験法として期待されている。

このような背景から、本研究では、MEUGの一般毒性、*in vivo* 変異原性ならびに発がん性を包括的に評価する目的で、F344 系 *gpt* delta ラットを用いた 90 日間反復投与毒性試験を行った。

B. 研究方法

5 週齢の F344 *gpt* delta ラット雌雄各 40 匹を約 1 週間の馴化飼育後、雌雄とも各群 10 匹ずつ 4 群に分け、検体投与群には 10、30 または 100 mg/kg の MEUG を強制経口投与した。また、対照群には溶媒を同様に投与した。実験終了後、全生存動物を剖検し、脳、心臓、肺、腎臓、副腎、胸腺、脳、脾臓、肝臓及び精巣の重量を測定した。また、腹大動脈血より、血液学、血清生化学の検索を行った。さらに、全身諸臓器について病理組織学的検査を行った。発がん標的臓器である肝臓においては、ラット肝前がん病変マーカーである GST-P 陽性肝細胞巣の量的解析のための免疫染色を行った。また、肝臓の一部を *gpt* assay 用に採取し、液体窒素により凍結し, 測定まで-80℃ で保存した。

（倫理面への配慮）

実験動物は、国立医薬品食品衛生研究の動物実験ガイドラインに準拠し、実験動物委員会の承認に基づき実施した。特に、動物愛護の精神に則って動物飼育を行い、動物処置には倫理基準に充分配慮し、実験終了時、安楽死においても深麻酔下、苦痛に配慮した。また、申請者ならびに研究協力者の健康保持のため、本研究で被験物質として使用する化合物、各実験で使用する薬品は、安全キャビネット等で厳重に注意して取り扱った。

C. 研究結果

試験期間中に雌の投与群で 3 匹が誤投与により死亡した。その他、動物の一般状態については特記すべき変化は認められなかった。実験期間中の体重推移では、雌雄ともに投与群と対照群の間に有意な差はなかった。(Fig. 1)。雌雄の摂餌量を Fig. 2 と Fig.3 に示す。実験期間中の摂餌量は雌雄ともに有意な変化はなかった。臓器重量においては、雄の 100 mg/kg投与群で肝臓の絶対および相対重量と雌の 100 mg/kg投与群の相対肝重量が対照群に比べ有意に増加した。雄の 100 mg/kg投与群の絶対腎重量、30 mg/kg以上の投与群の相対腎重量および 100 mg/kg投与群の相対精巣重量が対照群に比べて有意に増加した（Table 1)。雌では、30 mg/kg以上の投与群の絶対心重量と 100 mg/kg投与群の腎臓と脳の相対重量が対照群に比べ有意に増加した (Table 2)。血液学的検査においては、雌の 30 mg/kg投与群の WBC と 100 mg/kg投与群の Plt 数が対照群に比べ有意に増加した (Table 3)。 血清生化学的検査では、雄の 30 mg/kg以上の投与群で Cl（塩素）と AST（aspartate aminotransferase）が対照群に比べ有意に減少した。さらに、30 mg/kg投与群の phospholipid と 100 mg/kg投与群の A/G 比は有意に増加し、100 mg/kg投与群の CRN （ creatinine ） と ALT （ alanine aminotransferase）は有意に減少した。雌では、100 mg/kg投与群の Alb と CRN が対照群に比べて有意に減少し、Cl と IP (無機リン)は有意に増加した（Table 4)。さらに、30 mg/kg以上の投与群では T-Bil が有意に減少した（Table 4)。また、肝臓における GST-P

免疫染色においては、100 mg/kg投与群で雌雄ともにGST-P陽性肝細胞巣の数、面積ともに対照群に比べ有意に増加した（Fig. 4）。

D. 考察

本実験では、MEUGの*gpt* deltaラットを用いた短期包括試験を実施し、一般毒性、*in vivo*変異原性ならびに発がん性を評価した。雄の100 mg/kg投与群で肝臓の絶対および相対重量と雌の100 mg/kg投与群の相対肝重量が対照群に比べ有意に増加した。長期発がん性試験において、肝臓がMEUGの標的臓器であることから、これらの変化はMEUGの投与に起因した変化であると考えられた。さらに、雄の100 mg/kgまたは30 mg/kg以上の投与群において腎臓の絶対及び相対重量、100 mg/kg投与群の相対精巣重量が対照群に比べて有意に増加した。雌では、30 mg/kg以上の投与群の絶対心重量と100 mg/kg投与群の腎臓と脳の相対重量が対照群に比べ有意に増加した。しかし、血液学的または血清生化学的検査において、これらと関連する変化が認められていない。現在、病理組織学的検査が終了してないことから、これらの結果を踏まえてその毒性学的意義については評価をする必要があると考えられる。血清生化学的検査では、雄の30 mg/kg以上の投与群でClとASTが有意に減少した。さらに、30 mg/kgまたは100 mg/kg投与群でphospholipid、A/G比、CRNおよびALTが有意に減少した。雌では、100 mg/kg投与群においてAlb、CRN、Cl、IPが対照群に比べて有意に減少した。これらの変化についても、病理組織学的検査の結果と合わせて、その毒性学的意義について考察する予定である。肝臓におけるGST-P免疫染色では、雌雄ともに100mg/kg投与群でGST-P陽性肝細胞巣の数、面積ともに対照群に比べ有意に増加した。MEUGは長期発がん性試験において37mg/kg以上の投与群で肝腫瘍の発生率が有意に増加したことが報告されており、今回の結果はそれを裏付けるものであると考えられた。

今後、MEUGの遺伝毒性を検索するために肝組織を用いて*gpt*ならびに*red/gam*変異頻度の解析を行い、MEUGの*in vivo*変異原性を明らかにし、一般毒性試験ならびに発がん性試験結果と合わせて、MEUGの包括的な評価を行う予定である。

E. 結論

gpt deltaラットに90日間反復投与毒性試験を実施して、一般毒性、in vivo変異原性ならびに発がん性を包括的に評価することを目的に実施した本試験から、これまで報告されているMEUGの肝毒性ならびに肝前がん病変が有意に増加したことから、その発がん性を示唆する結果を得ることができた。今後、*gpt*ならびに*red/gam*変異頻度の解析を行い、MEUGの*in vivo*変異原性を検索し、包括的に毒性評価と共に、その発がんメカニズムを明らかにする予定である。

F. 健康危険情報

特になし

G. 研究成果

なし

H. 知的財産権の出願・登録状況

なし

【参考文献】

1) National Toxciology Program (NTP) 2000. Toxicology and carcinogenesis studies of methyleugenol (CAS No. 93 15 12) in F344/n rats and B6C3F1 mice (gavage studies). DRAFT NTP-TR-491; NIH Publication No.98 3950

Figure 1. Growth curves for male and female *gpt* delta rats given MEUG for 13 weeks.

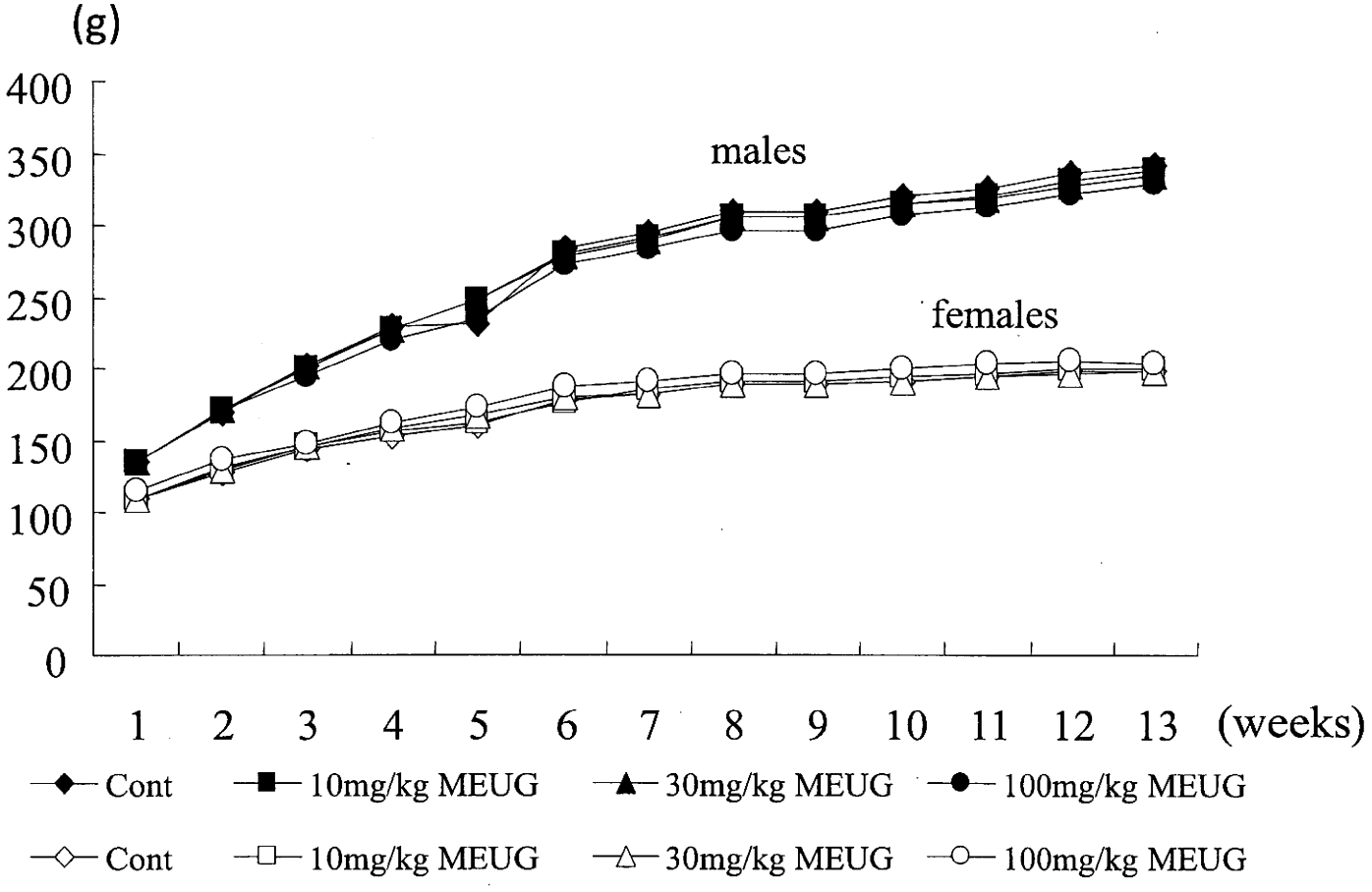

Fig. 2. Food consumption curves for male *gpt* delta rats given MEUG for 13 weeks.

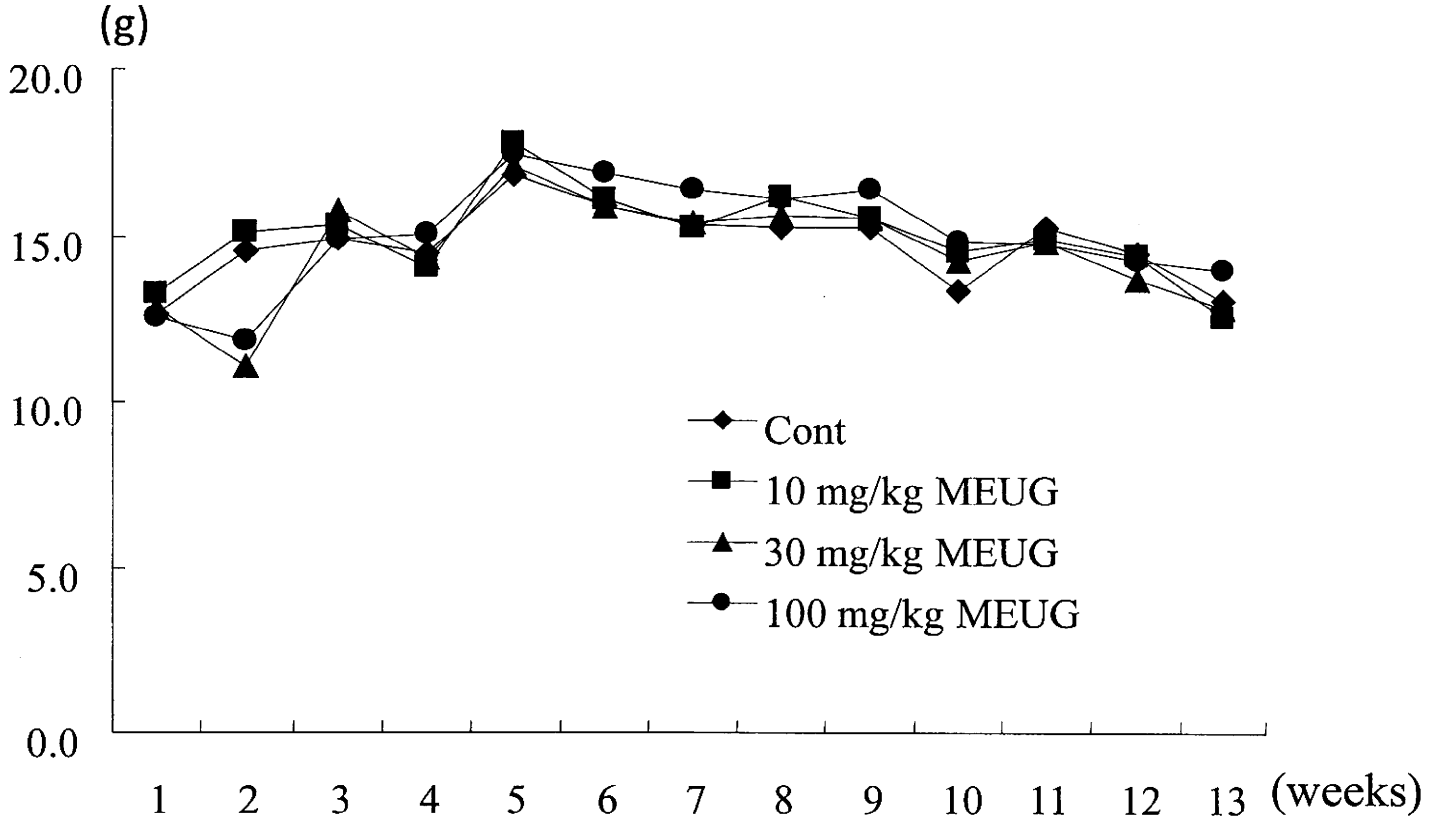

Fig. 3. Food consumption curves for male *gpt* delta rats given MEUG for 13 weeks.

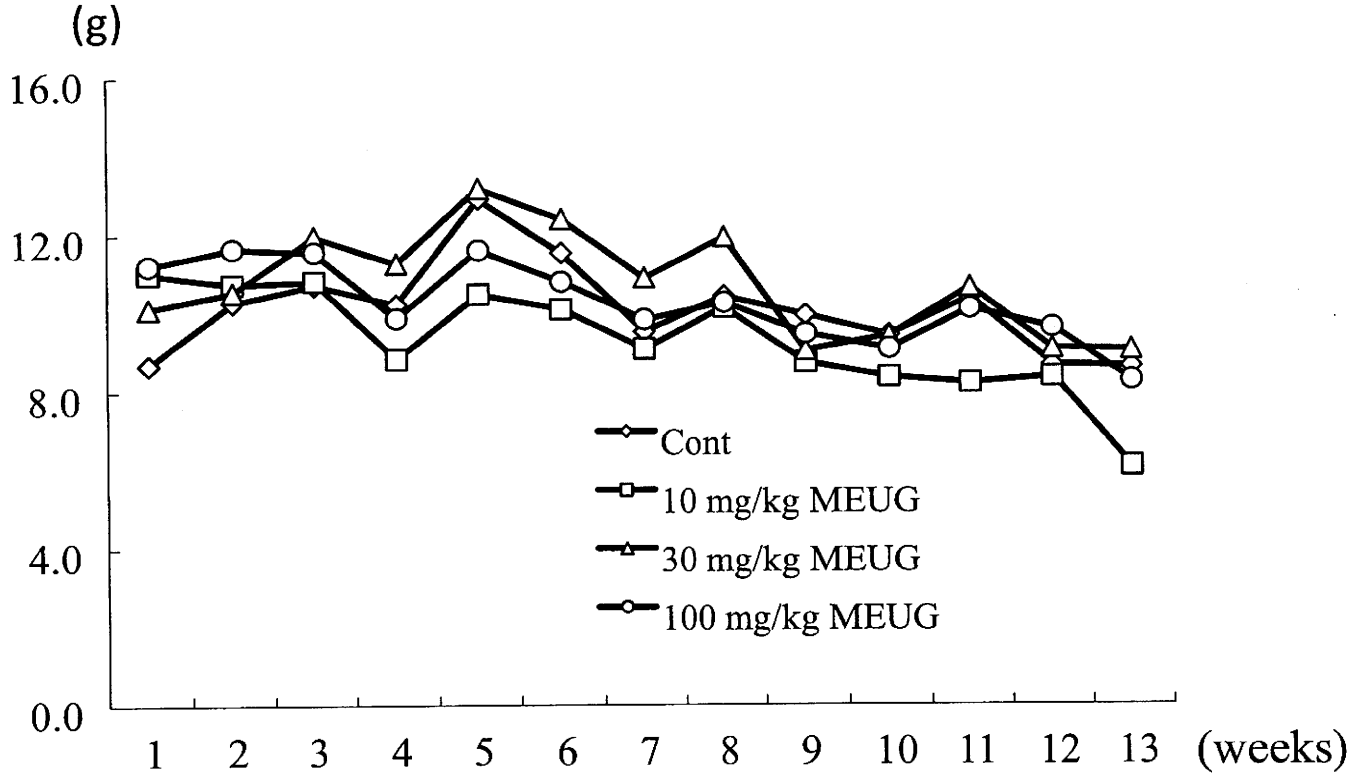

Figure 4. Number and area of GST-P positive foci (≧3cell) in male and female *gpt* delta rats given MEUG for 13 weeks

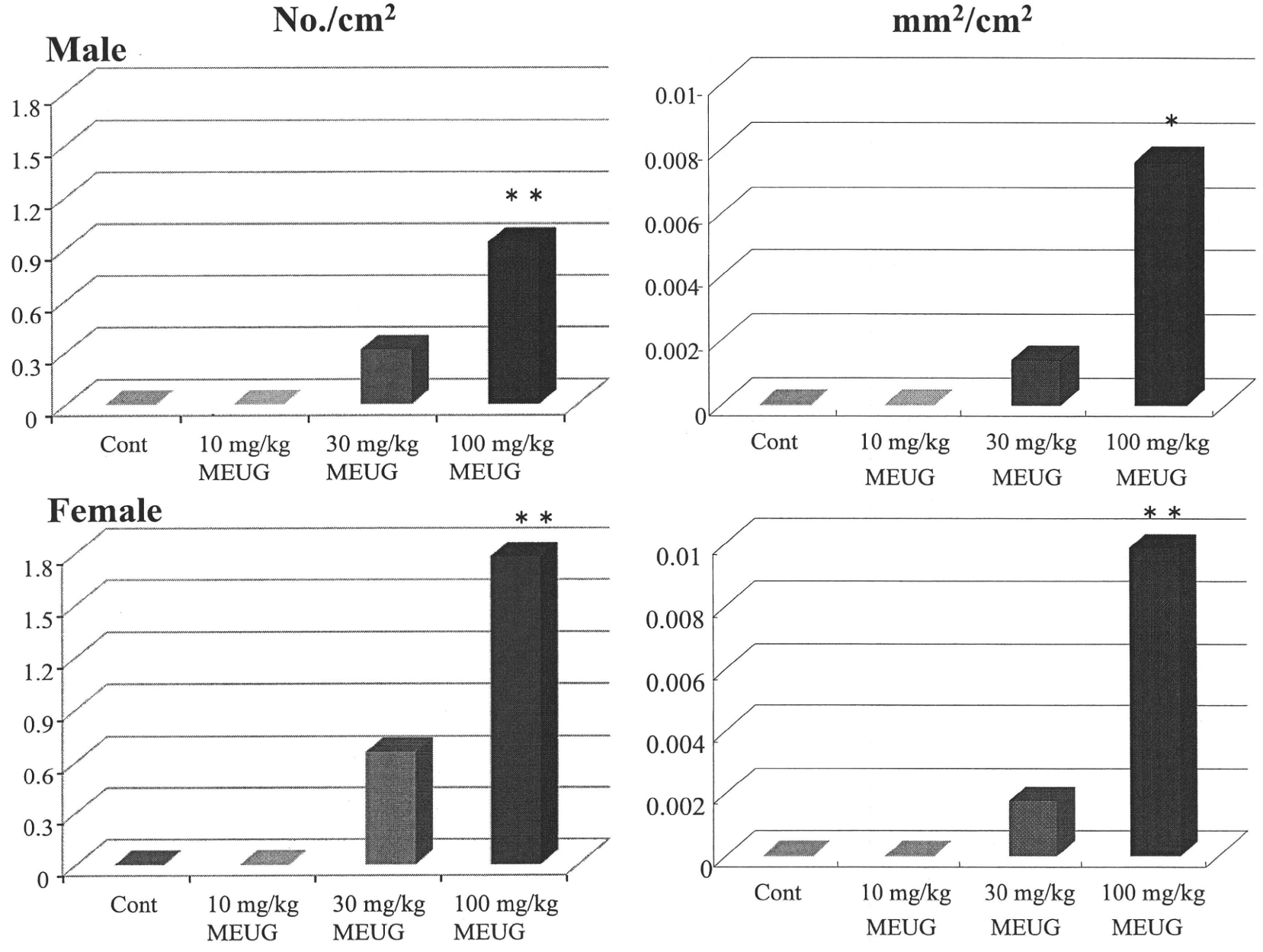

Table 1. Final body and organ weights for male *gpt* delta rats given MEUG for 13 weeks

Group	Control	10 mg/kg MEUG	30 mg/kg MEUG	100 mg/kg MEUG
No. of animal	10	10	10	10
Body weight	300.0±17.4[a]	325.0±10.4	322.4±13.7	310.2±35.4
Absolute(g)				
Liver	8.09±0.49	8.22±0.41	8.54±0.41	9.43±0.70 **
Lung	1.16±0.14	1.15±0.06	1.16±0.11	1.16±0.10
Kidney	1.82±0.11	1.78±0.07	1.84±0.07	1.93±0.08 *
Brain	1.93±0.05	1.94±0.03	1.96±0.03	1.96±0.04
Spleen	0.65±0.04	0.65±0.03	0.65±0.03	0.67±0.03
Thymus	0.24±0.05	0.20±0.02	0.22±0.05	0.21±0.03
Heart	0.85±0.06	0.84±0.01	0.84±0.04	0.84±0.04
Adrenal	0.06±0.06	0.04±0.01	0.05±0.00	0.05±0.00
Gonad	2.93±0.11	2.91±0.09	2.96±0.13	3.00±0.08
Relative(g/100g B.W.)				
Liver	2.45±0.07	2.53±0.08	2.65±0.08	3.09±0.45 **
Lung	0.35±0.03	0.35±0.03	0.36±0.03	0.38±0.05
Kidney	0.55±0.01	0.55±0.01	0.57±0.01 *	0.63±0.08 **
Brain	0.59±0.03	0.60±0.02	0.61±0.03	0.64±0.10
Spleen	0.20±0.01	0.20±0.01	0.20±0.01	0.22±0.04
Thymus	0.07±0.02	0.06±0.01	0.07±0.02	0.07±0.01
Heart	0.26±0.01	0.26±0.01	0.26±0.01	0.28±0.04
Adrenal	0.02±0.02	0.01±0.00	0.01±0.00	0.02±0.00
Gonad	0.89±0.05	0.89±0.03	0.92±0.03	0.99±0.15 *

*,**: Significantly different from the controls at the leverls of $p<0.05$ and $p<0.01$, respectively (Dunnet's test) [a] Mean±SD.

Table 2. Final body and organ weights for female *gpt* delta rats given MEUG for 13 weeks

Group	Control	10 mg/kg MEUG	30 mg/kg MEUG	100 mg/kg MEUG
No. of animal	10	9	9	9
Body	189.0±5.6	190.3±8.3	187.6±5.3	176.5±9.7
Absolute(g)				
Liver	4.26±0.22	4.34±0.22	4.29±0.22	4.38±0.20
Lung	0.84±0.10	0.80±0.05	0.83±0.09	0.80±0.07
Kidney	1.09±0.04	1.09±0.03	1.09±0.04	1.07±0.04
Brain	1.78±0.03	1.80±0.04	1.77±0.05	1.75±0.04
Spleen	0.41±0.02	0.42±0.02	0.40±0.03	0.40±0.02
Thymus	0.19±0.02	0.19±0.02	0.18±0.02	0.18±0.02
Heart	0.55±0.03	0.54±0.02	0.52±0.02 *	0.50±0.02 **
Adrenal	0.05±0.01	0.05±0.00	0.05±0.00	0.05±0.01
Relative(g/100g B.W.)				
Liver	2.26±0.12	2.28±0.14	2.29±0.11	2.48±0.08 **
Lung	0.45±0.06	0.42±0.04	0.44±0.05	0.45±0.05
Kidney	0.58±0.02	0.58±0.02	0.58±0.02	0.61±0.03 *
Brain	0.94±0.03	0.95±0.04	0.94±0.03	1.00±0.06 *
Spleen	0.22±0.01	0.22±0.01	0.21±0.01	0.23±0.02
Thymus	0.10±0.01	0.09±0.04	0.10±0.01	0.10±0.01
Heart	0.29±0.01	0.29±0.01	0.28±0.01	0.28±0.01
Adrenal	0.03±0.00	0.02±0.00	0.03±0.00	0.03±0.00

*,**: Significantly different from the controls at the leverls of $p<0.05$ and $p<0.01$, respectively (Dunnet's test) [a] Mean±SD.

Table 3. Hematological data for male and female *gpt* delta rats given MEUG for 13 weeks

Item	Dose of ME 0	10 mg/kg	30 mg/kg	100 mg/kg
Male				
No. of animals examined	10	10	10	10
WBC ($x10^2/\mu\ell$)	34.9 ± 5.6	46.1 ± 7.7	46.5 ± 5.8	48.7 ± 7.5
RBS ($x10^4/\mu\ell$)	923.5 ± 31.9	964.1 ± 42.1	919.1 ± 33.9	945.7 ± 34.3
Hb (g/dL)	15.8 ± 0.6	15.6 ± 0.2	15.4 ± 0.5	15.8 ± 0.6
Ht (%)	51.7 ± 2	51.1 ± 2.1	50.0 ± 1.8	51.4 ± 1.8
MCV (fL)	56.0 ± 0.5	53.0 ± 0.4	54.3 ± 0.3	54.4 ± 0.5
MCH (pg)	17.2 ± 0.2	16.2 ± 0.6	16.7 ± 0.4	16.7 ± 0.4
MCHC (g/dL)	30.6 ± 0.5	30.5 ± 1	30.8 ± 0.7	30.8 ± 0.8
Plt ($x10^4/\mu\ell$)	74.6 ± 8.1	66.1 ± 4.2	71.6 ± 5.7	70.3 ± 3.3
Differential leukocyte counts (%)				
Band form neutrophils	1.6 ± 1.1	1.4 ± 1.0	2.1 ± 1.1	1.6 ± 1.0
Segmented neutrophils	28.5 ± 9.4	35.4 ± 8.5	29.5 ± 6.3	29.9 ± 6.2
Eosinophils	1.5 ± 1.0	1.0 ± 0.3	1.2 ± 0.5	0.8 ± 0.7
Basophils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes	68.0 ± 8.7	61.9 ± 8.1	66.7 ± 6.2	67.7 ± 6.2
Monocytes	0.3 ± 0.4	0.2 ± 0.3	0.5 ± 0.7	0.2 ± 0.3
Reticulocytes	1.4 ± 1.2	2.6 ± 1.5	2.7 ± 2.1	4.0 ± 2.1
Female				
No. of animals examined	10	9	9	9
WBC ($x10^2/\mu\ell$)	34.9 ± 5.6	34.6 ± 10.6	44.8 ± 8.9*	31.7 ± 5.6
RBS ($x10^4/\mu\ell$)	923.5 ± 31.9	916.5 ± 31.0	896.7 ± 29.2	877.0 ± 56.9
Hb (g/dL)	15.8 ± 0.6	15.5 ± 0.6	15.4 ± 0.4	15.5 ± 0.8
Ht (%)	51.7 ± 2	50.9 ± 1.8	50.0 ± 1.6	49.1 ± 2.8
MCV (fL)	56.0 ± 0.5	55.6 ± 0.4	55.8 ± 0.4	56.7 ± 0.2
MCH (pg)	17.2 ± 0.2	17.0 ± 0.2	17.2 ± 0.4	17.9 ± 0.5
MCHC (g/dL)	30.6 ± 0.5	30.5 ± 0.3	30.8 ± 0.7	31.5 ± 1.0
Plt ($x10^4/\mu\ell$)	74.6 ± 8.1	72.4 ± 2.7	59.3 ± 3.3	84.7 ± 5.4**
Differential leukocyte counts (%)				
Band form neutrophils	1.1 ± 0.7	1.3 ± 0.7	1.9 ± 0.7	2.0 ± 1.0
Segmented neutrophils	21.5 ± 4.6	25.1 ± 4.1	20.3 ± 5.0	20.7 ± 5.3
Eosinophils	1.1 ± 0.7	1.3 ± 0.6	1.0 ± 0.9	0.7 ± 0.5
Basophils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes	75.9 ± 5.2	72.1 ± 4.2	76.1 ± 4.9	76.2 ± 5.7
Monocytes	0.4 ± 0.4	0.2 ± 0.3	0.6 ± 0.5	0.4 ± 0.4
Reticulocytes	4.4 ± 2.7	4.4 ± 3.0	5.9 ± 2.5	6.6 ± 4.2

Abbreviations: WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; Ht, hematocrit; MCV, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Plt, platelet.

[a] Mean ± SD. *: Significantly different from the controls at the levels of $p<0.05$ (Dunnett's test)

Table 4. Serum biochemistry for male and female *gpt* delta rats given MEUG for 13 weeks

Item	Dose of methyl eugenol (P.O.)			
	Cont	10 mg/kg	30 mg/kg	100 mg/kg
Male				
No. of animals examined	10	10	10	10
TP (g/dl)	6.56 ± 0.14[a]	6.57 ± 0.14	6.65 ± 0.12	6.45 ± 0.20
A/G	1.84 ± 0.05	1.96 ± 0.14	1.93 ± 0.09	2.05 ± 0.12**
Alb (g/dl)	4.25 ± 0.08	4.35 ± 0.10	4.38 ± 0.08	4.33 ± 0.16
T-Bil (mg/dl)	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Glucose (mg/dl)	172.00 ± 14.72	159.50 ± 16.33	150.70 ± 14.71	160.10 ± 20.52
TG (mg/dl)	134.70 ± 30.59	168.30 ± 21.06	193.90 ± 68.33	188.00 ± 62.09
Phospholipid (mg/dl)	114.10 ± 7.37	119.00 ± 4.64	129.20 ± 10.79*	125.70 ± 15.78
TC (mg/dl)	70.40 ± 2.67	70.10 ± 4.89	72.70 ± 5.21	66.20 ± 7.48
BUN (mg/dl)	18.61 ± 1.55	19.57 ± 1.15	19.86 ± 1.84	19.45 ± 2.40
CRN (mg/dl)	0.33 ± 0.03	0.33 ± 0.02	0.31 ± 0.02	0.29 ± 0.02**
Na (mEQ/l)	144.20 ± 0.79	144.80 ± 0.42	144.20 ± 0.92	144.00 ± 1.25
Cl (mEQ/l)	104.50 ± 0.85	103.70 ± 0.82	103.30 ± 1.06*	103.20 ± 1.40*
K (mEq/l)	4.35 ± 0.21	4.23 ± 0.19	4.26 ± 0.16	4.16 ± 0.21
Ca (mg/dl)	10.09 ± 0.17	10.07 ± 0.23	10.18 ± 0.20	10.21 ± 0.41
IP (mg/dl)	4.80 ± 0.57	5.18 ± 0.67	5.20 ± 0.52	5.75 ± 0.34**
AST (IU/l)	107.50 ± 23.80	92.60 ± 15.69	83.40 ± 19.96*	78.40 ± 7.83**
ALT (IU/l)	75.00 ± 21.09	63.90 ± 11.11	62.00 ± 22.14	55.40 ± 7.60*
ALP (IU/l)	410.80 ± 22.70	427.10 ± 26.33	409.20 ± 25.68	383.50 ± 35.19
Female				
No. of animals examined	10	9	9	9
TP (g/dl)	7.11 ± 0.33	6.96 ± 0.23	6.83 ± 0.29	6.67 ± 0.27
A/G	2.27 ± 0.15	2.24 ± 0.17	2.28 ± 0.18	2.24 ± 0.11
Alb (g/dl)	4.93 ± 0.22	4.80 ± 0.15	4.74 ± 0.24	4.61 ± 0.17**
T-Bil (mg/dl)	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01*	0.05 ± 0.01**
Glucose (mg/dl)	129.50 ± 18.82	115.78 ± 11.69	133.67 ± 13.35	131.00 ± 18.48
TG (mg/dl)	56.40 ± 22.13	43.00 ± 13.81	69.56 ± 40.40	57.56 ± 21.23
Phospholipid (mg/dl)	176.60 ± 20.15	169.67 ± 12.63	177.89 ± 14.84	177.11 ± 14.26
TC (mg/dl)	103.30 ± 11.20	101.56 ± 8.71	103.89 ± 6.83	102.00 ± 7.21
BUN (mg/dl)	17.11 ± 2.22	15.89 ± 1.22	15.96 ± 1.50	17.01 ± 0.82
CRN (mg/dl)	0.33 ± 0.03	0.34 ± 0.02	0.30 ± 0.02	0.29 ± 0.01**
Na (mEQ/l)	144.40 ± 0.52	144.67 ± 0.71	145.00 ± 0.87	145.11 ± 1.54
Cl (mEQ/l)	104.90 ± 0.88	105.67 ± 0.87	105.56 ± 0.88	106.78 ± 1.99*
K (mEq/l)	4.06 ± 0.22	3.98 ± 0.17	3.92 ± 0.10	3.94 ± 0.22
Ca (mg/dl)	10.36 ± 0.31	10.09 ± 0.17	10.39 ± 0.21	10.37 ± 0.24
IP (mg/dl)	4.47 ± 0.46	4.40 ± 0.39	4.49 ± 0.56	4.96 ± 0.33**
AST (IU/l)	71.70 ± 13.28	72.89 ± 8.91	64.56 ± 7.58	66.78 ± 9.81
ALT (IU/l)	39.50 ± 5.13	35.44 ± 5.50	35.56 ± 3.81	35.44 ± 6.54
ALP (IU/l)	266.80 ± 34.54	273.78 ± 40.76	275.11 ± 30.70	266.22 ± 29.80

Abbreviations: TP, total protein; Alb, albumin; T-Bil, total bilirubin; TG, triglyceride; TC, Total cholesterol; BUN, blood urea nitrogen; CRN, creatinine; Na, sodium; Cl, chlorine; K, potassium; Ca, calcium; IP, inorganic phosphate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase *,**: Significantly different from the controls at the levels of $p<0.05$ and $p<0.01$, respectively (Dunnett's test) [a] Mean±SD

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ishii Y, Suzuki Y, Hibi D, Jin M, Fukuhara K, Umemura T, Nishikawa A,	Detection and quantification of specific DNA adducts by liquid chromatography-tandem mass spectrometry in the livers of rats given estragole at the carcinogenic dose	Chem. Res. Toxicol.	24	532-541	2011

研究成果の刊行物・別刷

Chemical Research in Toxicology

ARTICLE

pubs.acs.org/crt

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

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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,

Received: November 27, 2010
Published: March 08, 2011

   dx.doi.org/10.1021/tx100410y | Chem. Res. Toxicol. 2011, 24, 532–541

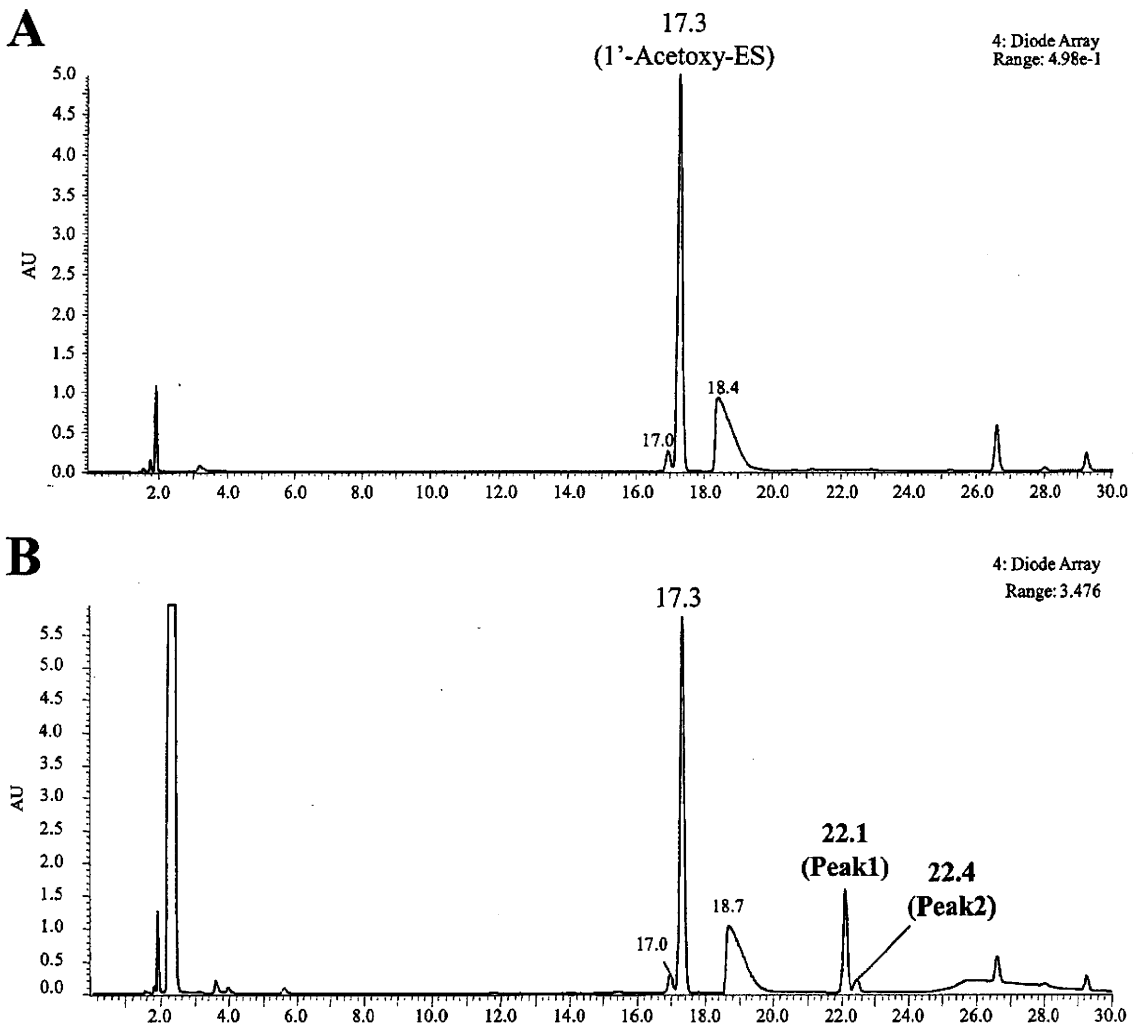

Figure 1. Typical LC-PDA chromatograms (280 nm) in the reaction of (A) 1′-acetoxy-ES alone and (B) 1′-acetoxy-ES with dA. LC-PDA conditions are described in the Materials and Methods.

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

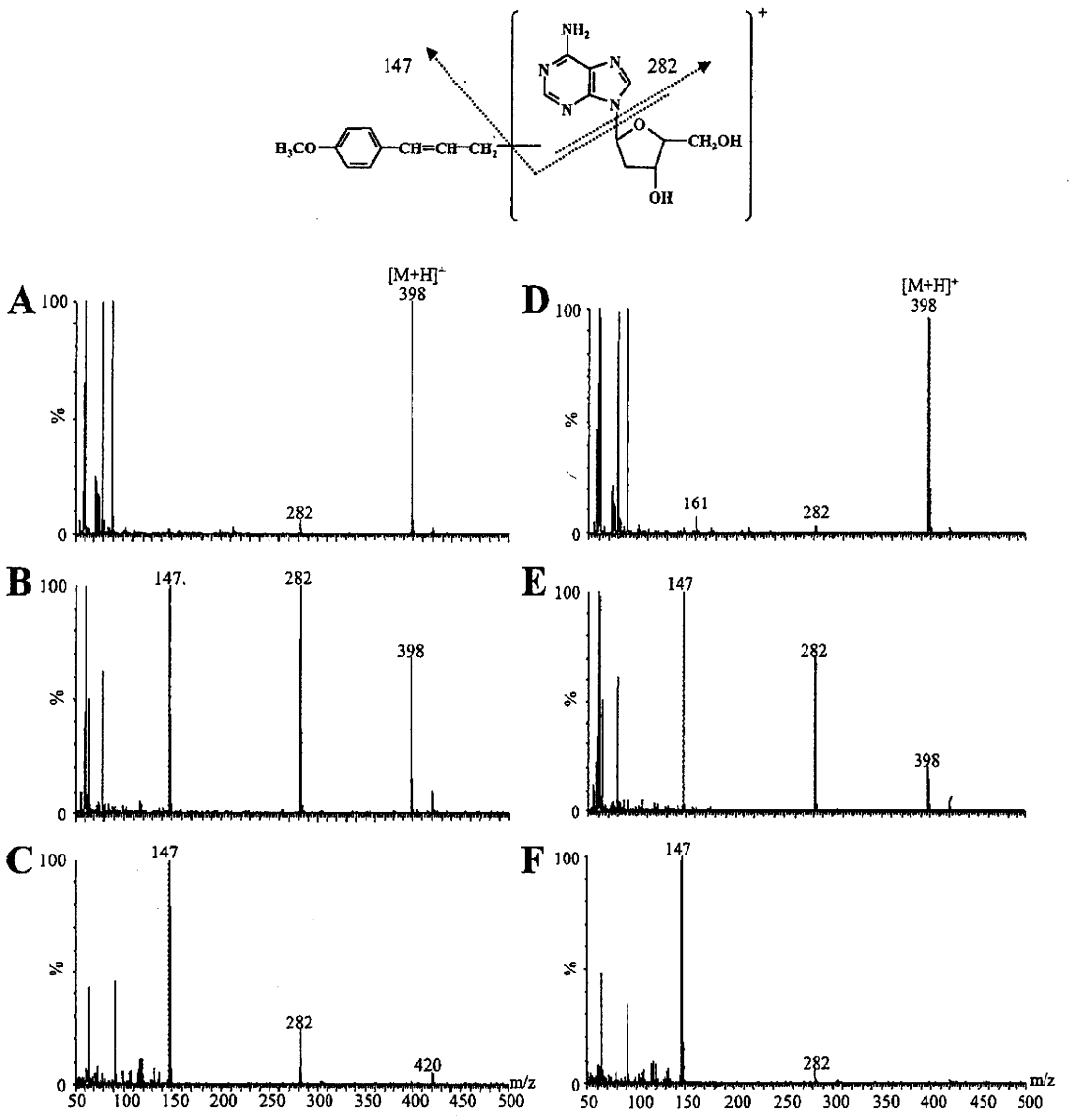

Figure 2. Mass spectra of peaks 1 (A–C) and 2 (D–F) obtained from LC-ESI/MS analysis for the reaction of 1′-acetoxy-ES with dA. Mass analysis was performed in scan mode. The cone voltages were 20 (A and D), 40 (B and E), and 60 V (C and E) in positive ion mode. LC-ESI/MS conditions are described in the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

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 (1H,bs)
H-2	8.21 (1H, bs)
H-8	8.37 (1H, s)
N^6-H	8.07 (1H,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

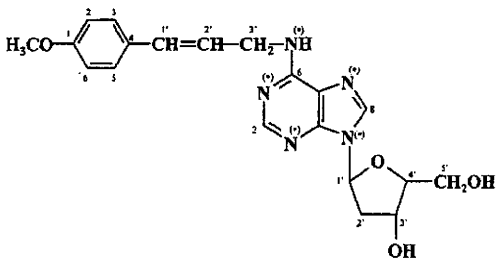

Figure 3. Chemical structure of ES-3′-N^6-dA adducts and the stable isotopically labeled compounds. The asterisk (*) indicates nitrogen [^{15}N]-labeling.

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

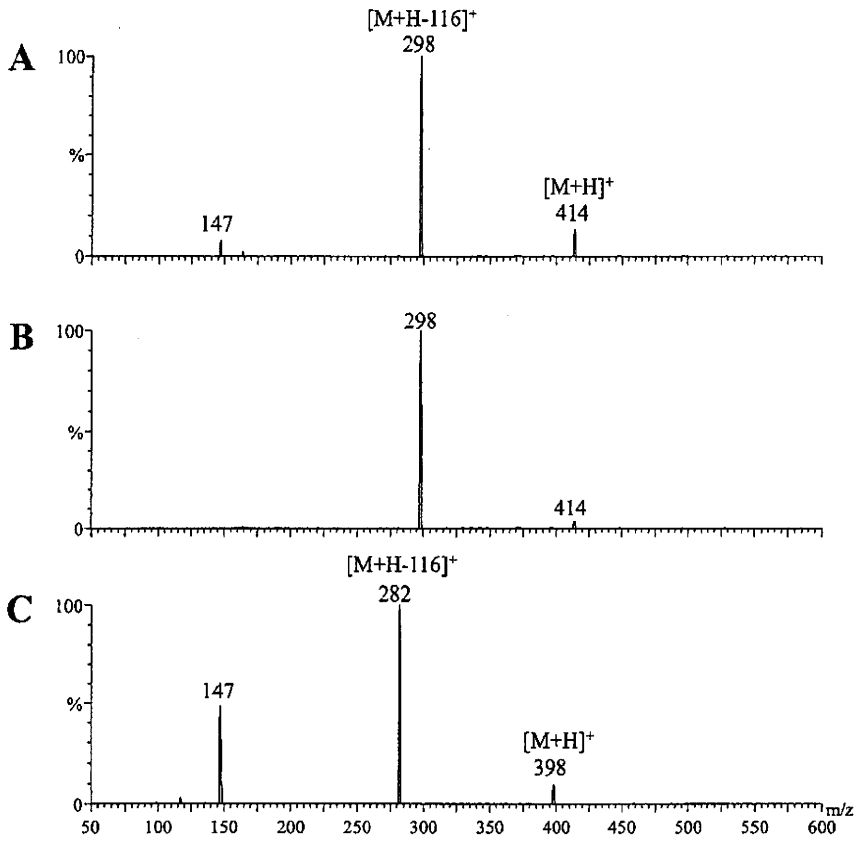

Figure 4. Product ion spectra of (A) ES-3′-C8-dG, (B) ES-3′-N^2-dG, and (C) ES-3′-N^6-dA. The cone voltages and collision energies were set at the optimum conditions for each compound in negative ion mode. LC-ESI/MS/MS conditions are described in the Materials and Methods.

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.

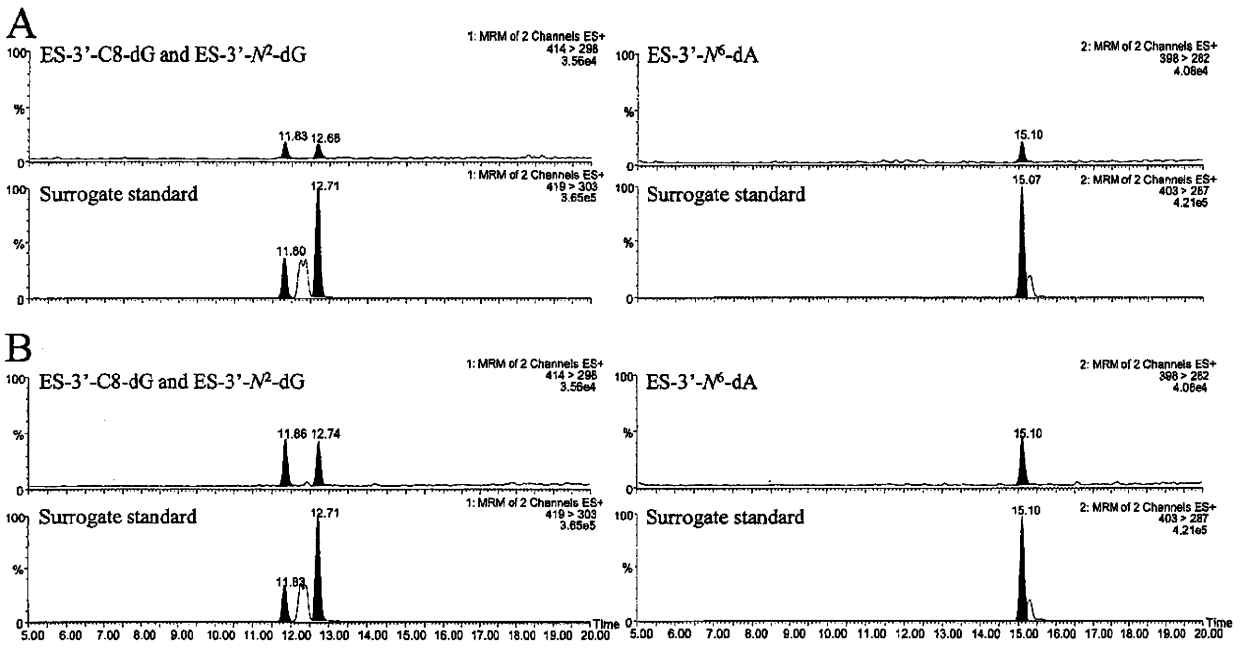

Figure 5. MRM chromatograms of LOD (A) and LOQ (B) levels of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA adducts and each surrogate standard from extracted DNA samples.

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,

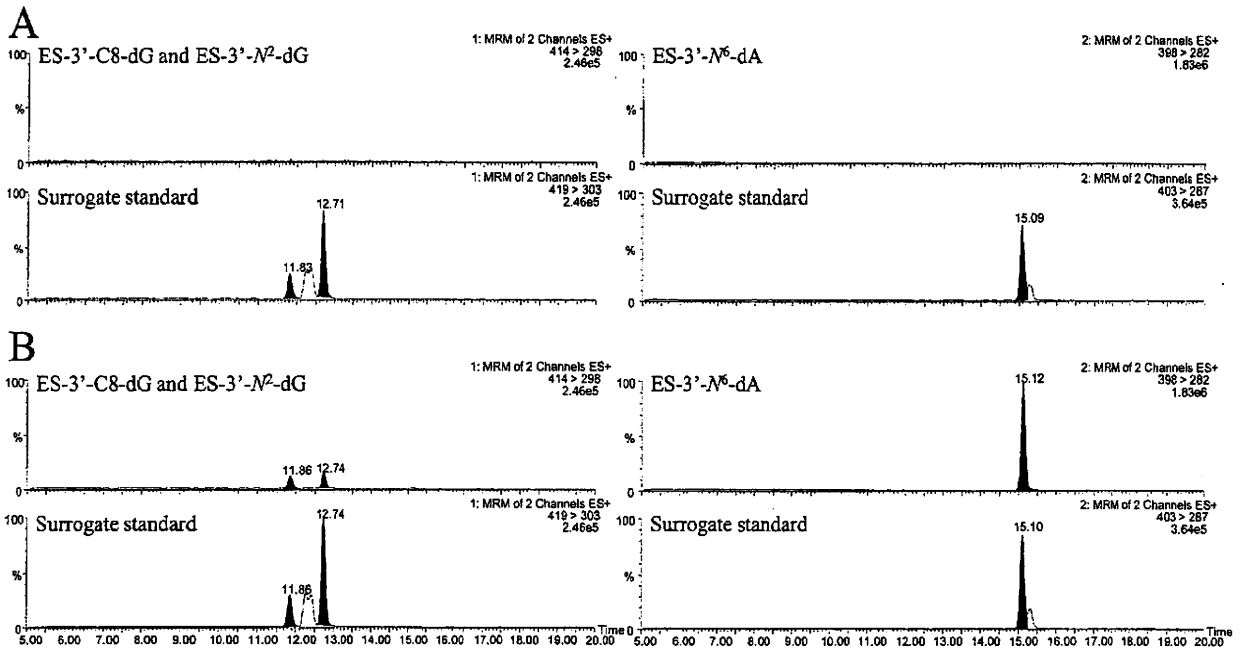

Figure 6. MRM chromatograms of ES-3′-C8-dG, ES-3′-N^2-dG, and ES-3′-N^6-dA adducts and each surrogate standard in rat liver DNA samples. (A) Liver from rat administrated with vehicle as a control group. (B) Liver from rat orally administrated with 600 mg/kg ES for 4 weeks.

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