectively.

4. Discussion

In this study, we used the adductome approach to detect the DNA damage caused by the compounds that gave positive results in the MN test condition. Three categories of compounds with different MOA for MN induction were selected. All tested carcinogens were confirmed to form DNA adducts; in contrast, three non-carcinogens yielded no DNA adduct peaks.

In the group A compounds consisting of DNA alkylating agents, O^6- and N^7-methyl-dG and O^6- and N^7-ethyl-dG were detected in the MNNG- and EMS-treated cells, respectively. Although N^3-methyl-dA and N^3-ethyl-dG have been found in other chromatographic analyses [6,7], these adducts were not detected in this adductome analysis, which was probably due to their instability. Another minor lesion, 1-methyl dG, was not detected because its amount was considered to be lower than the detection limit. These results indicate that alkylation of O^6 and N^7 positions of dG would be proof of DNA damage by the group A compounds in the MN-positive experimental condition.

In the group B compounds producing DNA bulky adducts, each compound yielded at least two DNA adduct peaks in the adductome analysis. PhIP yielded two peaks at *m/z* 450 and 490; the former peak is one of unidentified minor adducts [8], but the latter peak is coincident with PhIP-8-dG, the major adduct formed through a reactive intermediate *N*-acetoxy-PhIP [8]. B[*a*]P yielded two peaks at *m/z* 570, which are coincident with the molecular ions of the major adducts B[*a*]P-DE-N^2-dG consisting of four types of stereoisomers [9]. DMBA yielded twelve possible adduct peaks, which agrees with the report showing at least eight DNA adducts induced by DMBA with the ^{32}P-post-labeling analysis [11]. Three DMBA-induced peaks at *m/z* 574 would be stereoisomers of the DMBA-dG adduct, and a peak at *m/z* 558 is coincident with the molecular ion of DMBA-dA, but other peaks are unknown adducts. Six possible DNA adduct peaks were detected in the 4-NQO-treated cellular DNA. Two peaks at *m/z* 410 and 426 correspond to 4-AQO-dG and 4-AQO-dA adducts, respectively, in which several types of 4-NQO binding to C8, N^2, and N^6 of dG and dA are included [12–15], and other peaks cannot be identified because 4-NQO produces various base lesions with different half-life periods [16,17]. These results indicate that the adductome analysis can detect various types of DNA bulky adducts that were identified with the existing methods by other investigators. The efficiency of the adduct peak detection is different between nuclease P1 and MCN/SPD digestion methods in each compound because their enzyme activities on adducted-base excision would vary dependent on the adduct structures. The use of both digestion methods is necessary to detect DNA adducts when new chemicals are tested.

None of the group C compounds, caffeine, maltol, and sodium chloride, which are non-carcinogens but known to produce MN, yielded adduct peaks. Caffeine may interact with DNA repair enzymes and/or nucleotide precursor pools [19], and shows positive results in various genotoxicity tests [18]. Despite a great number of investigations over the past 50 years, the MOA of these compounds is not well understood. The cytotoxic effect of maltol can be explained by its pro-oxidant properties; the maltol/metal complex generates reactive oxygen species (ROS) causing the production of hydroxyl radicals and leading to the formation of DNA base adducts [20]. However, no ROS-related DNA adducts were detected in the present analysis. Sodium chloride increased the incidence of MN cells at extremely high concentrations (c.a. 128 mM). Hyperosmotic medium can cause chromosomal aberrations in CHO cells, mutations at the TK locus in L5178Y mouse lymphoma cells, and at the HPRT locus in V79 cells [21]. However, the mechanisms by which abnormalities are induced in cells subjected to high osmotic pressure are unknown. Although the failure to detect DNA adducts with the non-carcinogens does not mean necessarily that DNA adducts were not formed, DNA adductome is the promising

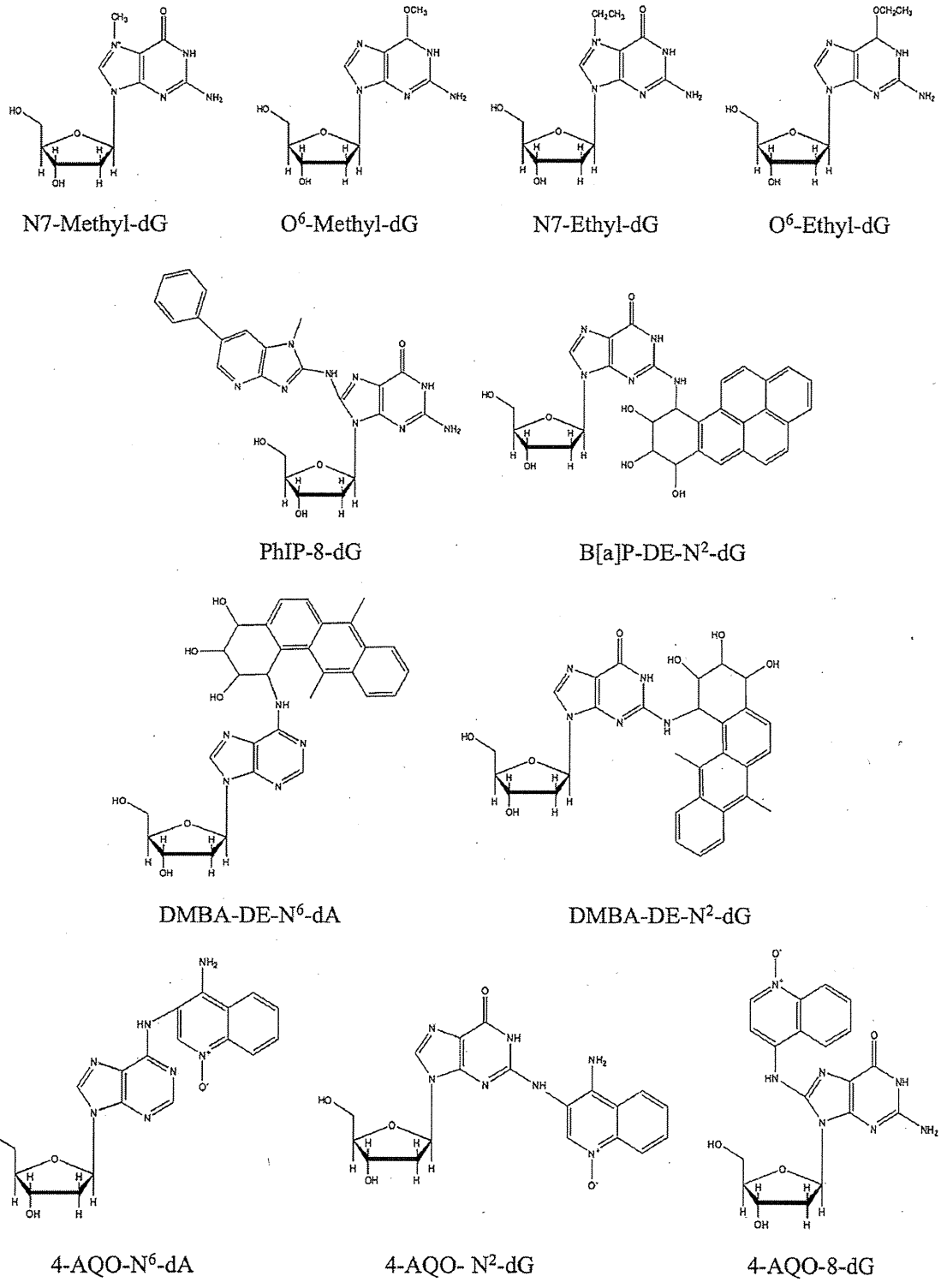

Fig. 2. Structures of DNA adducts estimated from their detected *m/z* values indicated in Table 2. The structures of DMBA-DE-N^6-dG and DMBA-DE-N^2-dG were estimated by the adduction pattern of other PAH compounds.

approach to distinguish false-positive genotoxic compounds from MN-positive compounds. The reliability of this approach will be improved more if the sensitivity of LC/MS/MS equipment is increased and the adductome protocol is more sophisticated.

In summary, with the conditions in which the test compounds significantly increased the frequency of MN cells, only carcinogens (groups A and B) yielded adduct peaks as expected (Table 2 and Fig. 3). The advantages of this adductome approach are as follows: (1) multiple types of DNA adducts can be detected comprehen-

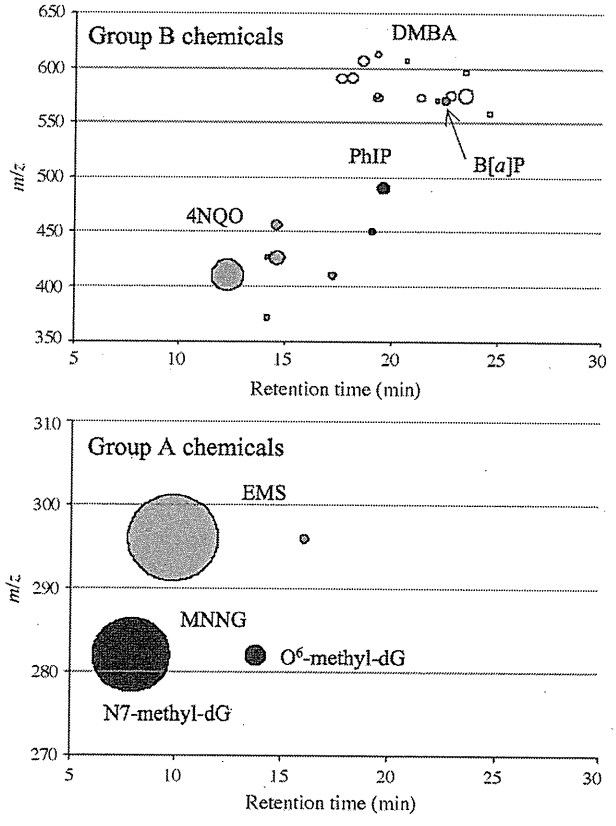

Fig. 3. DNA adductome maps of MN test positive carcinogens. CHL/IU cells were treated with Group A chemicals (carcinogens causing DNA alkylation) or Group B chemicals (carcinogens producing bulky DNA adducts), and the extracted DNA was digested by the MCN/SPD method (MNNG, EMS, B[*a*]P, DMBA) or nuclease P1 method (4-NQO and PhIP). The size of each bubble represents the "normalized peak area" shown in Table 2. Group A chemicals: EMS, pink; MNNG, brown. Group B chemicals: PhIP, blue; B[*a*]P, red; DMBA, yellow; 4-NQO, green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sively, (2) the structures of the detected adducts can be identified from their *m/z* and their analytical standards, and (3) various experimental designs can be applied to both *in vitro* and *in vivo* samples. These experimental features resolve some limitations of the existing methods for analyzing DNA adduct formation.

This study is a pilot experiment to confirm the usefulness of the adductome approach to detect DNA adduct s produced by the compounds showing positive results in the MN test with different MOA. This approach enables detection of various types of DNA adducts formed by typical carcinogens, and does not enable detection of any adducts for non-carcinogens. We conclude that the adductome approach would be applicable to assess the DNA-damaging capability of many types of *in vitro* MN test-positive compounds, and also be useful for understanding MOA of the test compounds.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrgentox.2010.11.012.

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