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Letter

Assessment of bladder DNA damage due to 2-acetylaminofluorene, diuron, and terephthalic acid by the rat comet assay

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ABSTRACT — Chemicals that induce tumors in the urinary bladder in long-term toxicity tests often induce hyperplasia, a pre-cancerous lesion in subacute toxicity tests. Determining whether or not the mechanism by which hyperplasia induced in the urinary bladder is due to genotoxicity at an early stage is important for the extrapolation of carcinogenicity to humans. An *in vivo* comet assay is suitable for short-term evaluation of urinary bladder genotoxicity. 2-Acetylaminofluorene (2-AAF), diuron, and terephthalic acid (TPA) are known to induce tumors in rat bladder by different mechanisms in long-term toxicity tests, but so far there are no reports on the bladder comet assay for the evaluation of these compounds in short-term toxicity tests. Here, a comet assay in rat urinary bladder for short-term evaluation was assessed by using rat strains in which hyperplasia induction has been reported for diuron and TPA, both of which induce hyperplasia in subacute toxicity tests, and rat strains in which tumors have been observed in long-term toxicity tests of 2-AAF. Regarding the respective DNA damaging properties of these compounds, the result was positive for 2-AAF, a typical genotoxic urinary bladder carcinogen, but negative for diuron and TPA, which induce hyperplasia through the chemical action of metabolites and through physical cytotoxicity due to urinary crystals, respectively. Thus, the comet assay of rat urinary bladder as a short term *in vivo* genotoxicity test is considered to be a useful and convenient method for the short-term evaluation of the genotoxicity of compounds which induce hyperplasia in the urinary bladder.

Key words: Comet assay, TPA, Urinary bladder, Diuron, 2-AAF, Hyperplasia

INTRODUCTION

2-Acetylaminofluorene (2-AAF), diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea], and terephthalic acid (TPA) have been reported to induce tumors in rat urinary bladder in respective long-term oral administration toxicity tests (Wilson *et al*., 1941; McDonald *et al*., 1962; Nascimento *et al*., 2006; OECD, 2001). However, the mode of action (MoA) differs among these bladder carcinogenic substances: the MoA has been reported to involve genotoxicity for 2-AAF, which is a representative aromatic amine (Heflich and Neft, 1994; Kirkland *et al*., 2005); chemical cytotoxicity by *in vivo* metabolites for diuron (da Rocha *et al*., 2010, 2014); and physical cytotoxicity due to urinary crystals for TPA (Dai *et al*., 2005b; Heck and Tyl, 1985; Chin *et al*., 1981). A comet assay is an *in*

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vivo test that can conveniently evaluate whether carcinogenicity of the urinary bladder is genotoxic or non-genotoxic. Its utility has been confirmed using several chemical substances (Wada *et al*., 2014), but to our knowledge there are no reports of a comet assay of the effects of 2-AAF and TPA on urinary bladder. In one study, diuron was administered for 20 weeks via the feed (Nascimento *et al*., 2006), but there are no reports on the comet assay in the urinary bladder after short-term single or multiple high-dose administration, as in the case of 2-AAF and TPA.

Hyperplasia refers to lesions with abnormal proliferation of cells and is considered to be a pre-cancerous lesion. Urinary bladder hyperplasia occurs frequently in rats and is often confirmed by using subacute toxicity tests of approximately 4 to 20 weeks in rodents (Fukushima *et al*., 1983; Hirose *et al*., 1976). Strains of rats have been reported to affect the development of tumors or hyperplasia (Fukushima *et al*., 1983; Nakanowatari *et al*., 1988). The causes of hyperplasia occurring in rats are roughly classified into those caused by genotoxicity owing to a direct action on DNA, and those caused by non-genotoxic effects that do not involve DNA. Evaluation of whether or not hyperplasia is caused due to genotoxicity at an early stage is an important consideration in the extrapolation of urinary bladder carcinogenesis to humans (Cohen and Lawson, 1995; Cohen, 1998). Here, therefore, a short-term comet assay for assessing genotoxicity in the urinary bladder was conducted by using strains of rats for which hyperplasia or tumors of the urinary bladder has been reported to occur due to the repeated administration of 2-AAF, diuron, and TPA, which have mutually different carcinogenic mechanisms. The aim was to determine whether the assay can accurately evaluate whether or not the cause of hyperplasia is DNA damage. We used Wistar and SD rats, in which hyperplasia has been observed for diuron and TPA, respectively, in subacute toxicity tests (Dai *et al*., 2005b; Nascimento *et al*., 2006). Because there are no reports of hyperplasia induction by 2-AAF in subacute toxicity tests, we used Wistar rats, in which tumors have been observed in a long-term toxicity test (McDonald *et al*., 1962).

MATERIALS AND METHODS

Materials

The test substances were purchased as follows: 2-AAF from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), diuron from Sigma-Aldrich Co. LLC. (SLT, USA), and TPA from Nacalai Tesque, Inc. (Kyoto, Japan). The positive control, ethyl methanesulfonate (EMS), was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was purchased from Dojindo Laboratories (Kumamoto, Japan). Low-melting agarose (LMA), standard melting agarose (SMA), Triton X-100, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. LLC. (SLT, USA). Sodium hydroxide (NaOH), tris-hydroxymethyl aminomethane (Tris), sodium lauroyl sarcosinate, and ethidium bromide (EtBr) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Animals

Male Wistar or Sprague-Dawley (SD) rats (aged 6 weeks) were purchased from Japan SLC, Inc. (Shizuoka, Japan), and were acclimatized for 7 days before the study. Rats were housed at a temperature of 24.5 ± 0.5°C under a 12 hr/12 hr light/dark cycle with free access to standard diet (CE-2, CLEA Japan, Inc., Tokyo, Japan) and tap water.

All animal experiments were conducted in accordance with the Animal Experiment Regulations of the University of Shizuoka.

Animal Treatment

2-AAF, diuron, and TPA were suspended in 0.5% sodium carboxymethyl cellulose solution (CMC). After pulverization of the test substance, a small amount of Tween 80 was added to improve suspension such that a final concentration of 0.2% to 1.0% was obtained. Physiological saline was used as solvent for the positive control.

Liver and stomach comet assays based on a maximum dose of 1000 mg/kg of 2-AAF have been reported, and a significant weight loss was confirmed at this dose (Kraynak, 2015). Therefore, this dose of 1000 mg/kg, the highest dose reported, was used as the highest dose for 2-AAF in the present study. Because the LD_{50} of diuron is 1017 mg/kg (Sigma-Aldrich, 2015), a dose-finding study based on approximately 50% and 80% of LD_{50} was conducted in three animals; and 500 mg/kg, which showed an adequate systemic effect, was set as the highest dose for diuron (Table 1). Lastly, the LD_{50} of TPA has been reported to exceed 2000 mg/kg (OECD, 2001); therefore, the maximum dose of this compound was set to 2000 mg/kg, which was the limit dosing for the *in vivo* comet assay (OECD, 2016). For each test substance, three dose groups were used, starting from the respective highest dose and using 2-fold dilutions. The administration dose of EMS, as a positive control group, was set to 200 mg/kg (Uno *et al*., 2015).

A volume of 10 mL/kg body weight of the test sub-

Table 1. The dose-finding study for comet assay of diuron.

Dose	Animal No.	Body weight (g)		Clinical signs
		1st adm.	2nd adm.	
500 mg/kg	1101	208.2	193.9	Listless, abnormal gait, prone position
	1102	195.3	187.5	Listless, abnormal gait, prone position, hypothermia
	1103	186.3	175.1	Listless, ptosis, prone position, hypothermia, chromodacryorrhea
800 mg/kg	1201	202.5	186.1	Listless, ptosis, abnormal gait, piloerection, lateral position, irregular respiration, hypothermia, chromodacryorrhea, moribund
	1202	204.1	186.3	Listless, ptosis, abnormal gait, piloerection, irregular respiration, hypothermia, chromodacryorrhea, moribund
	1203	201.8	187.8	Listless, ptosis, abnormal gait, irregular respiration, hypothermia, chromodacryorrhea, moribund

stance or its vehicle was administered twice, at 3 and 24 hr before autopsy, by oral gavage. Oral gavage was also used to administer the positive control substance. For each test, there were 4 animals in each group.

Comet Assay

The animals were exsanguinated under deep anesthesia with diethyl ether and then euthanized, and the urinary bladder and a portion of the left lateral lobe of liver were removed. The urinary bladder was cut open with dissection scissors and spread out as a sheet. Using a No. 14 scalpel blade (Futaba Co., Ltd., Tokyo, Japan), the epithelial cells were scraped off several times, and the cells were suspended by immersing the scalpel blade tip in 500 µL of cold mincing buffer (30 mM EDTA-2Na, 0.9% (w/v) potassium chloride (KCl) solution) prepared in a 1.5-mL microtube. The urinary bladder was also placed in a microtube and suspended for approximately 2-3 sec using a vortex mixer. The large cell mass was precipitated by allowing the urinary bladder to stand for approximately 15-30 sec, and 10 µL of the supernatant was collected in a clean 1.5-mL microtube. From the left lateral lobe of the liver, three or four pieces of approximately 5 mm square in size were excised and then minced in 3 mL of cold mincing buffer for about 2 min using dissection scissors. Ten microliters of the cell suspension was collected in a clean 1.5 mL microtube. Next, 90 µL of 0.5% LMA prepared with phosphate-buffered saline was added and mixed by pipetting. The mixture was added dropwise onto the MAS-GP slide glass (Matsunami Grass Ind. Ltd., Osaka, Japan) on which a base layer was formed with 0.7% SMA, and then covered with a new slide glass spreading the gel uniformly. Each slide was left to stand at 4°C for 20-30 min to solidify the gel. After solidification of the gel, 90 µL of 0.5% LMA was added dropwise onto the slide again to embed the cell layer fully in the agarose using another slide, and the slide was again left to stand at 4°C for 20-30 min. Each slide was immersed in a lysis solution (pH 10, 2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris, 1% (w/v) sodium lauroyl sarcosinate, 1% (v/v) Triton X-100, 10% (v/v) aqueous solution of DMSO) and left at 4°C overnight in the dark. The following day, the slide was soaked in a cold alkaline solution (0.3 M NaOH, 1 mM EDTA-2-Na aqueous solution) for 20 min to unwind double-stranded DNA into single strands, and then electrophoresis was carried out for 20 min at 300 mA, 25 V (0.96 V/cm electrode spacing). The slide was immersed in cold 0.4 M Tris-HCl buffer (pH 7.5) for 10 min, dehydrated in ethanol (≧ 99.6%) for 10 min, and then stained with 20 µg/mL of EtBr. The DNA ratio in comet tails (% tail DNA) of 50 cells was measured for each slide using a Comet Analyzer (Youworks Co., Tokyo, Japan). Those with an obvious abnormal shape (such as hedgehog) and those with % tail DNA of 90% or more were excluded from observation. Two slides were prepared per animal and the average value of the median of % tail DNA of the slide was taken as the % tail DNA per animal. The mean % tail DNA per group was calculated from this average median value.

Statistical Analysis

Differences in results between the negative control group and each test substance group were statistically compared by Dunnett's multiple comparison tests (two-sided) using the statistical analysis software Pharmaco Basic (Scientist Press Co., Ltd., Tokyo, Japan). Variance analysis was performed for the positive control group with an *F*-test, and then significance was tested by using Student's *t*-test

(one-sided) or Aspin-Welch's *t*-test (one-sided). In all cases, a level of $p < 0.05$ was taken to be significant.

RESULTS

The complete data set for all test substances is shown in Table 2.

2-AAF

A decrease in animal weight was observed at the time of the second administration as compared with the first administration, which confirmed that an appropriate dose was being administered. For the urinary bladder, the % tail DNA was 5.32 ± 0.79, 6.79 ± 1.92 and 9.49 ± 1.85 for the 250, 500 and 1000 mg/kg groups, respectively, as compared with 3.50 ± 0.70 for the negative control group; the values were significantly higher in the groups administered 500 mg/kg of 2-AAF or more. For the liver, the respective values were 3.72 ± 1.77, 6.76 ± 4.55 and 10.62 ± 1.82 as compared with 1.56 ± 0.39 for the negative control group; again, the values were significantly higher in the groups given 500 mg/kg or more. In addition, a dose-response relationship was observed for the increase in % tail DNA in both the urinary bladder and liver. In the positive control group, a significant increase in % tail DNA was observed in both the urinary bladder and liver.

Diuron

A decrease in animal weight was observed at the time of second administration relative to the first administration, which confirmed that an appropriate dose was being administered. For the urinary bladder, the % tail DNA was 5.33 ± 3.25, 3.87 ± 0.74 and 4.40 ± 1.66 for the 125, 250 and 500 mg/kg groups, respectively, as compared with 5.15 ± 1.72 for the negative control group; and no significant increase with dose was observed. For the liver, the respective values were 2.26 ± 0.60, 2.11 ± 0.42 and 4.07 ± 3.33 as compared with 1.97 ± 0.42 for the negative control group; and again, no significant increase with dose was observed. In the positive control group, a signif-

Table 2. Comet assay results for all test substances.

Treatment	% tail DNA (Mean ± S.D.)		Body weight (before administration)		Necropsy
	Bladder	Liver	1st	2nd	
2-Acetylaminofluorene					
Vehicle	3.50 ± 0.70	1.56 ± 0.39	190.9 ± 6.0	196.5 ± 6.6	Normal
2-AAF, 250 mg/kg	5.32 ± 0.79	3.72 ± 1.77	188.1 ± 9.7	184.1 ± 7.6	Normal
2-AAF, 500 mg/kg	6.79 ± 1.92*	6.76 ± 4.55*	187.4 ± 7.4	180.7 ± 7.3	Normal
2-AAF, 1000 mg/kg	9.49 ± 1.85**	10.62 ± 1.82**	186.5 ± 5.0	181.7 ± 5.3	Normal
EMS, 200 mg/kg	11.48 ± 2.48**	16.77 ± 3.08**	193.7 ± 4.5	186.0 ± 4.2	Normal
Diuron					
Vehicle	5.15 ± 1.72	1.97 ± 0.42	193.6 ± 2.9	197.9 ± 5.0	Normal
Diuron, 125 mg/kg	5.33 ± 3.25	2.26 ± 0.60	186.4 ± 9.3	181.5 ± 10.4	Normal
Diuron, 250 mg/kg	3.87 ± 0.74	2.11 ± 0.42	192.3 ± 3.2	183.0 ± 2.5	Normal
Diuron, 500 mg/kg	4.40 ± 1.66	4.07 ± 3.33	192.9 ± 1.5	183.2 ± 2.7	Normal
EMS, 200 mg/kg	23.57 ± 12.20*	16.09 ± 3.65**	192.1 ± 7.0	182.7 ± 7.4	Normal
Terephthalic acid					
Vehicle	5.64 ± 0.29	1.79 ± 0.31	270.4 ± 8.1	280.1 ± 7.6	Normal
TPA, 500 mg/kg	3.18 ± 0.82	1.75 ± 0.20	263.5 ± 6.1	272.6 ± 6.8	Normal
TPA, 1000 mg/kg	5.54 ± 2.01	1.66 ± 0.46	268.0 ± 7.3	276.3 ± 8.8	Cecum: distention (1/4), darkening (2/4)
TPA, 2000 mg/kg	8.28 ± 4.66	1.88 ± 0.31	266.9 ± 4.8	272.2 ± 5.6	Cecum: distention(2/4), darkening (1/4), Bladder: soft calculus (1/4)
EMS, 200 mg/kg	16.15 ± 7.62*	12.36 ± 4.65**	265.9 ± 11.5	262.9 ± 11.7	Normal

* : The mean % tail DNA was significantly higher than the negative control at $p < 0.05$.
** : The mean % tail DNA was significantly higher than the negative control at $p < 0.01$.

icant increase in % tail DNA was observed relative to the negative control in both the urinary bladder and liver.

TPA

In the 1000 and 2000 mg/kg administration groups, distention and darkening of the cecum were observed. And soft calculus in the urinary bladder was observed in the 2000 mg/kg administration group (Fig. 1). For the urinary bladder, the % tail DNA was 3.18 ± 0.82, 5.54 ± 2.01 and 8.28 ± 4.66 for the 500, 1000 and 2000 mg/kg groups, respectively, as compared with 5.64 ± 0.29 for the negative control group; and no significant increase with dose was observed. For the liver, the respective values were 1.75 ± 0.20, 1.66 ± 0.46, and 1.88 ± 0.31 as compared with 1.79 ± 0.31 for the negative control group; again, no significant increase with dose was observed. In the positive control group, significant increases in % tail DNA were observed in both the urinary bladder and liver.

DISCUSSION

2-AAF is a representative aromatic amine that shows positive results in various genotoxicity tests (Heflich and Neft, 1994; Kirkland *et al*., 2005). Similar to *N*-butyl-*N*-(4-hydroxybutyl) nitorosamine (BBN), a typical genotoxic urinary bladder carcinogen, 2-AAF has been defined as a genotoxic urinary bladder carcinogen in rodents and humans (Cohen, 1995). Hyperplasia was not confirmed in the urinary bladder when 2-AAF was administered for 40 weeks via the feed at a dose (50 ppm) that was observed to affect body weight (Tsuda *et al*., 1977). However, tumors were observed in the urinary bladder and liver after administration of a dose of 600 ppm in the feed for 6-9 months (McDonald *et al*., 1962). Carcinoma was found to have been induced in a subacute toxicity study of 2-AAF (Wilson *et al*., 1941). A few cases of nodule hyperplasia have been reported in tests based on administration via the feed for 25 weeks, although the rat strain was different (Maeura *et al*., 1984). Furthermore, in a study of a recently developed immunostaining procedure, where a phosphorylated form of histone (*γ*H2AX) that localizes at the break site of DNA double-strands acts as an indicator, 2-AAF led to positive results in the urinary bladder (Toyoda *et al*., 2015). As compared with that genotoxicity test, which requires an administration period of 28 days, the present study detected direct DNA damage caused by 2-AAF in the urinary bladder in a shorter period. 2-AAF has also been observed to cause tumors in the liver, and the general consensus for the liver comet assay was found to be positive; however, one laboratory has previously reported negative results (Uno *et al*., 2015). Even in tests with significant positive results, there have been a case where the assessment was borderline; for example, the increase in % tail DNA was small or the dose-response relationship was not clear (Kraynak *et al*., 2015). In the present study, there was a statistically significant increase in % tail DNA of the liver and a clear dose-response relationship was observed. The reason for this clear finding might be attributed to the fact that a trace amount of Tween 80 was used to pulverize the test substance, which might have had an impact on the result. This is because 2-AAF, which is insoluble in water and highly soluble in fat (National Library of Medicine, 2012), was emulsified due to the use of this surfactant. It was considered that this promoted the absorption of 2-AAF, markedly increasing the % tail DNA and dose response.

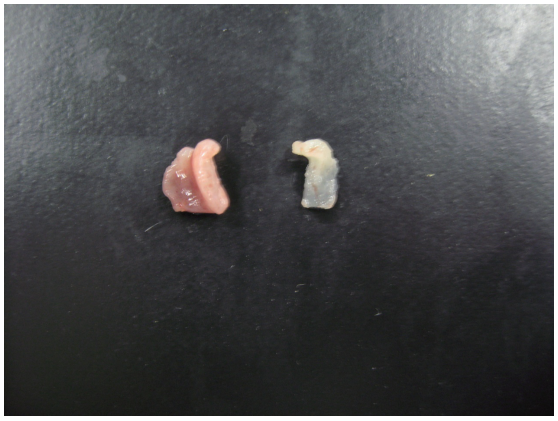

Fig. 1. Example of soft calculus observed in the urinary bladder in the group given TPA.

Diuron is a type of herbicide that was shown to cause carcinoma of the urinary bladder at a very high frequency in a 2-year rat carcinogenicity study (Nascimento *et al*., 2006), and its MoA involves chemical cytotoxicity of a metabolite (da Rocha *et al*., 2010, 2014). In a subacute toxicity test, hyperplasia occurred in the urinary bladder after administration of diuron at 2500 ppm via the feed for 20 weeks. Negative results in a comet assay of hyperplasia lesion sites have been reported, but to our knowledge there have been no reports on the effects of diuron in a short-term comet assay after high-dose administration. The results of the present study, in which higher doses were administered over a short period, were also negative, supporting the past findings. There was a trend towards higher values for the maximum dose in the liver, but no significant differences were noticed. Thus, the comet assay, a short-term *in vivo* genotoxicity test, revealed that hyperplasia specific to the urinary bladder

caused by diuron in a subacute toxicity test is not attributable to genotoxicity.

The chemical substance TPA is used as a raw material for polyester fibers, but tumors have been detected in the urinary bladder during a 2-year carcinogenicity test (OECD, 2001). In a subacute toxicity (90-day) test, TPA caused hyperplasia and the formation of calculi in the urinary bladder when administered in high doses via the feed (Dai *et al*., 2005b). It is a typical non-genotoxic urinary bladder carcinogen, which gives negative results in various genotoxicity tests (Zeiger *et al*., 1982; Lee *et al*., 2007). The hyperplasia, which appears with high frequency in males when administered at a high dose, is attributable to physical injuries caused by the urine crystals. Because α2μ-globulin contributes to the induction of crystals, fundamentally the MoA would not be extrapolated to humans (Cohen, 1998; Dai *et al*., 2005b). TPA is mostly excreted in urine (Hoshi *et al*., 1967), and the main component of crystals is CaTPA or calcium phosphate (Dai *et al*., 2005b; Heck and Tyl, 1985; Chin *et al*., 1981). The incidence of calculi and hyperplasia was found to be reduced by simultaneous administration of therapeutic agents for hypercalciuria (Dai *et al*., 2005a). Our tests using a scanning electron microscope (SEM) have confirmed that administration of TPA leads to the formation of crystals in the urinary bladder that originate from the TPA (data not shown). Because the short-term *in vivo* comet assay result of TPA was negative in the present study, the mechanism underlying the development of hyperplasia was not found to be attributable to genotoxicity. Although a soft calculus was observed in the urinary bladder, the shape was different from calculi reported previously. Small amounts of products from accessory reproductive glands, including secretions from the coagulating gland, have been observed in the urinary bladder as proteinaceous material (Greaves and Faccini, 1992). The soft calculus that was confirmed in the present study might possibly be due to these secretions. TPA is mainly excreted through the urine, is not directly involved in the distention or darkening of the cecum, which is considered to have been due to the negative effect of TPA on digestion.

For both diuron and TPA used in the present study, hyperplasia in the urinary bladder was observed in the subacute toxicity test, but the comet assay results were negative and were clearly different from the results of 2-AAF, which showed a low incidence of hyperplasia. Hyperplasia was previously observed for BBN in tests based on the same administration period as 2-AAF, but hyperplasia was not detected for 2-AAF (Tatematsu *et al*., 1977). In the urinary bladder comet assay of BBN with the same administration conditions as in the present study, the average % tail DNA was 24.74 ± 7.63 for a dose as low as 250 mg/kg (Wada *et al*., 2014). Because this % tail DNA is much higher than the value observed for the maximum dose of 2-AAF, it raises the possibility that the strength of the involvement of genotoxicity leads to differences in the induction of hyperplasia. It goes without saying that a comet assay of the urinary bladder is useful to evaluate whether the MoA of hyperplasia occurring in a subacute toxicity test is genotoxic or non-genotoxic; however, the assay is also an easy way to evaluate genotoxicity specific to the urinary bladder of a chemical when an existing genotoxicity test shows suspected positive genotoxicity but hyperplasia is not detected. One report has suggested that cell destruction due to cytotoxicity can be a possible cause of false-positive results in a comet assay (Burlinson *et al*., 2007), but our study showed negative results for both diuron and TPA, which induce hyperplasia due to cytotoxicity originating chemically or from urinary crystals. From these results, genotoxicity related to the threshold of carcinogenicity in long-term tests can be evaluated in a short period of time by conducting a comet assay in the urinary bladder of the relevant rat strains at the stage when hyperplasia is detected in a subacute toxicity tests. For compounds such as 2-AAF where hyperplasia is not detected in the subacute toxicity test, the assay may be able to predict the risk of carcinogenicity due to genotoxicity that is specific to the urinary bladder when a positive genotoxicity result is suspected in an existing genotoxicity test.

While species differences are into consideration sufficiently, strain differences are little taken into consideration in the comet assay. Because the frequency of hyperplasia and tumor occurrence in the urinary bladder was found to differ on the basis on rat strain for both genotoxic and non-genotoxic substances, detailed verification of the relationship between rat strains and genotoxicity is required to evaluate genotoxicity more accurately and rapidly in the future.

Conflict of interest---- The authors declare that there is no conflict of interest.

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