Table 4. *gpt* MFs in *p53* (+/-) *gpt* delta mice livers

Treatment	Animal No.	Cm^R colonies (x 10^5)	6-TG^R and Cm^R colonies	Mutant frequency (x 10^{-5})	Mean ± S.D.
Control	51	10.9	3	0.28	0.79 ± 0.36
	52	8.7	6	0.69	
	53	8.7	9	1.04	
	54	9.6	7	0.73	
	55	10.7	13	1.22	
MeIQx	61	10.5	119	11.30	7.40 ± 2.40*
	62	7.0	53	7.60	
	63	9.1	44	4.82	
	64	8.5	58	6.78	
	65	9.5	62	6.50	
CCl_4	71	14.8	8	0.54	0.65 ± 0.33
	72	5.7	6	1.05	
	73	13.7	2	0.15	
	74	8.1	6	0.74	
	75	8.0	6	0.75	
MeIQx + CCl_4	81	14.9	320	21.42	23.28 ± 8.19*,#,$
	82	5.0	152	30.43	
	83	6.6	189	28.77	
	84	12.0	150	12.48	

* ; Significantly different from the control group at $p < 0.01$.
# ; Significantly different from the MeIQx alone group at $p < 0.05$.
$; Significantly different from the CCl_4 alone group at $p < 0.01$.

report (Masumura *et al.*, 2003). Thus, the present data clearly demonstrated that co-treatment with CCl_4 could further enhance *gpt* MFs due to MeIQx; their mutation spectra were almost identical to those in the MeIQx alone group. It is well known that MeIQx incurs *N*-hydroxylation by CYP 1A1/2 followed by further activation due to *O*-acetyltransferase or sulfotransferase (Shimada *et al.*, 1989; Rich *et al.*, 1992; Turesky *et al.*, 1998), giving rise to *N*-(deoxyguanosine-8-yl)-MeIQx, *N*-(deoxyguaonosine-8-yl)-4,8-DiMeIQx and N^2-(deoxyguanosine-8-yl)-MeIQx (Schut and Snyderwine, 1999; Snyderwine *et al.*, 1993; Ochiai *et al.*, 1993). These bulky adducts for guanine are believed to be responsible for G:C to T:A transversions (Masumura *et al.*, 2003). On the other hand, MeIQx-DNA adduct formation was effectively inhibited by purpurin, an inducer of phase II enzymes, such as GST and UDP-glucuronyltransferase (Takahashi *et al.*, 2007), implying that MeIQx is conjugated and excreted by these enzymes. Our real-time RT-PCR analysis demonstrated that there were no changes in mRNA levels of *CYP1A2* and *GSTα4* between MeIQx-treated mice with and without CCl_4 treatment. Likewise, no differences in the *O*-acetyltransferase or sulfotransferase mRNA levels were observed regardless of CCl_4 injection (data not shown). Moreover, CYP1A2 protein levels were not elevated, rather decreased, in co-treatment group as com-

Table 5. Spi- MFs in *p53* (+/-) *gpt* delta mice livers

Treatment	Animal No.	Total population (x 10^5)	Total Spi- mutants	Mutant frequency (x 10^{-5})	Mean ± S.D.
Control	51	2.6	2	0.78	0.38 ± 0.29
	52	5.0	2	0.40	
	53	12.1	1	0.08	
	54	18.2	5	0.28	
MeIQx	61	9.3	13	1.40	0.63 ± 0.49
	62	7.5	5	0.67	
	63	4.9	1	0.20	
	64	8.7	6	0.69	
	65	5.7	1	0.17	
CCl_4	71	13.2	3	0.23	0.29 ± 0.09
	72	7.2	3	0.42	
	73	12.5	3	0.24	
	74	7.1	2	0.28	
MeIQx + CCl_4	81	16.4	35	2.14	1.75 ± 0.66*,#,$
	82	6.8	18	2.67	
	83	22.9	23	1.00	
	84	12.0	16	1.33	
	85	7.6	12	1.59	

* ; Significantly different from the control group at $p < 0.01$.
# ; Significantly different from the MeIQx alone group at $p < 0.05$.
$; Significantly different from the CCl_4 alone group at $p < 0.01$.

pared with MeIQx alone group and GSTα protein levels were not changed regardless of CCl_4 injection, suggesting no remarkable effects of co-administration of CCl_4 to enhance MeIQx metabolism/excretion.

CCl_4 is used to induce experimental hepatic injury. To mimic chronic hepatic inflammation, mice were intraperitoneally injected with 1 ml/kg CCl_4 solution once a week for 12 weeks. Elevated serum ALT levels return to a normal range 4 days after CCl_4 administration in the liver of C57BL/6 mice (Kato *et al.*, 2003). In addition, single *i.p.* administration of CCl_4 induces histological changes including inflammatory cell infiltration in the mouse liver (Kim *et al.*, 2009). Kupffer cell activation is a crucial event in hepatocyte injury induced by CCl_4 and activated Kupffer cells release cytokines such as TNF-α and IL-6, which are important factors for hepatocyte regeneration (Taub *et al.*, 1999). It is reported that MeIQx is an initiator in the resistant hepatocyte model, and induces γ-glutamyltransfertase positive hepatic foci in rat liver (Kleman *et al.*, 1989). Also, it has been demonstrated that co-administration with MeIQx and CCl_4 induced development of GST-P positive foci, as a precancerous lesion, in the liver compared to MeIQx only treated rats (Sone *et al.*, 1992; Iwai *et al.*, 2002). However, combined treatment with MeIQx and CCl_4 failed to enhance MeIQx-DNA adducts levels (Sone *et al.*, 1992). Iwai *et al.* (2002) demonstrated that repeated co-administration with CCl_4 enhanced the BrdU-labeling index in rat liver. These data suggest that development of precancerous lesions is caused by acceleration of cell turnover due to liver injury by CCl_4, but

Table 6. Mutation spectra of *gpt* mutant colonies in *p53* (+/+) mice

Treatment	Control	MeIQx	CCl_4	MeIQx + CCl_4
Base substitution				
Transversions				
G:C to T:A	3[a] (20.0)	34 (58.6)	8 (38.1)	54 (48.6)
G:C to C:G	2 (13.3)	2 (3.4)	0	6 (5.4)
A:T to T:A	1(6.7)	1 (1.7)	2 (9.5)	4 (3.6)
A:T to C:G	0	0	0	3 (2.7)
Transitions				
G:C to A:T	5 (33.3)	10 (17.2)	9 (42.9)	13 (11.7)
A:T to G:C	0	2 (3.4)	0	1 (0.9)
Deletion				
Single bp	2 (13.3)	8 (13.8)	2 (9.5)	21 (18.9)
Over 2 bp	0	0	0	2 (1.8)
Insertion	2 (13.3)	1 (1.7)	0	7 (6.3)
Complex	0	0	0	0
Total	15	58	21	111

( ); % of total colonies.
[a]: The number of colonies with independent mutations.

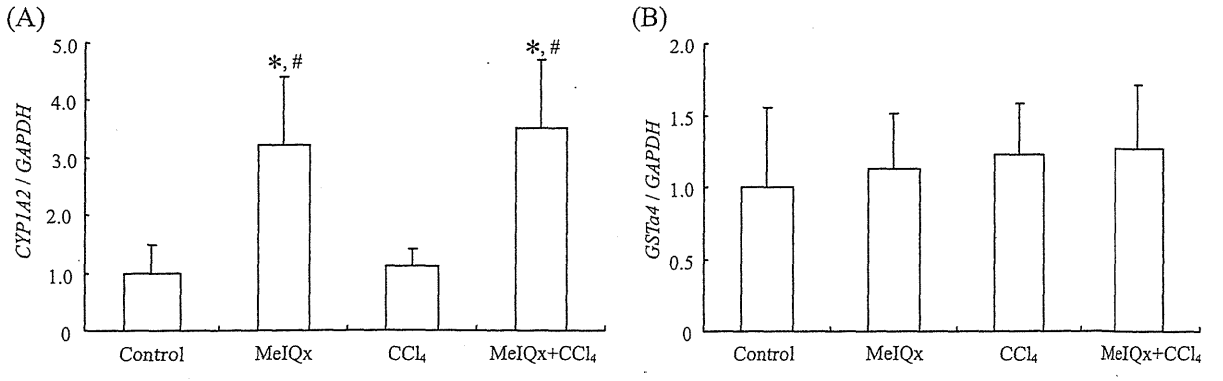

Fig. 2. Changes in mRNA expression level of *CYP1A2* (A) and *GSTα4* (B) in the liver of *p53*-proficient *gpt* delta mice. The values are mean ± S.D. of data for five animals. Normalization is to *GAPDH* mRNA levels. Significant increase (*: $p < 0.01$) from the control group. Significant increase (#: $p < 0.01$) from the CCl_4 alone group.

not caused by enhancement of *in vivo* mutagenicity of MeIQx. In the present study, *gpt* MFs were enhanced by concurrent administration with CCl_4 without biological significant changes in mRNA and protein levels related to MeIQx metabolism/excretion. The *gpt* mutant spectra analysis revealed increased clonal expansion of *gpt* colonies in the combined treatment group. Our data suggest that induction of cell proliferation may play an important role in mutagenicity of MeIQx. In the present study, slight inflammatory cell infiltration was observed in the liver of CCl_4 treated mice (data not shown), the change may suggest that the hepatic injury was caused by CCl_4

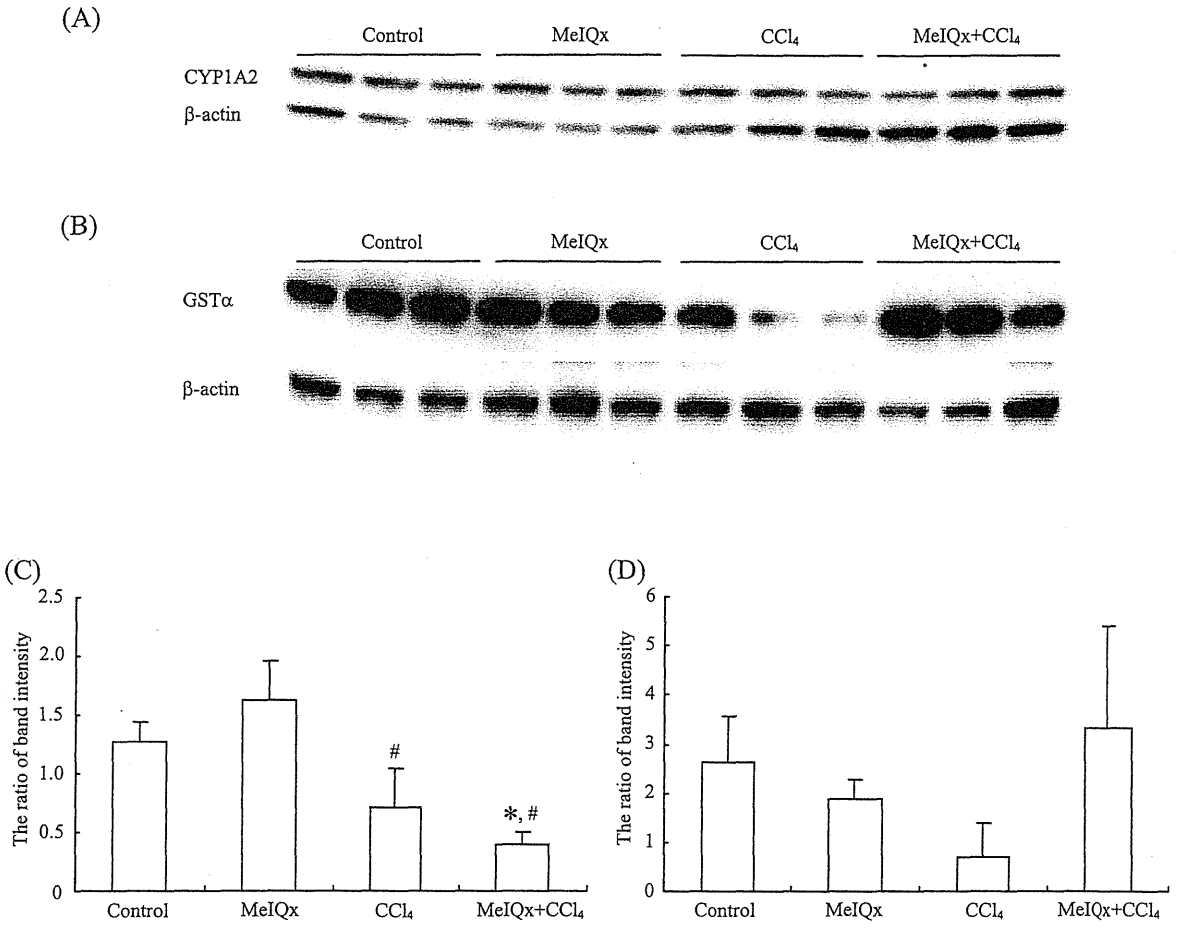

Fig. 3. Changes in protein expression level of CYP1A2 and GSTα in the liver of *p53*-proficient *gpt* delta mice. (A and B) Immunoblots of expression of CYP1A2 in liver microsomes (A) and GSTα in liver cytosols (B). Signals were quantified using β-actin as loading control from the liver. (C and D) The ratio of band intensity resolved by Western blotting of CYP1A2 (C) and GSTα (D) was quantified densitometrically for statistical comparison. Normalization is to β-actin levels. The values are mean ± S.D. of data for three animals. Significant decrease (*: $p < 0.05$) from the control group. Significant decrease (#: $p < 0.05$) from the MeIQx alone group.

injection. However, we could not detect induction of cell proliferation due to CCl_4 injection by immunohistochemical staining for proliferation cell nuclear antigen (data not shown). It was reported that the cell-proliferative activity is increased, and returns to a normal level 6 days after CCl_4 single injection in rats and mice (Zhou *et al.*, 2006). Because we have examined induction of cell proliferation at 7 day after final administration of CCl_4, it might have been a time lag to observe the cell proliferation.

In the Spi$^-$ mutation assay, MeIQx caused significant elevation of *red/gam* MFs in the livers of *p53*-proficient *gpt* delta mice. This result indicates that MeIQx caused not only point mutations but also deletion mutations in the mouse liver. Like the *gpt* assay, the same tendency was observed in the Spi$^-$ assay, in which MFs in the co-treatment group were higher than in the MeIQx alone group. Similar results were reported in the liver and colon in the *gpt* delta transgenic mice treated by other HCAs, such as 9-(4'-aminophenyl)-9*H*-pyrido[3,4-*b*]indole and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (Masumura *et al.*, 2000, 2003). Chronic inflammation causes DNA damage by reactive oxygen and nitrogen species produced by neutrophils and macrophages (Lu *et al.*, 2006). In the present study, since CCl_4 alone did not increase MFs, DNA damage did not occur with this CCl_4 dose. Therefore, the relation of hepatic injury and deletion mutations was clear, but it was thought that increased MFs of *red/gam* would have resulted from cell proliferation induced

by CCl_4.

p53 is a tumor suppressor protein and plays a key role in several pathways including inflammatory responses (Hussain and Harris, 2006). Inflammatory stress activates p53, and a DNA damaged cell is repaired or removed through induced pathways such as cell cycle arrest, DNA repair and apoptosis. In the present study, the *gpt* and Spi- assay of *p53*-deficient mice showed similar results to *p53*-proficient mice. Mice heterozygous for *p53* did not have sensitivity to carcinogenicity of MeIQx in a 1-year study (Park *et al.*, 1999), and our *in vivo* mutation assay results reflect this lack of sensitivity. The Spi- MFs in the co-treated group were not significantly different as compared with the MeIQx alone or control group. These data suggested that the *p53*-deficient mice had a different sensitivity for the type of mutation induced by MeIQx treatment.

In conclusion, repeated hepatic injury due to CCl_4 might have increased *gpt* and *red/gam* mutation frequencies in the livers of *gpt* delta mice treated with MeIQx. Although further studies are needed to investigate molecular mechanisms underlying the enhancing effects of CCl_4-induced liver injury on MeIQx genotoxicity, our data clearly demonstrate that this model could be a powerful tool for correlating events between inflammation and carcinogenesis.

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Chemical Research in Toxicology

ARTICLE

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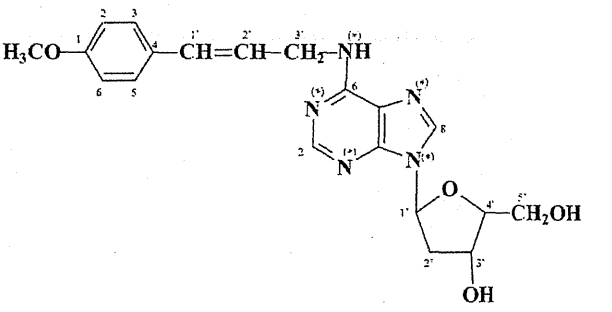
Detection and Quantification of Specific DNA Adducts by Liquid Chromatography−Tandem Mass Spectrometry in the Livers of Rats Given Estragole at the Carcinogenic Dose

Yuji Ishii,*,† Yuta Suzuki,† Daisuke Hibi,† Meilan Jin,† Kiyoshi Fukuhara,‡ Takashi Umemura,† and Akiyoshi Nishikawa†

†Division of Pathology and ‡Division of Organic Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

ABSTRACT: Estragole (ES) is a natural constituent of several herbs and spices that acts as a carcinogen in the livers of rodents. Given that the proximal electrophilic form of ES with a reactive carbocation is generated by cytochrome P450 and a sulfotransferase metabolizing pathway, there is a possibility that the resultant covalent adducts with DNA bases may play a key role in carcinogenesis. The existence of ES-specific deoxyguanosine (dG) and deoxyadenosine (dA) adducts has already been reported with the precise chemical structures of the dG adducts being confirmed. In the present study, we examined ES-specific dA adduct formation using LC-ESI/MS after the reaction of dA with 1′-acetoxy-ES produced by a sulfotransferase metabolic pathway mimic. Although two peaks were observed in the LC-ESI/MS chromatogram, the identification of ES-3′-N^6-dA as the measurable peak was determined by NMR analysis. To confirm ES-specific dG and dA adduct formation in vivo, an isotope dilution LC-ESI/MS/MS method applicable to in vivo samples for ES-3′-N^6-dA together with the two major dG adducts, that is, ES-3′-C8-dG and ES-3′-N^2-dG, was developed using selected ion recording. The limit of quantification was 0.2 fmol on column for ES-3′-C8-dG and ES-3′-N^2-dG and 0.06 fmol on column for ES-3′-N^6-dA, respectively. Using the developing analytical method, we attempted to measure adduct levels in the livers of rats treated with ES at a possible carcinogenic dose (600 mg/kg bw) for 4 weeks. ES-3′-C8-dG, ES-N^2-dG, and ES-3′-N^6-dA were detected at levels of 3.5 ± 0.4, 4.8 ± 0.8, and 20.5 ± 1.6/10^6 dG or dA in the livers of ES-treated rats. This quantitative data and newly developed technique for adduct observation in vivo might be helpful for ES hepatocarcinogenesis investigations.



INTRODUCTION

Estragole (4-allyl-1-methoxybenzene; ES) is a natural constituent of essential oils of various herbs and spices (including tarragon, basil, fennel, and anise) present in food.[1] Previous studies have revealed that ES has genotoxicity and carcinogenicity in the livers of mice, concluding that ES is a naturally occurring genotoxic carcinogen.[2−4] ES is metabolized to the proximate carcinogen, 1′-hydroxy-ES, by cytochrome P450 enzymes (P450), and sulfotransferase (SULT) converts 1′-hydroxy-ES to 1′-sulfooxy-ES.[2−5] The ultimate electrophilic carbocation structure, which can form covalent adducts with DNA bases, is formed from 1-sulfooxy-ES through dissociation of the sulfate group.[6,7] Because postlabeling analysis demonstrated the formation of four different adducts such as ES-3′-N^2-dG (deoxyguanosine), ES-3′-C8-dG, ES-1′-N^2-dG, and ES-3′-N^6-dA (deoxyadenosine) in the livers of mice administered the proximate carcinogenic metabolite 1′-hydroxy-ES, it has been accepted that the carcinogenicity of ES is caused by the formation of these adducts.[8−11] However, the precise amount of these adducts formed in vivo under continuous administration with a carcinogenic dose of ES still remains unknown. Transient base modifications of DNA do not always result in gene mutations because of chemical DNA instability[12] and the existence of specific DNA repair systems.[13] Because the specific activity of repair enzymes is primarily dependent on stereochemical structures and concentrations of their substrates, confirmation of DNA base modifications and precise quantification are necessary for understanding their biological significance.[14,15]

The dG modifications have generally been reported as the major adduct,[16,17] because dG is the most potent nucleophilic nucleoside that can efficiently react with the electrophilic carbocation form.[18] Furthermore, the findings of Y-family polymerase κ, which selectively acts on translesion synthesis on N^2-dG adducts, showed the presence of a linkage between dG modification and mutation in the chemical carcinogenicity.[19−21] However, several carcinogens including aristolochic acid[22,23] and polyaromatic hydrocarbons[24] preferentially form dA adducts,

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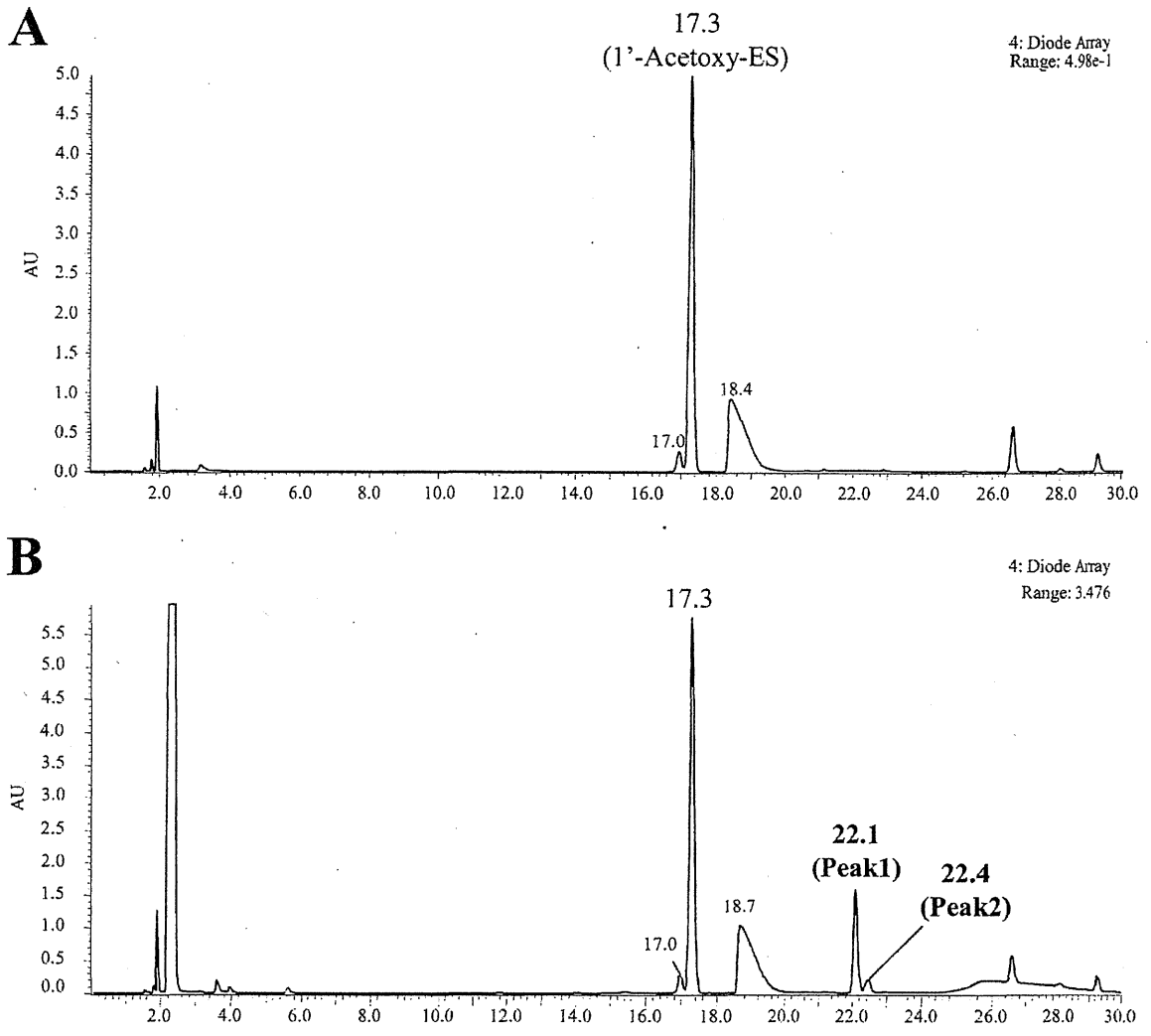

Figure 1. Typical LC-PDA chromatograms (280 nm) in the reaction of (A) 1′-acetoxy-ES alone and (B) 1′-acetoxy-ES with dA. LC-PDA conditions are described in the Materials and Methods.

thereby predominantly inducing dA mutations.[25,26] Thus, quantitative analysis for each ES-modified base is necessary to investigate the role of specific DNA adducts in ES carcinogenesis.

Although the exact chemical structure of three dG adducts has already been confirmed by mass spectrometry (MS) techniques, that of the dA adduct has not been identified. In this study, ES-dA adduct formation by reaction of dA with 1′-acetoxy-ES (used as a 1′-sulfooxy-ES mimic) was investigated by liquid chromatography (LC)-electron spray ionization (ESI)/MS analysis. The precise chemical structures of the detected adducts were identified using nuclear magnetic resonance (NMR). Subsequently, a quantitative analytical method using LC-ESI/tandem mass spectrometry (MS/MS) for ES-specific dG and dA adducts was developed. After the evaluation of applicability to in vivo samples, our LC-ESI/MS/MS method was applied to quantify ES-specific DNA adducts in the livers of rats treated with 600 mg/kg bw of ES for 4 weeks.

MATERIALS AND METHODS

Chemicals and Reagents. ES, *p*-anisaldehyde, vinylmagnesium bromide, tetrahydrofuran (THF), dG, dA, and alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclease P1 was from Yamasa Shoyu Co. (Chiba, Japan). Stable isotope-labeled [$^{15}N_5$]-dG and [$^{15}N_5$]-dA were obtained from Cambridge Isotope Laboratories (Cambridge, MA). Acetic anhydride ammonium carbonate, dimethylsulfoxide (DMSO), dichloromethane, *N,N*-dimethylformamide (DMF), diethyl ether, isopropyl alcohol, and DNA extractor TIS kit were purchased from Wako Pure Chemicals (Tokyo, Japan). All other chemicals used were of specific analytical or HPLC grade.

Synthesis of 1′-Hydroxyestragole. 1′-Hydroxy-ES was synthesized from *p*-anisaldehyde as described by Punt et al.[7] The synthesis of 1′-hydroxy-ES encompassed a Grignard reaction, using vinylmagnesium bromide as the Grignard reagent (1 M solution in THF). Briefly, *p*-anisaldehyde (0.0165 mol) was dissolved in 10 mL of dry THF, and this solution was added dropwise over a period of 30 min to the Grignard reagent (0.035 mol) while stirring at 50 °C in anhydrous conditions under a nitrogen atmosphere. The reaction mixture was further incubated for 90 min, and the resulting solution was added to a solution of 4.5 g of ammonium chloride in 200 mL of ice cold water. The emulsion was stirred for several minutes, and 1′-hydroxy-ES was extracted with diethyl ether. The organic solution was dried with magnesium sulfate, and the yield was 94%. The identity of the product was confirmed by ^{1}H NMR. ^{1}H NMR spectra were recorded with a Varian 500 MHz NMR system

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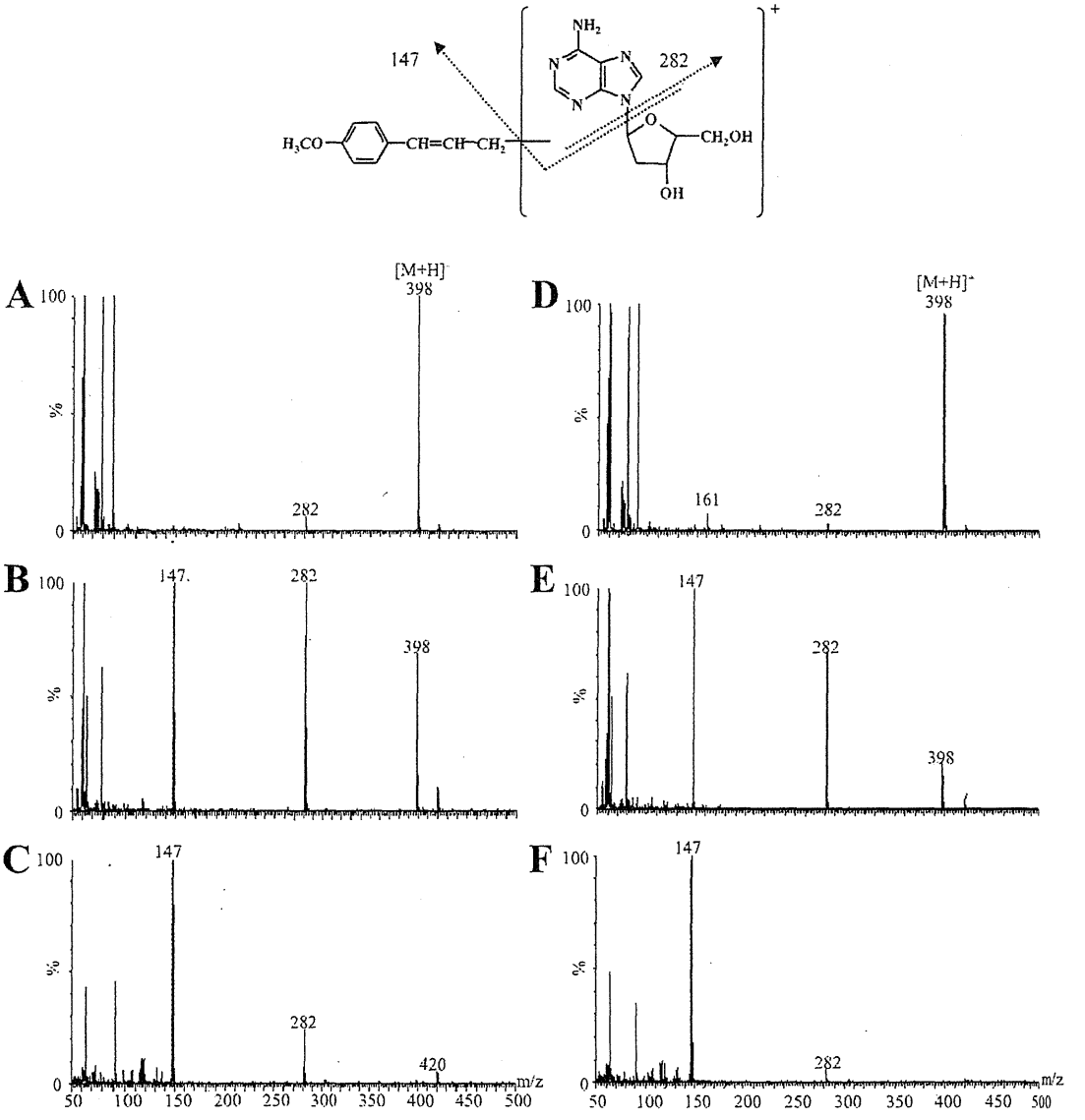

Figure 2. Mass spectra of peaks 1 (A–C) and 2 (D–F) obtained from LC-ESI/MS analysis for the reaction of 1′-acetoxy-ES with dA. Mass analysis was performed in scan mode. The cone voltages were 20 (A and D), 40 (B and E), and 60 V (C and E) in positive ion mode. LC-ESI/MS conditions are described in the Materials and Methods.

(Varian Inc. Corp.). 1′-Hydroxy-ES: ^{1}H NMR (DMSO-d_6): δ 7.22–7.29 (m, 2H, Ar), 6.82–6.89 (m, 2H, Ar), 5.98–6.10 (m, 1H, $-CHCH_2$), 5.26–5.35 [m, 2H, $-C(OH)CH-$], 5.10–5.20 (m, 2H, $-CHCH_2$), 3.79 (s, 1H, $-OCH_3$), 2.24 [s, 1H, $-C(OH)-$].

Synthesis of 1′-Acetoxyestragole. 1′-Acetoxy-ES was synthesized from 1′-hydroxy-ES as described by Punt et al.[7] and Drinkwater et al.[2] In brief, 1′-hydroxy-ES (50 mg) was dissolved in 200 μL of pyridine. Acetic anhydride (33 μL) was added dropwise to this solution, and the reaction mixture was stirred for 5 h at room temperature after which 400 μL of dichloromethane was added. The reaction mixture was extracted several times with aliquots of 200 μL of 1 N HCl. When the aqueous phase reached pH 2–3, the organic layer then immediately was extracted with 400 μL of 1 M sodium carbonate solution (pH 7.6). The organic solution was dried with magnesium sulfate, and the solvent was evaporated in a nitrogen atmosphere. The identity of the product was confirmed by ^{1}H NMR. 1′-Hydroxy-ES: ^{1}H NMR (DMSO-d_6): δ 7.26–7.28 (m, 2H, Ar), 6.92–6.95 (m, 2H, Ar), 5.99–6.07 (m, 1H, $-CHCH_2$), 5.26–5.27 [m, 2H, $-CH(OH)CH-$], 5.19–5.23 (m, 2H, $-CHCH_2$), 3.75 (s, 1H, $-OCH_3$), 2.05 (s, 1H, $-CO-CH_3$).

Determination of ES-Specific dA Adduct. ES-specific dA adducts were determined from reactions between 1′-acetoxy-ES and dA based on the protocol of Phillips et al.[8] The reaction products containing 1′-acetoxy-ES were diluted 50-fold in DMF from which 200 μL was added to 1.8 mL of 10 mM dA solution in 50 mM sodium phosphate buffer (pH 7.4). The reaction mixture was stirred for 24 h at 37 °C. The reaction mixture was then passed through an HLC-DISK syringe filter (Kanto Chemical Co Inc., Tokyo) and was separated on a LC-photodiode array (PDA)-ESI/MS system. LC-PDA-ESI/MS analyses were performed using an Alliance HT model 2695 liquid chromatographic system coupled to a 996 PDA detector and Micromass ZQ, a single quadrupole, MS system (Waters Corp., Miliford, MA) equipped with an ESI source through a splitter. Twenty microliters of the reaction mixture was injected directly onto a reverse-phase C_{18} column (Mightysil RP-18, 4.6 mm × 150 mm, 5 μm, Kanto Chemical Co.,

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Table 1. ^{1}H NMR Chemical Shifts of Peak 1[a]

H-1′	6.34 (1H, m)
H-2′	2.70 (1H, m)
H-2″	2.25 (1H, m)
H-3′	4.40 (1H, m)
H-4′	3.87 (1H, m)
H-5′	3.61 (1H, m)
H-5″	3.51 (1H, m)
OH-3′	5.31 (1H, s)
OH-5′	5.22 (lH,bs)
H-2	8.21 (1H, bs)
H-8	8.37 (1H, s)
N^6-H	8.07 (lH,bs)
ESH-1′	6.42 (1H, m)
ESH-2′	6.21 (1H, m)
ESH-3′	4.22 (2H, bs)
ESH-2,6	7.30 (2H, m)
ESH-3,5	6.85 (2H, m)
$ESOCH_3$	3.75 (3H, m)

[a] m, multiplet; s, singlet.

Inc.) maintained at 40 °C. Solvent A was water, solvent B was methanol, and solvent C was 0.1% formic acid. The column was equilibrated with a mixture of solvent A/solvent B/solvent C (70/10/20, v/v). A linear gradient was applied from 30 to 90% methanol over 40 min, kept at 90% for 10 min, lowered to 20% over 2 min, and re-equilibrated at the initial conditions for 15 min. Mass analysis was performed in scan mode. The cone voltages were 20, 40, and 60 V in the positive ion mode.

Synthesis of ES-Derived dA Adduct. ES-dA adducts were synthesized from a reaction between 1′-acetoxy-ES and dA. Four milliliters of 30 mM 1′-acetoxy-ES solution in dichloromethane was diluted 50-fold in DMF from which 200 mL was added to 1800 mL of 10 mM dG or dA solution in 50 mM sodium phosphate buffer (pH 7.4). The reaction was stirred for 24 h at 50 °C. The yield of peak 1 was 86 mg (7.1%). The reaction was repeated several times as needed to acquire enough products for NMR analysis.

Purification was performed using the combined reaction mixtures that were evaporated and reconstituted in 50% methanol/water. The concentrated reaction mixture was separated by a LC system equipped with a UV detector (LC-UV) (PU-2080 Plus Intelligent HPLC Pump, AS-2057 plus Intelligent Sampler, CO-966 Intelligent Column Thermostat, and UV-970 Intelligent UV/vis Detector; Jasco Co., Tokyo). Two milliliters of sample was injected directly on to a reverse phase C_{18} column (Mightysil RP-18 GP, 20 mm × 250 mm, 5 μm, Kanto Chemical Co., Inc.) maintained at 40 °C. Solvent A was 0.01% formic acid, and solvent B was methanol containing 0.01% formic acid. The column was equilibrated with a mixture of solvent A/solvent B (70/30, v/v). A linear gradient was applied from 30 to 90% methanol over 30 min, kept at 90% for 10 min, lowered to 30% over 2 min, and equilibrated at the initial conditions for 15 min. Products eluting at 22.5 and 31.2 min (UV absorbance at 280 nm; flow rate, 10 mL/min) were collected. The fractions were dried in vacuo and weighed. The identity of the product was confirmed by LC-ESI/MS and ^{1}H NMR. ^{1}H NMR spectra were recorded with a Varian 600 MHz NMR system (Varian Inc. Corp., Palo Alto, CA). Chemical shifts are expressed in ppm downfield shift from trimethylsilane (TMS) (δ scale). The synthesis of stable isotopically labeled surrogate standards was also performed on a small scale by the same method. The reaction products were purified using the LC-UV system.

Standard Solutions. ES-3′-C8-dG and ES-3′-N^2-dG were synthesized from 1′-acetoxy-ES and dG as described by Punt et al.[7] The yields

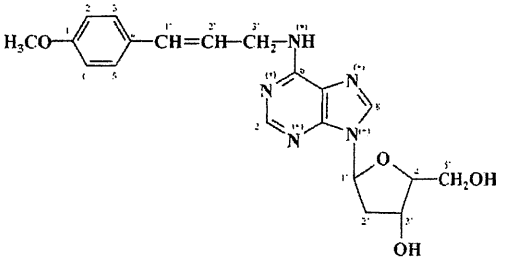

Figure 3. Chemical structure of ES-3′-N^6-dA adducts and the stable isotopically labeled compounds. The asterisk (*) indicates nitrogen [^{15}N]-labeling.

of ES-3′-C8-dG and ES-3′-N^2-dG were 63.4 (5.2%) and 103.5 mg (8.56%), respectively, in the reaction. The syntheses of [$^{15}N_5$]-ES-3′-8-dG, [$^{15}N_5$]-ES-3′-N^2-dG, and [$^{15}N_5$]-ES-3′-N^6-dA as stable isotopically labeled surrogate standards were also performed on a small scale by the same method. $^{15}N_5$-labeled ES-dG adducts including four isomers and ES-3′-N^6-dA were repeatedly purified using LC-UV system until 98% and over.

One millimolar solutions of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA were prepared in methanol and immediately diluted with methanol/HPLC grade water (50/50, v/v) to 10 μM (stock solution). Working solutions for calibration (0.01−10 nM for ES-3′-C8-dG, ES-3′-N^2-dG, and 0.003−10 nM for ES-3′- N^6-dA) were prepared by the addition of an adequate amount of surrogate standard and diluted with methanol/HPLC grade water (50/50, v/v) to appropriate concentrations.

LC-ESI/MS/MS Conditions. LC-ESI/MS/MS analyses were performed using a Quattoro Ultima (Micromass) coupled to a Hewlett Packard 1100 series (G1322A, degasser; G1312A, bin pump; G1316A, Colcom; G1329A, ALS; Agilent Technologies, Palo Alto, CA). The mass spectrometer was operated using an ESI source in the positive ion mode for multiple reaction monitoring (MRM). An aliquot (20 μL) of the sample was injected into a Mightysil C18-GP (2.0 mm × 150 mm, 5 μm; Kanto Chemical Co., Tokyo, Japan) maintained at 40 °C. Solvent A was 0.001% formic acid, and solvent B was 0.001% formic acid containing acetonitrile. The column was equilibrated with a mixture of solvent A/solvent B (75/25, v/v). The mobile phase consisted of a mixture of 0.001% formic acid/0.001% formic acid containing acetonitrile at an initial ratio of 75/25, employing a linear gradient to a final ratio of 30/70 (v/v) over 30 min, at a constant flow rate of 0.2 mL/min.

In the assay for ES-3′-N^2-dG and ES-3′-C8-dG, the precursor ion ([M + H]$^+$) was m/z 414, and the selected product ion [M + H − glycoside]$^+$ was m/z 298. Correspondingly, for [^{15}N]-labeled ES-3′-N^2-dG and ES-3′-C8-dG, the precursor ion was m/z 419, and the selected product ion was m/z 303. The cone voltage used was 18 V, and the collision energy was 10 eV. In the assay for ES-3′-N^6-dA, the precursor ion ([M + H]$^+$) was m/z 398, and the selected product ion [M + H − glycoside]$^+$ was m/z 282. Correspondingly, for [^{15}N]-labeled ES-3′-N^6-dA, the precursor ion was m/z 403, and the selected product ion was m/z 287. The cone voltage used was 12 V, and the collision energy was 18 eV. The source block temperature was 120 °C, and the desolvation temperature was 400 °C. The flow rate of the cone gas was set at 150 L/h, while that of the desolvation gas was set at 600 L/h. Under these conditions, the standard retention times were 11.8, 12.7, and 15.1 min for ES-3′-N^2-dG, ES-3′-C8-dG, and ES-3′-N^6-dA, respectively.

Recovery. The recovery was evaluated by calculating the mean of the response at each concentration. The spiked concentrations (low, middle, and high dose) of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA were determined from the concentrations of each compound in the liver DNA of control rats, using LC-ESI/MS/MS. A standard sample was

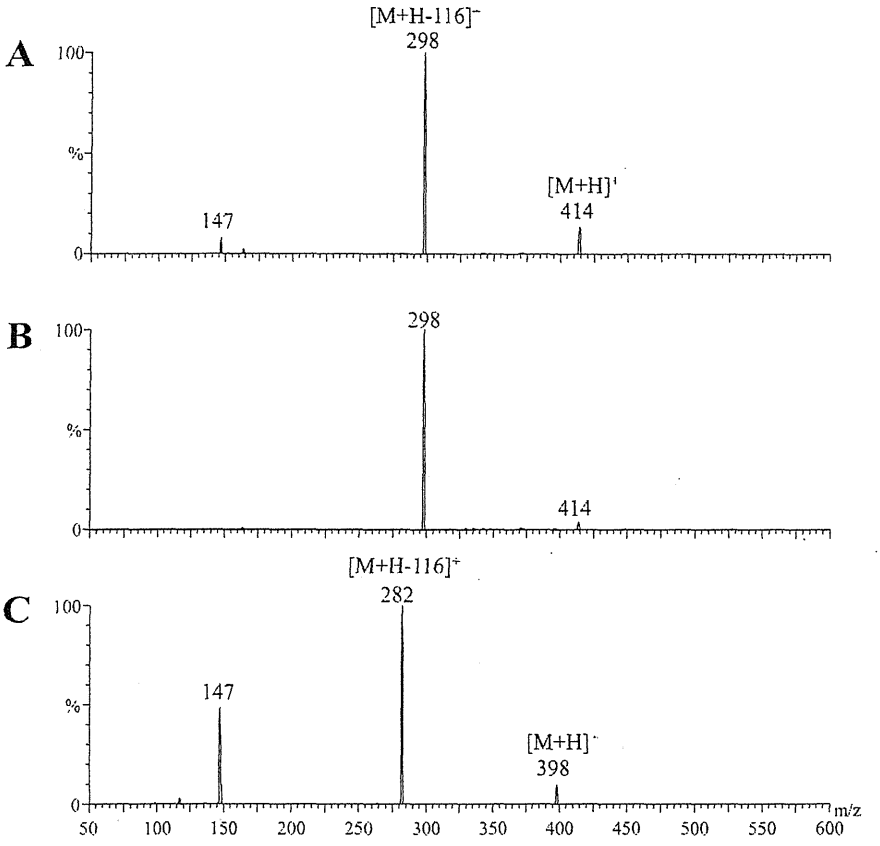

Figure 4. Product ion spectra of (A) ES-3′-C8-dG, (B) ES-3′-N^2-dG, and (C) ES-3′-N^6-dA. The cone voltages and collision energies were set at the optimum conditions for each compound in negative ion mode. LC-ESI/MS/MS conditions are described in the Materials and Methods.

added together with an adequate amount of surrogate standards to 20 mM sodium acetate buffer (pH 4.2) for DNA digestion so that the final concentration might be set to 0.05, 0.5, and 5 nM. The extracted DNA pellets of rat liver were redissolved in this buffer and digested according to the protocol. The sample was analyzed by LC-ESI/MS/MS, and the recovery rates were calculated.

Animal and Treatment. The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo, Japan). Five week old male F344 rats were obtained from Japan SLC (Shizuoka, Japan). Ten rats were housed in polycarbonate cages (five rats per cage) with hardwood chips for bedding in conventional temperature (23 ± 2 °C), humidity (55 ± 5%), air change (12 times per hour), and lighting (12 h light/dark cycle) and were given free access to CRF-1 basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Groups of 10 rats were given ES by gavage in corn oil at 600 mg/kg bw per day, 5 days/week. All rats were killed at 4 weeks by exsanguination under ether anesthesia, and the livers were immediately removed and weighed. Samples were frozen with liquid nitrogen and stored at −80 °C until measurement of ES-specific DNA adducts.

DNA Isolation and Enzymatic Digestion. DNA extraction and digestion were performed according to the method of Nakae et al.[27] and our previous report.[28] The samples were homogenized with lysis buffer including commercial DNA isolation kit. The mixture was centrifuged at 10000*g* for 20 s at 4 °C. The deposit was dissolved in 200 μL of enzyme reaction buffer. After treatment with RNase and protease K, the DNA pellet was obtained by washing with 2-propanol and ethanol and centrifugation.

The dried DNA pellet was dissolved in surrogate standard containing 20 mM sodium acetate buffer, pH 4.8, and was incubated with 4 μL of nuclease P1 (2000 U/mL) at 70 °C for 15 min. Then, 20 μL of 1.0 M Tris-HCl buffer, pH 8.2, was added, and the sample was incubated with 4 μL of alkaline phosphatase (2500 U/mL) at 37 °C for 60 min. After the addition of 3.0 M sodium acetate buffer, pH 5.1, the digested DNA samples were used for adduct analysis and base analysis. Then, 50 μL of the digested sample for dG and dA analysis was passed through 100000 NMWL filter (Millipore, Bedford, MA) and injected into the LC-UV. One hundred microliters of digested sample for adduct analysis was diluted with an equal volume of methanol and injected into the LC-ESI/MS/MS.

LC-UV Analysis for dG and dA. dG and dA were determined by an LC-UV system (Jasco Co.: PU-980 Intelligent HPLC Pump, AS-950-10 Intelligent Sampler, CO-1560 Intelligent Column Thermostat, MD-1515 Multiwavelength Detector, Tokyo, Japan). Two milliliters of sample was injected directly on to a reversed phase C18 column (Ultrasphere ODS, 4.6 mm × 250 mm, 5 μm, Beckman Coulter, Inc.) maintained at 40 °C. Solvent A was 0.01% formic acid containing water, and solvent B was 0.01% formic acid containing methanol. The column was equilibrated with a mixture of solvent A/solvent B (98/2, v/v). The compounds were eluted at a flow rate of 1.0 mL/min. A linear gradient was applied from 2 to 10% methanol over 20 min, kept at 10% for 5 min, lowered to 2% over 2 min, and equilibrated at these initial conditions for 15 min. The wavelength of the UV detector was set at 280 nm for the detection of dG and dA.

RESULTS

Identification of Luc-N^6-dA. The reaction mixtures for 1′-acetoxy-ES with/without dA were separated by LC-PDA-ESI/MS.

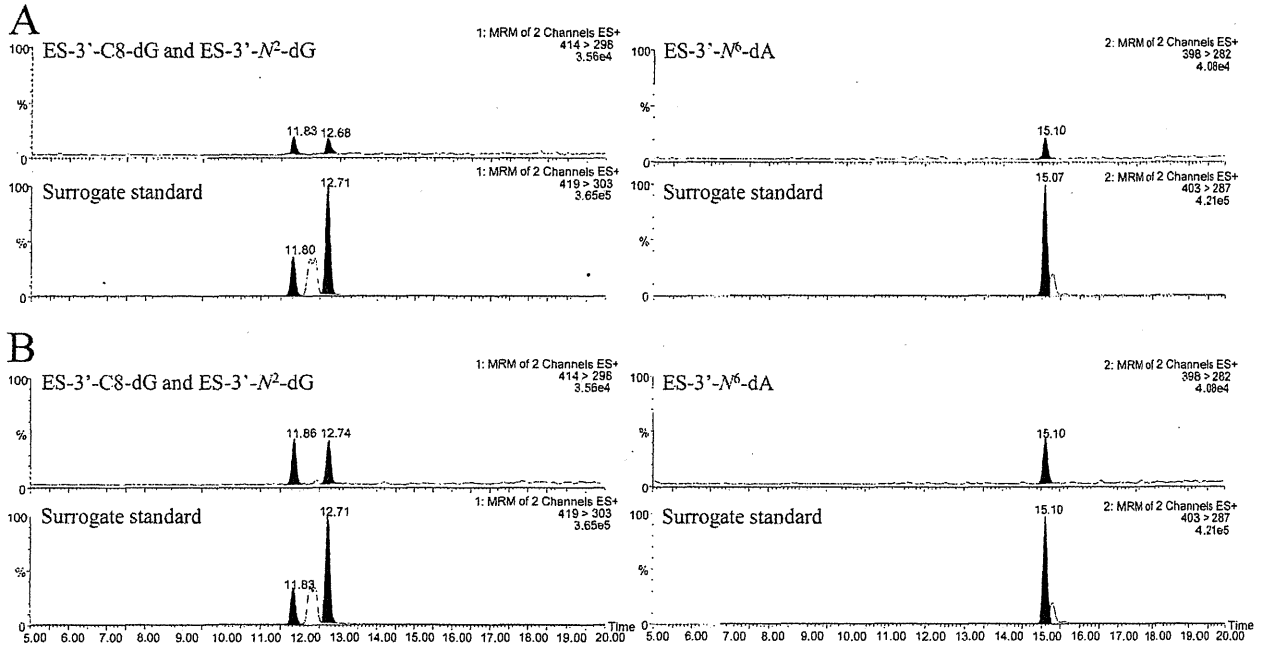

Figure 5. MRM chromatograms of LOD (A) and LOQ (B) levels of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA adducts and each surrogate standard from extracted DNA samples.

Table 2. Recoveries of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA Adducts in Rat Liver Samples

compounds	added (pmol/L)	concentration (pmol/L)	recovery (%)	RSD (%)
ES-3′-C8-dG (n = 5)	50	50.0 ± 2.3	98.0 ± 4.6	4.7
	500	501.1 ± 26.5	100.3 ± 5.3	5.3
	5000	4938.8 ± 135.5	98.8 ± 2.7	2.7
ES-3′-N^2-dG (n = 5)	50	50.6 ± 3.7	101.3 ± 7.5	7.4
	500	497.2 ± 18.6	99.4 ± 3.7	3.7
	5000	4981.2 ± 79.2	99.6 ± 1.6	1.6
ES-3′-N^6-dA (n = 5)	50	51.7 ± 0.77	103.5 ± 1.5	1.5
	500	509.9 ± 7.29	102.0 ± 1.5	1.4
	5000	4988.0 ± 109.8	99.8 ± 2.2	2.2

Typical LC-PDA chromatograms are shown in Figure 1A,B. Two unknown peaks were observed in the PDA chromatogram (280 nm) after the reaction of 1′-acetoxy-ES with dA (Figure1B). Simultaneously, mass spectra and UV−vis absorption spectra were obtained by ESI/MS (cone voltage 20, 40, and 60 V) and PDA. MS spectra of all unknown peaks were analyzed according to the ES and dA structure, and then, two peaks were analyzed in the chromatograms. One of the two peaks (peak 1) was observed at 22.1 min in the chromatogram of dA reaction (Figure 1B). The UV−vis absorption spectrum of peak 1 had λ_{max} at 260 nm. In the mass spectra of peak 1 when the cone voltage was set at 20 V (Figure 2A), the precursor ion ($[M + H]^+$, m/z 398) and product ion (m/z 282), corresponding to an ES-adenine adduct following glycoside bond cleavage, were clearly observed. A product ion (m/z 147) corresponding to 2-allyl-4-mthoxybenzene structure was observed in the mass spectra at 40 and 60 V (Figure 2B,C). The other peak (peak 2) was observed at 22.4 min in the chromatogram for the reaction of 1′-acetoxy-ES and dA (Figure 1B). The UV−vis absorption spectrum was virtually identical to peak 1 and also had at λ_{max} at 260. In the mass spectra of peak 2 when the cone voltage was set at 20 and 40 V (Figure 2D,E), the precursor ion ($[M + H]^+$, m/z 398) and product ion (m/z 282), corresponding to ES-adenine adduct following glycoside bond cleavage, were clearly observed. A product ion (m/z 147) corresponding to 2-allyl-4-mthoxybenzene structure was also observed in the mass spectra of 40 and 60 V (Figure 2E,F).

1′-Acetoxy-ES was used as an electrophilic synthon in large scale synthesis to identify the chemical structures of peaks 1 and 2 from dA, respectively. These adducts were synthesized repeatedly by reaction with each base until the quantity was sufficient for ^{1}H NMR after HPLC-UV chromatography. However, peak 2 could not be collected in sufficient amounts for structural analysis. ^{1}H NMR chemical sifts of peak 1 redissolved in DMSO-d_6 are shown in Table 1. On the basis of these data, we judged that peak 1 was ES-3′-N^6-dA. Chemical structures of ES-3′-N^6-dA and stable isotopically labeled compound as a surrogate standard for LC-ESI/MS/MS analysis are shown in Figure 3.

Optimal Conditions for LC-MS/MS Detection. Figure 4 shows the product ion spectra for ES-3′-C8-dG, ES-3′-N^2-dG,

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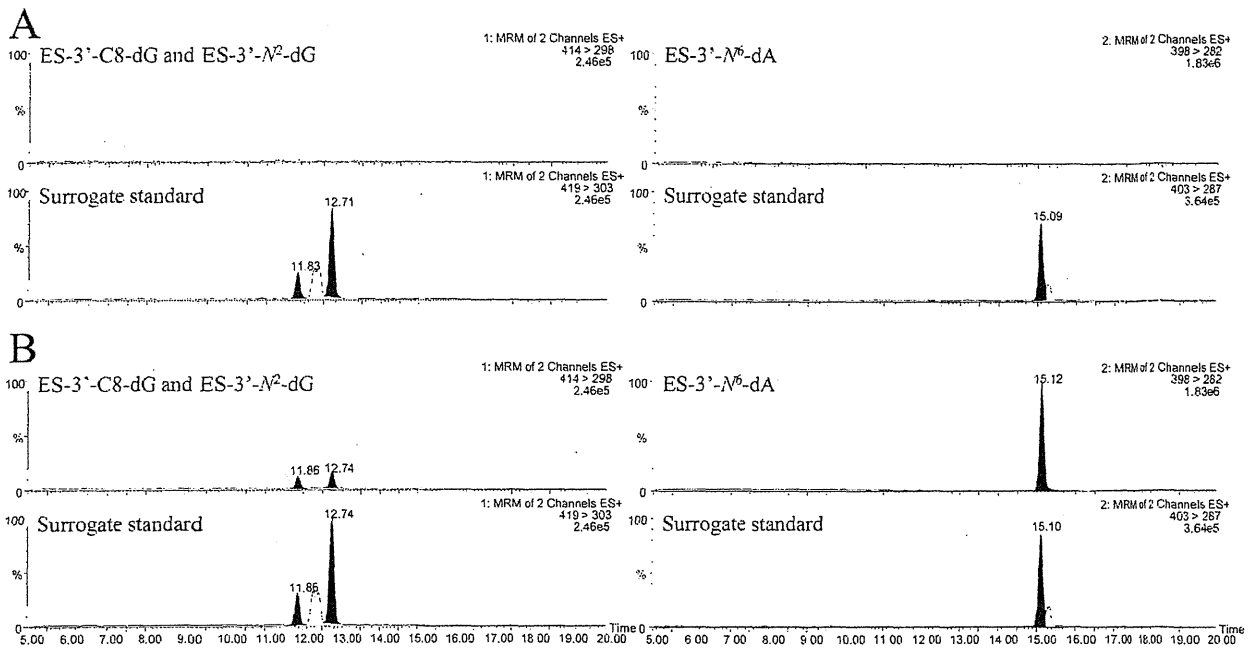

Figure 6. MRM chromatograms of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA adducts and each surrogate standard in rat liver DNA samples. (A) Liver from rat administrated with vehicle as a control group. (B) Liver from rat orally administrated with 600 mg/kg ES for 4 weeks.

Table 3. LC-MS/MS Analysis of Liver DNA Samples Obtained from Rats Treated with 600 mg/kg bw ES for up to 4 Weeks

group	sample no.	ES-3′-C8-dG/10^6dG	ES-3′-N^2-dG/10^6dG	ES-3′-N^6-dA/10^6dA
control	1	ND[a]	ND	ND
	2	ND	ND	ND
	3	ND	ND	ND
	4	ND	ND	ND
	5	ND	ND	ND
	average			
ES (600 mg/kg)	6	3.02	4.16	18.96
	7	3.53	4.28	20.05
	8	3.42	4.37	21.22
	9	4.12	6.18	22.89
	10	3.33	5.07	20.47
	average	3.48 ± 0.40	4.81 ± 0.84	20.47 ± 1.61

[a] ND, not detected.

and ES-3′-N^6-dA. The mass spectrometer equipped with an ESI source using a crossflow counter electrode was run in positive ion mode to detect MRM of the transitions 414 > 298, 414 > 298, and 398 >282, respectively.

The crucial parameters affecting LC-ESI/MS/MS, namely, cone voltage, collision energy, and mobile phase, were investigated. To establish the optimum cone voltage and collision energy for the detection of these adducts, the signals at m/z 414 and 419 precursor ions versus cone voltage were investigated, respectively. The optimal cone voltages were 18, 18, and 12 V in the negative ion mode for ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA standard solutions, respectively. Then, the m/z 298 and 282 product ion signals versus collision energy were investigated, respectively. The optimal collision energies were 10, 10, and 18 eV for ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA standard solutions, respectively. The ionization of the samples at the LC-MS interface was affected by the mobile phase; hence, a mobile phase containing a volatile acid or salt was generally used. In this study, the responses were measured using 0−0.1% formic acid in water−acetonitrile (v/v) as the mobile phase. The responses of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA were increased by the addition of formic acid to the mobile phase. The increase in response reached a maximum and leveled off when 0.001% formic acid was added.

Validation of LC-MS/MS. The calculated instrument detection limit (IDL) of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA of the standard solutions was 3.0, 3.0, and 1.0 pM, respectively, for LC-MS/MS detection at the ratio of the compound's signal to the background signal (S/N) of 3. In addition, the instrument quantification limit (IQL) was calculated when S/N = 10 was 10, 10, and 3.0 pM, respectively. The limit of detection (LOD) and limit of quantification (LOQ) in the real sample were the same as IDL and

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Table 4. Interday Precision for the Determination of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA Adduct Levels in the Rat Liver DNA Samples on Five Different Days Using LC-MS/MS

	repeat analysis of ES-3′-8- dG adduct (nM)						
sample no.	day 1	day 2	day 3	day 4	day 5	mean (nmol/L)	RSD (%)
6	0.510	0.518	0.491	0.506	0.509	0.507 ± 0.010	1.96
7	0.431	0.443	0.450	0.432	0.459	0.443 ± 0.012	2.68
8	0.462	0.470	0.482	0.483	0.445	0.468 ± 0.016	3.39
9	0.474	0.490	0.480	0.458	0.476	0.476 ± 0.011	2.39
10	0.484	0.483	0.483	0.511	0.447	0.482 ± 0.023	4.74
	repeat analysis of ES-3′-N^2-dG adduct (nM)						
sample no.	day 1	day 2	day 3	day 4	day 5	mean (nmol/L)	RSD (%)
6	0.701	0.711	0.733	0.703	0.727	0.715 ± 0.014	1.97
7	0.522	0.597	0.597	0.586	0.546	0.569 ± 0.034	5.91
8	0.590	0.626	0.608	0.638	0.605	0.613 ± 0.019	3.06
9	0.711	0.706	0.705	0.678	0.661	0.692 ± 0.022	3.15
10	0.737	0.676	0.717	0.706	0.718	0.711 ± 0.022	3.13
	repeat analysis of ES-3′-N^6-dA adduct (nM)						
sample no.	day 1	day 2	day 3	day 4	day 5	mean (nmol/L)	RSD (%)
6	3.100	2.967	3.098	3.044	2.949	3.032 ± 0.071	2.36
7	2.286	2.333	2.236	2.339	2.250	2.289 ± 0.047	2.04
8	2.699	2.617	2.738	2.797	2.766	2.723 ± 0.069	2.55
9	2.444	2.455	2.546	2.506	2.526	2.495 ± 0.044	1.77
10	2.736	2.735	2.846	2.880	2.830	2.805 ± 0.066	2.36

IQL, respectively. The peak area ratio with respect to each surrogate standard was plotted, and the response was found to be linear (ES-3′-C8-dG, $y = 1.9707x - 0.0096$ and $y = 2.0318x - 0.0989$; ES-3′-N^2-dG, $y = 0.6359x + 0.0048$ and $y = 0.6336x - 0.0263$; and ES-3′-N^6-dA, $y = 1.8567x + 0.012$ and $y = 1.8205x + 0.065$) over the calibration range, from LOQ to 1.0 nM (low range) and from 0.1 to 10 nM (high range), with a correlation coefficient (r) of over 0.999. The average retention times of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA standards were 11.8 (RSD = 0.08%, n = 5), 12.7 (RSD = 0.12%, n = 5), and 15.1 min (RSD = 0.06%, n = 5), respectively. MRM chromatograms of the mixture of three adduct and their corresponding surrogate standards in 50% methanol at LOD and LOQ levels are shown in Figure 5.

As shown in Table 2, the average recoveries of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA from DNA sample in the livers of nontreated rats were 99.0, 100.1, and 101.7% for each adduct.

Analysis of ES-N^2-dG, C8-dG, and N^6-dA in the Livers DNA Extracted from Rats Treated with ES. The potential formation of ES adducts in livers of rats exposed to ES was assessed using the isotope dilution LC-ESI/MS/MS method. Typical selected ion recording (SIR) chromatograms are shown in Figure 6. No peaks indicative of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA were observed in liver DNA extracted from control rats. In contrast, ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA were detected at 11.8, 12.7, and 15.1 min in the liver DNA MRM chromatograms extracted from rats treated with 600 mg/kg ES for up to 4 weeks, respectively. Quantitative data are summarized in Table 3. The ES-3′-C8-dG, ES-3′-N^2-dG/10^6dG, and ES-3′-N^6-dA/10^6dA ratios were 3.5 ± 0.4, 4.8 ± 0.8, and 20.5 ± 1.6, respectively. The interday sample variation of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA adduct levels analyzed on different days resulted in an average coefficient of variation (CV) of 3.0, 3.4, and 2.2% for different samples that were analyzed (Table 4). All of these adducts were not detected in the control group.

DISCUSSION

Phillips et al. have suggested the chemical structure of four ES-specific DNA adducts, ES-3′-N^2-dG, ES-3′-C8-dG, ES-1′-N^2-dG, and ES-3′-N^6-dA, using radio isotope-labeled nucleosides. The formation of these adducts in mouse liver treated with [^{14}C]-labeled ES was demonstrated by radioactive detection after HPLC fractionation.[8] Subsequently, LC-MS analysis by Punt et al. demonstrated the formation of three ES-dG adducts, ES-3′-N^2-dG, ES-3′-C8-dG, and ES-1′-N^2-dG, after in vitro nucleoside reaction.[7] However, the existence of the dA adduct reported previously and its chemical structure have not been confirmed using MS technique. In the present study, we examine dA adduct formation using LC-ESI/MS by taking advantage of reactive carbocation formation generated by acetylation of ES proposed by Punt et al.[7] In the reaction of dA with 1′-acetoxy-ES, two peaks including ions characteristic for the ES-dA adduct were observed by total ion chromatography. Subsequently, ^{1}H NMR analysis of large-scale synthesized and HPLC purified samples led to the conclusion that the major adduct was ES-3′-N^6-dA, in line with the previous report.[8] Although a minor and unknown ES-dA adduct was also found, its precise chemical structure could not be elucidated due to the low yield.

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ES has been reported to have genotoxicity and carcinogenicity in the livers of mice of both sexes.[2] ES treatment at a dose of 600 mg/kg by gavage for 16 weeks induced significant developments of glutathione S-transferase placenta form (GST-P) foci in the liver of rats (unpublished data). To examine the precise quantity of ES-specific adducts in ES-hepatocarcinogenesis, we used interim samples (4 weeks) for the carcinogenicity study mentioned above. On the basis of previous reports, we attempted to detect ES-3′-N^2-dG, ES-3′-C8-dG, and ES-3′-N^6-dA. Because appropriate sample preparations and a highly sensitive analytical method are necessary to detect modified DNA bases in genomic DNA, we developed a new analytical method by an isotope dilution LC-ESI/MS/MS method using SIR. ^{32}P-postlabeling analysis without internal standards for identifying the products neither provides structural characterization of adducts nor has sufficient selectivities. In addition, this assay requires radioactive γ-^{32}P-labeled ATP in the analytical process, which raises the necessity of rigorous handling. The LC-MS/MS technique using stable isotope adducts as internal standards achieves accurate quantification of DNA adducts along with structural characterization. The LOQs of ES-3′-N^2-dG, ES-3′-C8-dG, and ES-3′-N^6-dA in our method were determined to be 2−5 adducts/10^8 unmodified dG or dA bases from a 150 mg liver sample. In addition, the high recoveries of these adducts in the wide range from LOQ level indicated that our new method enables precise adduct determination with the use of surrogate standards and is applicable to the detection of these compounds in animal tissue samples. As a result, our method was able to quantify these three adducts in liver DNA of rats treated with 600 mg/kg ES for 4 weeks, and we observed that the ES-3′-N^6-dA adduct is predominant. Phillips et al. have suggested that ES-3′-N^2-dG was a major adduct in the livers of mice treated with single dose of 1′-hydroxy-ES (12 μmol/mouse), a metabolite of ES. Considering that the present experiment was performed under carcinogenic conditions, it is highly probable that the status of DNA adduct formation observed in the present study may be reflected in the ES carcinogenesis. The modification at the N^6-position of dA has been reported following treatment with other potent mutagens including aristolochic acid,[22,23] polyaromatic hydrocarbon, benzo[*c*]phenanthrene,[29,30] and 5,6-dimathylchrysene.[31] It is likely that the formation of various types of chemical-specific base modifications is dependent on accessibility of the chemical (or the proximate form) to the reactive amino group between dG and dA in the DNA helical structure.[32] Those mutagens predominantly induce AT-TA transversion and AT-GC transition mutations.[25,33,34] Therefore, our unpublished data that the AT-GC transition mutation was predominant in ES-treated rat livers allow us to hypothesize that N^6-dA adducts might play a key role in ES mutagenicity. Thus, information regarding the precise concentrations of ES-specific DNA adducts discovered in the present study would be very helpful for further research on ES hepatocarcinogenesis.

Minor adducts of dG (ES-1′-N^2-dG) and dA (not identified) were hardly detected in in vivo samples even though such adducts are detectable in the reaction of 1′-acetoxy-ES with deoxynucleoside.[7] Dissociation of the ester group from an ester of 1-hydroxy-ES generates an electrophilic ion in which the positive charge may reside on the double bond between the 2-, 3- and 1-, 2-positions on the allyl chain. Nucleophilic attack by the purine bases of DNA at the 1′-position of ES would account for ES-1′-N^2-dG formation, while attack at the 3′-position would account for ES-3′-N^2-dG. Because dG modification at the 3′-position of ES far from the benzene ring is predominant both in vivo and in vitro, it is conceivable that the priority of these reactions is determined by steric hindrance between DNA bases and ES. The suggestion that unknown minor dA adducts also might result from modification at the 1′-position of ES is in line with this hypothesis. Therefore, the fact that genomic DNA possesses more steric hindrance than nucleosides might explain the lack of detection of these minor adducts in vivo.

In conclusion, dA modified by ES was determined to be EG-3′-N^6-dA as the major adduct. ES-3′-N^2-dG, ES-3′-C8-dG, and ES-3′-N^6-dA adducts can be identified and quantified by this new method, which may prove useful in other related studies.

AUTHOR INFORMATION

Corresponding Author

*Tel: +81-3-3700-9819. Fax: +81-3-3700-1425. E-mail: y-ishii@nihs.go.jp.

ABBREVIATIONS

ES, estragole; dG, deoxyguanosine; dA, deoxyadenosine; LC, liquid chromatography; ESI, electron spray ionization; MS, mass spectrometry; NMR, nuclear magnetic resonance; MS/MS, tandem mass spectrometry; SIR, selected ion recording; LOQ_L limit of quantification; SULT, sulfotransferase; THF, tetrahydrofuran; DMSO, dimethylsulfoxide; DMF, dimethylformamide; TMS, trimethylsilane; MRM, multiple reaction monitoring; PDA, photodiode array; IDL, instrument detection limit; IQL, instrument quantification limit; LOD, limit of detection; GST-P, glutathione S-transferase placenta form.

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GENOTOXICITY AND CARCINOGENICITY

Possible involvement of genotoxic mechanisms in estragole-induced hepatocarcinogenesis in rats

Yuta Suzuki · Takashi Umemura · Daisuke Hibi · Tomoki Inoue · Meilan Jin · Yuji Ishii · Hiroki Sakai · Takehiko Nohmi · Tokuma Yanai · Akiyoshi Nishikawa · Kumiko Ogawa

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Abstract Estragole (ES) is a natural organic compound used frequently as a flavoring food additive. Although it has been reported to be tumorigenic and induce DNA adducts in the mouse liver, there have been no reports regarding ES hepatocarcinogenicity in rats. In the current study, we therefore examined potent carcinogenicity, DNA adduct formation and in vivo genotoxicity of ES in the livers of wild and reporter gene-carrying F344 rats. Males were administered 600 mg/kg bw ES by gavage and sequentially sacrificed at weeks 4, 8 and 16 for GST-P and PCNA immunohistochemistry and measurement of ES-specific DNA adducts by LC-MS/MS in the livers. GST-P-positive foci increased with time in ES-treated rats from week 4, PCNA-labeling indices being similarly elevated at both weeks 4 and 8. ES-specific DNA adducts such as ES-3′-N^2-dG, 3′-8-dG and 3′-N^6-dA were consistently detected, particularly at week 4. In a second study, male F344 *gpt* delta rats were administered 0, 22, 66, 200 or 600 mg/kg bw ES for 4 weeks. *Gpt* mutant frequency in the liver was increased in a dose-dependent manner, with significance at 200 and 600 mg/kg bw in good correlation with PCNA-labeling indices. Mutation spectra analysis showed A:T to G:C transitions to be predominantly increased in line with the formation of ES-3′-N^6-dA or 3′-8-dG. These results indicate that ES could be a possible genotoxic hepatocarcinogen in the rat, at least when given at high doses.

Keywords Estragole · Rat · Hepatocarcinogenesis · Genotoxicity

Y. Suzuki · T. Umemura · D. Hibi · T. Inoue · M. Jin · Y. Ishii · A. Nishikawa (✉) · K. Ogawa
Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
e-mail: nishikaw@nihs.go.jp

Y. Suzuki · D. Hibi · H. Sakai · T. Yanai
Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

H. Sakai · T. Yanai
Laboratory of Veterinary Pathology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

T. Nohmi
Division of Genetics and Mutagenesis, National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan

Introduction

Estragole (ES) is an allylbenzene compound, which is a natural constituent of several herbs, including basil, fennel, anise and tarragon. It has found use as a flavoring agent although acting as a hepatocarcinogen in various strains of mice (Drinkwater et al. 1976; Miller et al. 1983; Wiseman et al. 1987). DNA adducts were also detected in livers of mice following intraperitoneal injection of ES (Randerath et al. 1984; Phillips et al. 1984), and ES-specific DNA adducts are strongly implicated in carcinogenesis (Wiseman et al. 1985, Fennell et al. 1985). In in vitro assays, ES was generally non-mutagenic in *Salmonella typhimurium*, *Escherichia coli* WP2 uvrA and *Bacillus subtilis* (Zeiger et al. 1987; Sekizawa and Shibamoto 1982), although mutagenicity was demonstrated in strain TA1535 of *S. typhimurium* without metabolic activation (Swanson et al. 1979) and ES induced DNA damage in an in vitro unscheduled DNA synthesis (UDS) test in rat hepatocytes (Nesslany et al. 2010). In vivo, while the UDS assay in rats was also positive, a rat bone-marrow micronucleus test was

negative (Nesslany et al. 2010). Although DNA adduct formation has been reported in mouse livers (Randerath et al. 1984; Phillips et al. 1984), equivocal results have been generated in terms of genotoxicity and the mechanism of ES hepatocarcinogenesis is unclear and remains to be elucidated.

In 2001, the Scientific Committee on Food (SCF) of the European Union reported a scientific opinion that ES is both genotoxic and carcinogenic. Accordingly, SCF recommended reduction in exposure levels and restrictions on use (SCF 2001). On the other hand, the expert panel of the Flavor and Extract Manufacturers Association (FEMA) concluded that dietary exposure to ES from spice consumption does not pose a significant cancer risk to humans because several studies clearly established that profiles of metabolism, metabolic activation and covalent binding were dose dependent at high levels but diminished markedly at lower levels of exposure (Smith et al. 2002).

In the present study, to assess the carcinogenic potential of ES in rats, quantitative immunohistochemical analyses of glutathione *S*-transferase placental form (GST-P)-positive hepatocytes were performed in F344 rats given ES at a high dose for up to 16 weeks. ES-specific DNA adducts were analyzed by LC-MS/MS, a method as sensitive as ^{32}P-post-labeling (Singh and Farmer 2006). Additionally, to understand the molecular mechanisms underlying genotoxicity and carcinogenicity of ES, mutation assays were conducted using the reporter gene-carrying *gpt* delta transgenic rat, considered as a useful tool for estimating in vivo genotoxic and carcinogenic risks of environmental chemicals (Nohmi et al. 2000; Kanki et al. 2005; Umemura et al. 2007; Nishikawa et al. 2008).

Materials and methods

Chemicals and animals

ES (CAS No. 140-67-0) and diethylnitrosamine (DEN, CAS No. 55-18-5) were purchased from Tokyo Kasei (Tokyo, Japan). Five-week-old male F344 rats and *gpt* delta rats carrying approximately 10 tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan), housed in polycarbonate cages with hardwood chips for bedding in a conventional animal facility and maintained under conditions of controlled temperature (23 ± 2 °C), humidity (55 ± 5 %), air change (12 times per hour) and lighting (12 h light/dark cycle). The animals were given free access to CRF-1 basal diet (Oriental Yeast, Tokyo) and tap water and were used after a 1-week acclimation period.

Animal treatments

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo).

Experiment I After acclimation, eighty F344 male rats were randomly divided into three groups, with 30 rats each in two groups (control and ES-treated groups), and 20 in the DEN-treated group. Control animals received corn oil vehicle alone. ES-treated rats received 600 mg/5 mL/kg body weight (bw) ES in corn oil by gavage 5 days per week. Dose level and frequency of dose were determined based on an NTP technical report on the 3-month toxicity of ES (NTP 2011) in which 600 mg/kg bw ES showed potent carcinogenicity based on the occurrence of one hepatocellular adenoma in the liver of one of 10 male rats. The DEN-treated positive control group was given 10 ppm DEN dissolved in the drinking water for 16 weeks. Five animals each of the control and ES groups were euthanized at week 4 and 8, and the other 20 animals each were euthanized at 16 weeks after the beginning of the experiment. At each time point, removed livers were weighed and portions were immediately fixed in buffered formalin and routinely processed for embedding in paraffin, sectioning, and hematoxylin and eosin (H&E) and immunohistochemical staining. The remaining liver tissues were frozen in liquid nitrogen and stored in a deep freezer at −80 °C until measurement of ES-DNA adducts.

Experiment II The dosage levels, experimental design and dosing method were selected based on the results of experiment I and the NTP report (NTP 2011). Groups of five male *gpt* delta rats were given ES by gavage at concentrations of 0, 22, 66, 200 or 600 mg/5 mL/kg bw 5 days per week for 4 weeks. Controls (0 mg/kg bw) received the corn oil vehicle alone. At the end of the experimental period, the animals were euthanized and livers were immediately removed and weighed. Then, portions were fixed in buffered formalin and processed for routine histological and histochemical examinations similarly to Experiment I. The remaining liver tissues were frozen for measurement of ES-DNA adducts, as for Experiment I and in vivo mutation assays.

Immunohistochemistry for GST-P and PCNA

Immunohistochemistry for GST-P and proliferating cell nuclear antigen (PCNA) was conducted in liver tissue sections of all groups of animals, using anti-GST-P rabbit polyclonal antibodies (Medical and Biological Laboratories, Aichi, Japan) at a dilution of 1:1,000 and an anti-PCNA monoclonal antibody (Dako Cytomation A/S, Glostrup, Denmark) at a dilution of 1:100. A streptavidin-

biotin-peroxidase complex kit (Dako Cytomation) was employed with the chromogen 3,3′-diaminobenzidine for demonstration of binding, followed by counterstaining with hematoxylin. Numbers and areas of GST-P-positive foci consisting of 5 or more nucleated hepatocytes in cross-section were evaluated with the aid of an image analyzer (IPAP, Sumika Technoservice, Hyogo, Japan). For quantification of cell proliferation, sections from five rats in each group were analyzed at 200× magnification, with 5 fields chosen at random. The number of PCNA-positive hepatocytes in at least 2,000 cells in normal-looking areas was counted, and the PCNA-positive index was expressed as the percentage value.

In vivo mutation assay

6-Thioguanine (TG) selection was carried out as previously described (Nohmi et al. 2000). Briefly, genomic DNA was extracted from the liver, and lambda EG10 DNA (48 kb) was rescued as a lambda phage by in vitro packaging. For 6-TG selection, the packaged phage was incubated with *E. coli* YG6020, expressing Cre recombinase, and converted to a plasmid-carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol, and 6-TG. The *gpt* gene of *E. coli* encodes guanine phosphoribosyltransferase that catalyzes phosphoribosylation of guanine and also 6-TG. Since 6-TG is toxic to cells when incorporated into DNA, *E. coli* cells having wild-type *gpt* gene cannot survive on plates containing 6-TG, but only *gpt* mutants can be selected to form colonies on plates containing 6-TG. The gene name *gpt* is derived from the enzyme guanine phosphoribosyltransferase. In order to determine the total number of rescued plasmids, infected cells were also poured on plates containing chloramphenicol without 6-TG. The plates were incubated at 37 °C for the selection of 6-TG resistant colonies, and the *gpt* mutant frequency (MF) was calculated by dividing the number of *gpt* mutants after clonal correction by the number of rescued phages. For characterizing the mutation spectra of *gpt* mutants, a 739-bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as described previously (Nohmi et al. 2000). For mutation spectrum, *gpt* mutants (6-TG^R and Cm^R colonies) shown in Table 5 were all analyzed. Sequencing analysis was performed at Takara Bio Inc. (Mie, Japan), and then, colonies sharing the same mutation regarded as demonstrating clonal growth were eliminated from the total number of mutant colonies.

Quantification of ES-DNA adduct levels in livers

The level of ES-specific DNA adducts in livers was quantified with our newly established method using LC-MS/MS (Ishii et al. 2011). Briefly, samples were homogenized with the lysis buffer included in a commercial DNA isolation kit. The mixture was centrifuged, and the deposit was dissolved in enzyme reaction buffer. After treatment with RNase and protease K, a DNA pellet was obtained. The dried DNA pellet was dissolved in surrogate standard containing sodium acetate buffer, pH 4.8, and incubated with nuclease P1. This was followed with the addition of Tris–HCl buffer, pH 8.2, and the solution was incubated with alkaline phosphatase. After the addition of sodium acetate buffer, pH 5.1, samples were injected into the LC-MS/MS. LC-MS/MS analysis was performed using a Quattoro Ultima (Micromass, Beverly, MA, USA) coupled to a HEWLETT PACKARD 1100 series (Agilent technologies, Palo Alto, USA). The mass spectrometer was operated using an electrospray ionization source in the positive ion mode (ESI$^+$) for multiple reaction monitoring (MRM).

Statistics

The significance of differences in the results of Experiment I was evaluated using Student's *t* test, and in Experiment II with ANOVA, followed by the Dunnett's multiple comparison test or the multiple Student-Welch test with Pharmaco Analyst II (Hakuhousha, Tokyo, Japan).

Results

Sequential analysis of rats given 600 mg/kg bw ES for up to 16 weeks

Body and liver weights measured in Experiment I are shown in Fig. 1 and Table 1. Body weights were significantly ($P < 0.01$) decreased in the group treated with ES as compared with the control group from week 1. Absolute liver weights in the ES group were significantly ($P < 0.01$) decreased at week 4, but increased at week 16. Relative liver weights in the ES group were significantly ($P < 0.01$)

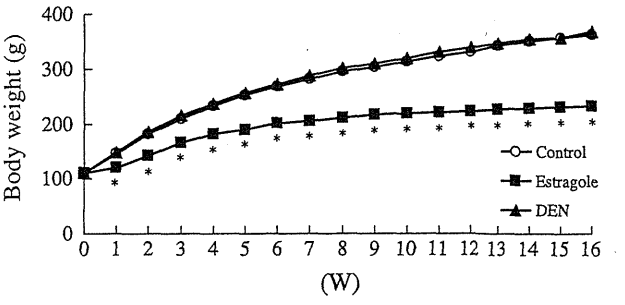

Fig. 1 Growth curves for F344 rats treated with 600 mg/kg estragole or 10 ppm DEN for 16 weeks. *$P < 0.01$ versus control

Table 1 Mean body and liver weights of F344 rats treated with 600 mg/kg bw estragole or 10 ppm DEN for 4, 8 or 16 weeks

Group	Rats	Body weights (g)	Absolute liver weights (g)	Relative liver weights (g %)
4 weeks				
Control	5	231.3 ± 11.8[a]	8.40 ± 0.54	3.63 ± 0.12
Estragole	5	176.6 ± 8.1*	6.99 ± 0.51*	3.95 ± 0.13*
		(−24 %)	(−17 %)	(+9 %)
8 weeks				
Control	5	297.5 ± 8.2	9.80 ± 0.50	3.30 ± 0.12
Estragole	5	211.9 ± 13.4*	9.18 ± 0.89	4.33 ± 0.16*
		(−29 %)		(+31 %)
16 weeks				
Control	20	361.6 ± 17.1	10.48 ± 0.66	2.90 ± 0.08
Estragole	20	228.3 ± 22.3*	21.29 ± 2.77*	9.33 ± 0.84*
		(−37 %)	(+103 %)	(+222 %)
DEN	20	366.8 ± 17.5	12.16 ± 0.75*	3.31 ± 0.09*
			(+16 %)	(+14 %)

Values in the parenthesis indicate percentage of change against the mean control values (+: increase, −: decrease)

* $P < 0.01$ versus control

[a] Means ± SD

Table 2 Histopathological findings for the livers of F344 rats treated 600 mg/kg bw estragole

Week	4	8	16
No. of animals examined	5	5	20
Lymphocytic aggregation (±/+/++)	5 (5/0/0)	5 (1/4/0)	20 (0/5/15)
Hepatocyte hypertrophy (++)	0	0	20
Oval cell proliferation (±/+)	0	1 (1/0)	16 (10/6)
Bile duct proliferation (±/+)	0	1 (1/0)	16 (10/6)
Cholangiofibrosis (±/+/++)	0	1 (1/0/0)	12 (6/4/2)

±: minimal, +: mild, ++: moderate

increased compared with the control group at weeks 4, 8 and 16. The absolute and relative liver weights in the DEN group were also significantly ($P < 0.01$) increased at week 16.

Histopathological findings in the liver are summarized in Table 2. Mild to moderate lymphocytic aggregation was observed in livers of ES-treated animals throughout the experiment, becoming more severe over time. Centrilobular hepatocellular hypertrophy was evident only at week 16. Oval cell and bile duct proliferation was noted in one of five and sixteen of twenty ES-treated rats at weeks 8 and 16, respectively. Cholangiofibrosis was detected in one and twelve ES-treated rats at weeks 8 and 16, respectively. The results regarding GST-P positive liver cell foci are shown in Table 3. At weeks 4 and 8, GST-P positive foci were induced in the ES group. At week 16, the mean number and area of GST-P positive foci were significantly ($P < 0.01$) increased in the ES or DEN groups as compared to those in the control group. Data for immunohistochemical expression of the cell proliferation marker PCNA are summarized in Fig. 2. PCNA-positive indices (%) of the normal-looking hepatocytes in the control and ES-treated livers were 1.15 ± 0.49 and 5.25 ± 1.14 at week 4; and 0.73 ± 0.10 and 5.34 ± 1.01 at week 8, respectively. Values were significantly ($P < 0.01$) increased in the ES group as compared with the control group at both time points.

ES-specific DNA adducts, 3′-8-dG, 3′-N^2-dG and 3′-N^6-dA, were detected by the LC-MS/MS method in the rat liver treated with 600 mg/kg bw ES for 4 and 8 weeks (Fig. 3). No ES-specific DNA adducts were detected in the livers of control group. The level of DNA adducts detected at week 4 was significantly ($P < 0.01$) higher than that seen at week 8.

Dose–response analysis of rats given 22, 66, 200 or 600 mg/kg bw ES for 4 weeks

Three of 5 rats in the 600 mg/kg ES group died with severe body weight loss at 1 or 2 weeks after the start of experiment. The cause of deaths could not be clarified because of autolysis or cannibalism although somewhat higher susceptibility to liver toxicity of *gpt* delta rats than wild F344 rats might be suggested. Therefore, data for the 600 mg/kg ES group were excluded from the effective animal numbers. Data for final body weights and absolute and relative liver weights measured in Experiment II are summarized in Fig. 4 and Table 4. Body weights were significantly ($P < 0.01$) decreased in the 200 mg/kg bw treated group as compared with the control group from week 3. Relative liver weights were significantly ($P < 0.01$) increased in the 200 mg/kg bw treated group as compared with the control group. Final body weights and liver weights were comparable between control and 66 mg/kg bw or lower ES-treated groups.