Table 9. Initial and final body weights and kidney weight of rats after treatment with renal carcinogens, or non-carcinogenic renal toxicants

Group	Number of animals	Initial body weight (g)	Final body weight (g)	Kidney weight	
				Absolute (g)	Relative (g/100g BW)
Day 3					
CONT	10	131.7±8.7[a]	150.6±10.1	1.22±0.09	0.81±0.03
NFT	11	131.1±9.9	122.8±11.7**	1.14±0.09	0.94±0.09**
ADAQ	11	130.2±9.8	143.9±9.2	1.25±0.07	0.87±0.04*
TCP	11	132.0±11.4	124.5±9.5**	1.19±0.10	0.96±0.04**
CP	10	131.9±7.0	124.8±7.9**	1.09±0.09**	0.87±0.05*
TAT	10	132.3±8.5	149.1±8.7	1.21±0.08	0.81±0.02
CBX	10	131.8±6.3	148.8±8.2	1.19±0.08	0.80±0.02
Day 7					
CONT	10	133.8±8.5	176.2±10.8	1.43±0.08	0.81±0.04
NFT	11	133.9±11.7	116.9±8.8**	1.12±0.10**	0.96±0.07**
ADAQ	11	133.3±9.6	168.8±11.6	1.40±0.14	0.83±0.03
TCP	11	134.7±8.2	133.9±10.7**	1.24±0.10**	0.93±0.03**
CP	10	135.6±8.2	130.4±7.0**	1.14±0.10**	0.87±0.04**
TAT	10	133.0±10.0	168.2±11.7	1.31±0.13*	0.78±0.03
CBX	10	132.6±7.8	171.2±11.3	1.35±0.07	0.79±0.03
Day 28					
CONT	10	118.6±6.8	240.9±5.7	1.66±0.08	0.69±0.03
NFT	11	119.5±6.2	131.0±9.7**	1.31±0.14**	1.00±0.10**
ADAQ	11	119.5±6.1	228.9±16.3	1.76±0.13	0.77±0.03**
TCP	11	121.0±6.6	173.4±6.4**	1.55±0.07	0.89±0.03**
CP	10	119.0±7.4	177.1±13.5**	1.54±0.09*	0.87±0.03**
TAT	10	119.5±6.4	234.8±10.8	1.61±0.12	0.68±0.03
CBX	10	118.9±8.8	230.1±13.3	1.71±0.13	0.74±0.02**

Abbreviations: ADAQ, 1-amino-2,4-dibromoantraquinone; CBX, carboxin; CONT, untreated controls; CP, 1-chloro-2-propanol; NFT, nitrofurantoin; TAT, triamterene; TCP, 1,2,3-trichloropropane.

[a] Values are expressed as mean ± SD.

* $P < 0.05$, ** $P < 0.01$ vs. untreated controls (Dunnett's or Steel's test).

Table 10. Relative transcript levels in the OSOM of rats treated with NFT, ADAQ, TCP or CBX at day 3, day 7 and day 28

Gene	Relative transcript level normalized to *Actb*				Relative transcript level normalized to *Gapdh*			
	NFT[a]	ADAQ[a]	TCP[a]	CBX[a]	NFT[a]	ADAQ[a]	TCP[a]	CBX[a]
Day 3								
Cdkn1a	4.11±1.69*	1.35±0.35	4.79±0.69**	2.38±0.42*	2.60±0.95**	3.85±0.48**	4.25±0.54**	2.17±0.47**
Chek1	1.46±0.18**	0.71±0.12*	1.55±0.21**	1.42±0.19**	0.94±0.16	0.67±0.07**	1.25±0.19*	1.30±0.26*
Mad2l1	1.23±0.19*	0.77±0.17*	1.45±0.15**	1.27±0.11*	0.80±0.19	0.73±0.13	1.17±0.16	1.14±0.01
Mdm2	1.20±0.19	1.41±0.22**	2.13±0.21**	1.79±0.17**	0.78±0.18*	1.33±0.09*	1.71±0.12**	1.63±0.21**
Rbl2	1.27±0.21*	1.20±0.15	1.45±0.18**	1.20±0.07	0.85±0.31	1.13±0.03	1.17±0.14	1.09±0.13
Tp53	1.20±0.23	1.30±0.18	1.42±0.13*	1.79±0.35**	0.77±0.11*	1.24±0.20	1.15±0.16	1.64±0.43**
Day 7								
Cdkn1a	1.99±0.46*	0.45±0.16**	2.32±0.62*	0.89±0.10	1.49±0.34	0.38±0.13**	1.85±0.51*	0.83±0.12
Chek1	2.08±0.47**	0.89±0.13	2.22±0.28**	1.25±0.18	1.56±0.35**	0.76±0.13	1.78±0.30**	1.17±0.17
Mad2l1	1.37±0.18**	0.98±0.05	1.35±0.13**	1.13±0.14	1.03±0.14	0.83±0.05*	1.08±0.16	1.05±0.14
Mdm2	1.18±0.15	1.52±0.10**	1.98±0.23**	1.47±0.07**	0.89±0.10	1.29±0.11**	1.58±0.21**	1.37±0.11**
Rbl2	1.03±0.21	1.30±0.05**	1.45±0.10**	1.23±0.09*	0.77±0.12**	1.11±0.09	1.16±0.18	1.15±0.14
Tp53	1.50±0.26**	1.53±0.11**	1.68±0.18**	1.58±0.18**	1.13±0.21	1.30±0.08*	1.35±0.19**	1.47±0.18**
Day 28								
Cdkn1a	1.28±0.61	0.51±0.08**	4.30±1.16**	1.36±0.33	0.93±0.41	0.49±0.06**	4.27±1.31**	1.41±0.58
Chek1	1.44±0.48*	1.23±0.19	2.02±0.30**	1.84±0.25**	1.02±0.24	1.18±0.16	2.00±0.39**	1.89±0.57*
Mad2l1	1.17±0.32	1.25±0.11**	1.20±0.13*	1.38±0.09**	0.84±0.15	1.20±0.08	1.19±0.16	1.40±0.27**
Mdm2	0.96±0.16	1.40±0.11**	1.68±0.16**	1.38±0.09**	0.70±0.10*	1.36±0.14**	1.67±0.26**	1.40±0.27*
Rbl2	0.97±0.28	1.10±0.06	1.14±0.03*	0.99±0.12	0.70±0.17**	1.06±0.09	1.13±0.13	1.00±0.19
Tp53	1.10±0.13	1.24±0.12*	1.27±0.13**	1.50±0. 09**	0.80±0.11	1.20±0.14	1.25±0.07*	1.52±0. 27**

Abbreviations: *Actb*, actin beta; ADAQ, 1-amino-2,4-dibromoantraquinone; CBX, carboxin; *Cdkn1a*, cyclin-dependent kinase inhibitor 1A; *Chek1*, checkpoint kinase 1; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Mad2l1*, MAD2 mitotic arrest deficient-like 1 (yeast); *Mdm2*, MDM2 proto-oncogene, E3 ubiquitin protein ligase; NFT, nitrofurantoin; *Rbl2*, retinoblastoma-like 2; OSOM, outer stripe of the outer medulla; TCP, 1,2,3-trichloropropane; *Tp53*, tumor protein p53.

[a] n = 6.

[b] Values represent relative expression levels expressed as mean ± SD.

* $P < 0.05$, ** $P < 0.01$ vs. untreated controls (Dunnett's or Steel's test).

Table 11. Final body weight and liver weight of rats after treatment with hepatocarcinogens, hepatocarcinogenic promoters or non-carcinogenic hepatotoxicants at the post-initiation phase

Group	Number of animals	Final body weight (g)	Liver weight	
			Absolute (g)	Relative (g/100g BW)
Week 2				
DEN-alone	12	207.7±10.7	6.59±0.36	3.18±0.11
DEN+BNF	11	172.1±16.6**	8.89±1.50**	5.13±0.42**
DEN+CRB	11	182.8±13.5**	6.79±0.67	3.71±0.19**
DEN+LMG	12	171.2±8.2**	6.77±0.39	3.96±0.12**
DEN+APAP	9	183.0±11.7**	6.63±0.67	3.62±0.19**
Week 4				
DEN-alone	11	246.9±9.9	9.03±1.13	3.67±0.52
DEN+BNF	12	218.0±15.4**	13.87±1.69**	6.35±0.40**
DEN+CRB	11	219.3±12.5**	8.72±0.46	3.98±0.11**
DEN+LMG	11	218.2±7.5**	10.17±0.54**	4.66±0.15**
DEN+APAP	11	228.6±12.8**	8.71±0.50	3.96±0.52**
Week 6				
DEN-alone	14	279.9±7.5	8.94±0.45	3.19±0.15
DEN+BNF	15	258.0±10.5**	13.94±1.09**	5.40±0.34**
DEN+CRB	16	240.6±15.2**	9.29±0.69	3.77±0.16**
DEN+LMG	16	240.6±7.9**	10.79±0.43**	4.49±0.10**
DEN+APAP	15	257.7±11.9**	9.68±0.54**	3.76±0.12**

Abbreviations: APAP, acetaminophen; BNF, β-naphthoflavone; CRB, carbadox; DEN, *N*-diethylnitrosamine; LMG, leucomalachite green.
Values are expressed as mean ± SD.
* $P < 0.05$, ** $P < 0.01$ vs. DEN-alone group (Dunnett's or Steel's test).

Table 12. *gpt* MF and Spi$^-$ MF for *Nrf2* $^{+/+}$ *gpt* delta mice treated with NFT for 8 weeks

			***gpt* assay**				**Spi$^-$-assay**			
Genotype	Treatment	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	Mutant Frequency ($\times 10^{-5}$)	Mean ± SD	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA(P2)	Mutant Frequency ($\times 10^{-5}$)	Mean ± SD
Nrf2$^{+/+}$	Control	W2	14.09	15	1.06		24.57	2	0.08	
		W104	9.86	6	0.61		6.39	1	0.16	
		W105	64.17	30	0.47		53.01	14	0.26	
		W106	10.49	10	0.95	0.77 ± 0.28	10.17	2	0.20	0.17 ± 0.08
	NFT	W4	6.44	10	1.55		10.17	3	0.29	
		W5	11.70	19	1.62		12.51	1	0.08	
		W110	38.43	42	1.09		29.16	7	0.24	
		W111	12.42	14	1.13	1.35 ± 0.28 *	17.37	4	0.23	0.21 ± 0.09
Nrf2$^{-/-}$	Control	Ho5	17.87	12	0.67		18.99	2	0.11	
		Ho6	9.72	12	1.23		8.19	1	0.12	
		Ho7	12.56	9	0.72		12.60	2	0.16	
		Ho8	16.38	10	0.61	0.81 ± 0.29	18.18	2	0.11	0.12 ± 0.02
	NFT	Ho9	9.99	31	3.10		21.51	4	0.19	
		Ho10	10.67	31	2.91		21.33	5	0.23	
		Ho11	14.81	18	1.22		12.69	2	0.16	
		Ho12	9.14	18	1.97	2.30 ± 0.88 *	17.37	4	0.23	0.20 ± 0.04 *
	MeIQx	P1	8.24	60	7.29		7.47	10	1.34	

* Significantly different from the relevant control group at $p<0.05$.

Table 13. *gpt* MF and Spi⁻ MF for *Nrf2* $^{+/+}$ *gpt* delta mice treated with NFT or NFA for 13 weeks

Treatment	*gpt* assay					Spi⁻ assay			
	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	Mutant Frequency ($\times 10^{-5}$)	Mean ± SD	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA(P2)	Mutant Frequency ($\times 10^{-5}$)	Mean ± SD
Control	W1	25.02	7	0.28		20.34	4	0.20	
	W2	18.00	12	0.67		18.45	2	0.11	
	W3	18.09	11	0.61		11.70	3	0.26	
	W4	8.24	5	0.61		4.23	4	0.95	
	W5	36.99	12	0.32	0.50 ± 0.18	33.39	8	0.24	0.35 ± 0.34
NFT 35 mg/kg bw	W7	13.95	9	0.65		22.23	11	0.49	
	W8	23.09	8	0.35		10.35	6	0.58	
	W9	25.11	16	0.64		19.71	12	0.61	
	W10	12.33	5	0.41		7.29	5	0.69	
	W11	15.12	11	0.73	0.55 ± 0.17	13.95	2	0.14	0.50 ± 0.21
NFT 70 mg/kg bw	W13	20.61	5	0.24		19.35	5	0.26	
	W14	22.14	15	0.68		15.39	12	0.78	
	W15	17.64	13	0.74		22.77	4	0.18	
	W16	7.25	5	0.69		4.41	2	0.45	
	W17	3.96	7	1.77	0.82 ± 0.56	4.95	0	0.00	0.33 ± 0.30
NFA 21 mg/kg bw	W19	22.91	13	0.57		26.46	7	0.26	
	W20	11.61	5	0.43		10.98	4	0.36	
	W21	32.49	8	0.25		25.20	8	0.32	
	W22	19.80	7	0.35		16.74	7	0.42	
	W23	15.44	5	0.32	0.38 ± 0.12	10.89	4	0.37	0.35 ± 0.06
NFA 41 mg/kg bw	W25	35.15	4	0.11		36.09	4	0.11	
	W26	24.57	6	0.24		16.74	7	0.42	
	W27	41.09	8	0.19		34.56	15	0.43	
	W28	7.74	8	1.03		10.44	4	0.38	
	W29	16.02	13	0.81	0.48 ± 0.41	13.32	5	0.38	0.34 ± 0.13
MeIQx [a]	P1	3.69	141	38.21		2.52	39	15.48	

[a] 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, positive control

Table 14. *gpt* MF and Spi⁻ MF for *Nrf2* $^{-/-}$ *gpt* delta mice treated with NFT or NFA for 13 weeks

Treatment	*gpt* assay					Spi⁻ assay			
	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	Mutant Frequency ($\times 10^{-5}$)	Mean ± SD	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA(P2)	Mutant Frequency ($\times 10^{-5}$)	Mean ± SD
Control	Ho1	8.15	6	0.74		6.39	3	0.47	
	Ho2	24.93	16	0.64		19.62	5	0.25	
	Ho3	20.43	16	0.78		14.04	2	0.14	
	Ho4	11.43	11	0.96		10.53	6	0.57	
	Ho5	21.87	22	1.01	0.83 ± 0.15	20.34	5	0.25	0.34 ± 0.18
NFT 35 mg/kg bw	Ho7	12.15	12	0.99		13.14	7	0.53	
	Ho8	10.26	5	0.49		10.44	2	0.19	
	Ho9	28.80	17	0.59		26.01	7	0.27	
	Ho10	24.71	19	0.77		21.78	13	0.60	
	Ho11	20.34	13	0.64	0.69 ± 0.19	21.69	12	0.55	0.43 ± 0.18
NFT 70 mg/kg bw	Ho15	10.22	19	1.86		12.69	7	0.55	
	Ho16	10.22	14	1.37		12.24	5	0.41	
	Ho17	19.40	40	2.06		18.54	9	0.49	
	Ho18	18.23	24	1.32	1.65 ± 0.37 *	19.62	7	0.36	0.45 ± 0.09
NFA 21 mg/kg bw	Ho19	11.48	5	0.44		11.34	0	0.00	
	Ho20	16.56	27	1.63		13.86	5	0.36	
	Ho22	18.77	41	2.18		36.72	12	0.33	
	Ho23	11.16	11	0.99		14.13	4	0.28	
	Ho24	19.67	4	0.20	1.09 ± 0.82	15.66	12	0.77	0.35 ± 0.27
NFA 41 mg/kg bw	Ho25	16.74	12	0.72		17.64	8	0.45	
	Ho26	11.16	10	0.90		9.27	3	0.32	
	Ho28	4.10	4	0.98		3.69	3	0.81	
	Ho29	14.99	11	0.73		14.04	7	0.50	
	Ho30	18.14	15	0.83	0.83 ± 0.11	23.58	9	0.38	0.49 ± 0.19
MeIQx [a]	P1	4.46	189	42.42		3.51	33	15.48	

*Significantly different ($P<0.05$) from the control group by Dunnett's test.

[a] 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, positive control

Table 15. Final body and kidney weights for F344 *gpt* delta rats treated with NFT and antioxidants for 4 weeks

	Final body weight (g)	Kidney weights	
		Absolute (g)	Relative (g%)
Control	255.3 ± 27.7	1.79 ± 0.22	0.70 ± 0.02
NFT	233.7 ± 16.3 *	1.82 ± 0.12	0.78 ± 0.02 **
NAC	261.3 ± 11.4	1.97 ± 0.08	0.76 ± 0.03 *
SAA	256.0 ± 16.5	1.88 ± 0.16	0.73 ± 0.02
αTP	246.8 ± 18.3	1.72 ± 0.10	0.70 ± 0.02
NFT/NAC	231.8 ± 13.7 *	1.92 ± 0.10	0.83 ± 0.04 **
NFT/SAA	231.3 ± 16.5 *	1.89 ± 0.11	0.82 ± 0.03 **
NFT/αTP	247.3 ± 11.8	1.99 ± 0.11	0.80 ± 0.03 **

*,** Significantly different (P<0.05, 0.01) from the control group by Dunnett's test.

Table 16. Body and organ weights, daily food, water and estimated chemical intakes of rats given PBO, APO and/or NAC for 8 weeks after DEN initiation

Groups	DEN-alone	PBO	APO	NAC	PBO+APO	PBO+NAC
Number of rats	12	11	12	12	15	10
Final body weight (g)	279.9±13.9	205.1±7.4**	285.6±18.8	269.9±15.2	206.6±12.3**	194.6±14.7**
Food intake (g/rat/day)	12.7±0.9	11.7±2.4	12.9±1.0	12.1±1.1	11.7±2.5	11.5±2.6
Water intake (g/rat/day)	17.1±1.5	15.6±2.4	18.0±1.5	13.9±3.2	15.9±2.6	12.8±3.1
PBO intake (mg/kg BW/day)	–	37.63±2.15	–	–	37.28±2.77	35.45±2.82
APO intake (mg/kg BW/day)	–	–	0.95±0.08	–	0.64±0.06	–
NAC intake (mg/kg BW/day)	–	–	–	8.32±0.70	–	6.00±0.49
Absolute liver weight (g)	8.89±0.55	12.19±0.82**	8.96±0.75	8.94±1.25	12.61±1.21**	11.31 ±1.08**
Relative liver weight (% BW)	3.17±0.11	5.94±0.31**	3.13±0.08	3.32±0.54	6.10±0.43**	5.81±0.19**

Values are expressed as mean ± SD.

*$P < 0.05$ or **$P < 0.01$ compared with control group (Dunnett's or Steel's test).

Table 17. The result of blood biochemistry of rats given PBO, APO and/or NAC for 8 weeks after DEN initiation

Groups	DEN-alone	PBO	APO	NAC	PBO+APO	PBO+NAC
Number of rats	12	11	12	12	15	10
Total protein (g/dl)	6.62±0.22	7.42±0.21**	6.73±0.26	6.72±0.23	7.66±0.36**	7.52±0.30**
Albumin (g/dl)	4.73±0.16	5.55±0.18**	4.75±0.13	4.74±0.13	5.64±0.14**	5.62±0.19**
AST (GOT) (IU/l)	69.08±5.05	60.64±5.59*	69.50±3.48	68.36±4.06	61.71±5.65*	59.00±8.51*
ALT (GPT) (IU/l)	43.83±4.15	43.55±3.39	44.25±4.37	45.58±11.97	45.07±3.38	44.20±3.85
ALP (IU/l)	937.92±70.30	951.00±82.39	936.75±84.79	1020.58±249.03	972.07±167.62	896.90±74.38
Creatinin (mg/dl)	0.34±0.05	0.30±0.04	0.33±0.05	0.32±0.04	0.30±0.00	0.29±0.03
Urea nitrogen (mg/dl)	15.75±1.48	22.36±1.12**	15.83±1.11	14.83±1.03	21.27±1.39**	23.50±1.58**
Glucose (mg/dl)	192.75±27.64	147.45±17.87**	199.00±34.47	176.50±27.58	139.13±21.54**	140.10±18.45**
Triglyceride (mg/dl)	134.33±25.00	30.91±6.24**	112.33±19.98	104.92±22.66	38.93±38.56**	24.60±4.84**
Total cholesterol (mg/dl)	68.83±3.13	103.18±7.26**	74.67±4.31*	78.67±16.00*	110.40±14.70**	111.10±6.69**

Values are expressed as mean ± SD.

*$P < 0.05$ or **$P < 0.01$ compared with control group (Dunnett's or Steel's test).

Table. 18. GST-P-positive foci, and Ki-67 and TUNEL positive cell ratio in the liver of rats given PBO, APO and/or NAC for 8 weeks after DEN initiation

Groups	DEN-alone	PBO	APO	NAC	PBO+APO	PBO+NAC
Number of rats	12	11	12	12	15	10
GST-P positive foci						
Numbers (No./cm^2)	8.04± 2.53	23.79± 8.75**	7.97± 1.66	8.46± 2.15	17.82± 4.22**	17.85± 3.59**
Areas (mm^2/cm^2)	0.42± 0.14	1.82± 0.69**	0.42± 0.08	0.41± 0.12	1.66± 0.75**	1.75± 0.72**
Ki-67 positive cells (%)	4.89±0.55	12.19±0.82**	4.96±0.75	5.04±1.25	12.61±1.21**	11.31±1.08**
Caspase-3 positive cells (%)	1.17±0.11	3.94±0.31**	1.13±0.08	1.32±0.54	4.10±0.43**	3.81±0.19**

Values are expressed as mean ± S.D.

**$P < 0.01$ compared with control group (Dunnett's or Steel's test).

Table 19. Changes of transcript expression in the liver of rats given PBO, APO and/or NAC for 8 weeks after DEN initiation

	Group					
Genes	DEN-alone	PBO	APO	NAC	PBO+APO	PBO+NAC
Drug metabolizing enzymes						
Cyp1a1	1.03±0.31	330.19±116.78**	1.02±0.33	1.19±0.64	352.45±104.01**	348.38±284.67**
Cyp2b1/2	1.03±0.29	298.74±21.86**	1.06±0.29	1.08±0.63	283.34±30.41**	279.17±39.42**
Antioxidant enzymes						
Nqo1	1.01±0.12	4.69±0.53**	1.08±0.10	1.20±0.29	6.93±1.68**	6.10±1.68**
Gpx2	1.03±0.27	45.58±5.81**	0.84±0.09	4.51±8.07	48.30±7.54**	40.31±7.23**
NOX-related factors						
Cybb	1.01±0.15	0.98±0.15	1.16±0.26	1.52±0.84	1.24±0.36	1.60±0.15
Rac1	1.01±0.13	1.01±0.04	1.01±0.07	1.13±0.20	0.99±0.16	1.04±0.13

Values of mRNA expression level (normalized by *Actb*) are expressed as mean ± S.D. (N=6).

**$P < 0.01$ compared with control group (Dunnett's or Steel's test).

Table 20. Final body weight, liver weight, food intake, and water intake in rats treated with MG with or without APO or EMIQ after DEN initiation§

Group	CTL	MG	MG+APO	MG+EMIQ
No. of animals	11	13	9	12
Final body weight (g)	276.7±14.1	277.6±9.7	282.1±16.3	280.7±13.7
Absolute liver weight (g)	8.26±0.73	8.70±0.44	8.73±0.72	8.88±0.62
Relative liver weight (%BW)	2.98±0.17	3.13±0.08	3.10±0.20	3.16±0.15[a]
Food intake (g/rat/day)	9.5±3.4	9.3±3.4	9.4±3.2	9.6±3.1
Water intake (g/rat/day)	17.3±2.2	18.6±2.7	17.5±1.9	16.4±1.7

Abbreviations: CTL, control; MG, malachite green; APO, apocynin; EMIQ, enzymatically modified isoquercitrin; BW, body weight; DEN, N-diethylnitrosamine.

§: All animals were subjected to two-thirds partial hepatectomy at week 1 after starting MG promotion.

Data are shown as the mean ± standard deviation.

[a]: $p<0.05$ versus CTL (Tukey's or the Steel-Dwass test).

Table 21. Blood biochemistry in rats treated with MG with or without APO or EMIQ after DEN initiation§

Group	CTL	MG	MG+APO	MG+EMIQ
No. of animals	11	13	9	12
AST	82 ± 5	81 ± 4	80 ± 4	80 ± 6
ALT	48 ± 7	48 ± 4	47 ± 5	52 ± 6
ALP	1673 ± 124	1517 ± 71[a]	1582 ± 102	1389 ± 95[a,b,c]
GLU	160 ± 36	136 ± 18	150 ± 29	151 ± 19
T.CHOL	90 ± 10	80 ± 6[a]	78 ± 7	69 ± 5[a,b,c]
TG	316 ± 128	327 ± 71	293 ± 102	265 ± 92

Abbreviations: CTL, control; MG, malachite green; APO, apocynin; EMIQ, enzymatically modified isoquercitrin; DEN, N-diethylnitrosamine; AST, aspartate aminotransaminase; ALT, alanine aminotransaminase; ALP, alkaline phosphatase; GLU, glucoase; T.CHOL, total cholesterol; TG, triglyceride.

§: All animals were subjected to two-thirds partial hepatectomy at week 1 after starting MG promotion.

Data are shown as the mean ± standard deviation.

a: $p<0.05$ versus CTL (Tukey's or the Steel-Dwass test).

b: $p<0.05$ versus MG (Tukey's or the Steel-Dwass test).

c: $p<0.05$ versus MG+APO (Tukey's or the Steel-Dwass test).

Abbreviations: CTL, control group; HFD, high fat diet group; BW, body weight; DEN, N-diethylnitrosamine.

§: All animals were subjected to two-thirds partial hepatectomy at week 3 after DEN initiation.

Table 22. Final body weight, organ weight, food intake, water intake, and histopathological firings in rats fed with basal diet and high fat diet after DEN initiation§

Group	CTL	HFD
No. of animals	14	13
Final body weight (g)	282.0±13.9	300.3±8.1[a]
Absolute intraabdominal adipose tissue weight (g)	1.96±0.21	2.91±0.23[a]
Relative intraabdominal adipose tissue weight (%BW)	0.70±0.06	0.97±0.07[a]
Absolute liver weight (g)	8.29±0.50	8.44±0.37
Relative liver weight (%BW)	2.95±0.09	2.83±0.08[a]
Food intake (g/rat/day)	13.7±2.3	11.4±0.7
Water intake (g/rat/day)	20.3±2.8	16.5±1.5
Steatosis, hepatocellular (Score)	1.3±0.5	2.2±0.7[a]
Neutrophil, sinusoidal, liver (No./filed)	1.3±0.6	0.7±0.2
p67phox$^+$ cell, sinusoidal, liver (No./field)	3.5±1.4	3.4±1.8

Data are shown as the mean ± standard deviation.

[a]: $p<0.05$ vs CTL (Student's t test or Aspin-Welch test).

Table 23. Blood biochemistry in rats fed with basal diet and high fat diet after DEN initiation§

Group	CTL	HFD
No. of animals	14	13
AST (U/L)	90 ± 12	78 ± 4[a]
ALT (U/L)	55 ± 12	50 ± 5
ALP (U/L)	1305 ± 187	1461 ± 103[a]
GGTP (U/L)	0.89 ± 0.28	0.77 ± 0.21
GLU (mg/dL)	127± 17	135 ± 7
TP (g/dL)	7.4± 0.3	7.2 ± 0.2
ALUB (g/dL)	5.2± 0.1	5.2± 0.1
GLOB (g/dL)	2.2± 0.3	2.1 ± 0.1
A/G (ratio)	2.4± 0.3	2.5 ± 0.2
T.CHOL (mg/dL)	75± 6	80 ± 8
TG (mg/dL)	116 ± 26	226 ± 61[a]
T.BIL (mg/dL)	0.06 ± 0.01	0.11 ± 0.02[a]

Abbreviations: CTL, control group; HFD, high fat diet group; DEN, N-diethylnitrosamine; AST, aspartate aminotransaminase; ALT, alanine aminotransaminase; ALP, alkaline phosphatase; GGTP, γ-glutamyl transpeptidase; GLU, glucose; TP, total protein; ALUB, albumin; GLOB, globulin; A/G, albumin/globulin; T.CHOL, total cholesterol; TG, triglyceride; T.BIL, total bilirubin.

§: All animals were subjected to two-thirds partial hepatectomy at week 3 after DEN initiation.

Data are shown as the mean ± standard deviation.

a: $p<0.05$ versus CTL (Student's t test or Aspin-Welch test).

Table 24. Final body weight, liver weight, food intake, and water intake in rats treated with or without DRZ or APO after DEN initiation§

Group	CTL	HFD	HFD+DRZ	HFD+DRZ+APO
No. of animals	11	13	12	12
Final body weight (g)	278.0±7.3	285.7±24.3	267.4±14.7	259.6±17.2[b]
Absolute adipose tissue weight (g)	2.25±0.39	2.82±0.47[a]	2.44±0.49	2.44±0.50
Relative adipose tissue weight (%BW)	0.81±0.14	0.99±0.14[a]	0.91±0.16	0.93±0.16
Absolute liver weight (g)	9.48±0.64	9.00±0.63	8.59±0.62[a]	8.74±0.79
Relative liver weight (%BW)	3.41±0.20	3.16±0.15[a]	3.21±0.13	3.37±0.27[b]
Absolute testis weight (g)	2.99±0.11	3.00±0.28	1.33±0.13[a,b]	1.29±0.17[a,b]
Relative testis weight (%BW)	1.08±0.03	1.05±0.04	0.50±0.04[a,b]	0.50±0.07[a,b]
Absolute epididymis weight (g)	0.81±0.07	0.80±0.08	0.45±0.05[a,b]	0.46±0.03[a,b]
Relative epididymis weight (%BW)	0.29±0.03	0.28±0.02	0.17±0.01[a,b]	0.18±0.01[a,b]
Colon length (cm)	18.1±1.7	16.4±1.2[a]	16.3±1.1[a]	15.7±2.0[a]
Food intake (g/rat/day)	12.9±0.5	10.5±0.4[a]	10.4±0.5[a]	10.7±1.3[a]
Water intake (g/rat/day)	23.2±2.6	20.5±1.2	17.8±1.3[a]	17.1±1.6[a]
Steatosis, hepatocellular (Score)	1.3±0.5	2.8±0.7[a]	1.9±0.9[b]	2.1±0.8[b]
Neutrophil, sinusoidal, liver (No./filed)	1.8±0.5	1.6±0.4	1.8±0.7	1.9±0.6
p67phox+ cell, sinusoidal, liver (No./field)	15.4±3.0	17.5±4.2	16.9±3.3	17.8±5.3
Colitis, large intestine (Score)	2.6±0.9	2.4±0.6	2.3±0.3	2.7±0.8
Degeneration/atrophy, tubular, testis (Score)	0.0±0.0	0.0±0.0	2.5±0.7[a,b]	2.6±0.5[a,b]
Reduced sperm, luminal, epididymis (Score)	0.0±0.0	0.0±0.0	2.0±0.0[a,b]	2.0±0.0[a,b]

Abbreviations: CTL, control; HFD, high fat diet; DRZ, dimetridazole; APO, apocynin; BW, body weight; DEN, N-diethylnitrosamine.

§ : All animals were subjected to two-thirds partial hepatectomy at week 1 after starting DRZ promotion.

Data are shown as the mean ± standard deviation.

a: $p<0.05$ vs CTL (Tukey's or the Steel-Dwass test).

b: $p<0.05$ vs HFD (Tukey's or the Steel-Dwass test).

Table 25. Blood biochemistry in rats treated with or without DRZ or APO after DEN initiation§

Group	CTL	HFD	HFD+DRZ	HFD+DRZ+APO
No. of animals	10	13	12	11
AST (U/L)	84±5	92±9a	71±4[ab]	72±5[ab]
ALT (U/L)	48±6	57±9a	48±4[b]	49±5b
ALP (U/L)	1230±151	1379±428	1213±136	1232±116
GGTP (U/L)	1.3±0.2	1.2±0.8	1.2±0.3	1.2±0.4
GLU (mg/dL)	139±20	142±29	146±22	174±34[ab]
TP (g/dL)	7.24±0.20	7.28±0.28[a]	7.24±0.27[b]	7.14±0.32[b]
ALUB (g/dL)	5.01±0.08	5.04±0.25	5.08±0.17	4.98±0.25
GLOB (g/dL)	2.24±0.14	2.24±0.19	2.16±0.14	2.16±0.18
A/G (ratio)	2.25±0.12	2.26±0.24	2.36±0.14	2.31±0.22
T.CHOL (mg/dL)	70.6±4	78.4±10	84.3±9[a]	83.1±12
TG (mg/dL)	154±30	261±56[a]	218±72	299±92[a]
T.BIL (mg/dL)	0.043±0.01	0.102±0.03[a]	0.058±0.02[b]	0.068±0.01[a]

Abbreviations: CTL, control; HFD, high fat diet; DEN, N-diethylnitrosamine; AST, aspartate aminotransaminase; ALT, alanine aminotransaminase; ALP, alkaline phosphatase; GGTP, γ-glutamyl transpeptidase; GLU, glucose; TP, total protein; ALUB, albumin; GLOB, globulin; A/G, albumin/globulin; T.CHOL, total cholesterol; TG, triglyceride; T.BIL, total bilirubin.

§ : All animals were subjected to two-thirds partial hepatectomy at week 1 after starting DRZ promotion.

Data are shown as the mean ± standard deviation.

a: $p<0.05$ vs CTL (Tukey's or the Steel-Dwass test).

b: $p<0.05$ vs HFD (Tukey's or the Steel-Dwass test).

c: $p<0.05$ vs HFD+DRZ (Tukey's or the Steel-Dwass test).

Table 26. Hepatic gene expression in rats treated with or without DRZ or APO after DEN initiation§

Group	CTL	HFD	HFD+DRZ	HFD+DRZ+APO
No. of animals	6	6	6	6
P67phox	1.04±0.30	1.34±0.15	0.91±0.29	1.28±0.44
P22phox	1.04±0.33	1.17±0.24	0.72±0.27	1.13±0.32
Poldip2	1.06±0.45	1.61±0.23[a]	1.04±0.13[b]	1.06±0.19[b]
Ppara	1.02±0.21	1.16±0.25	1.25±0.30	1.32±0.33
Pparg	1.05±0.35	2.04±0.25[a]	2.15±0.52[a]	2.15±0.52[a]
Aox	1.01±0.17	1.39±0.21[a]	1.16±0.26	1.40±0.21[a]
Plin2	1.01±0.12	2.11±0.38[a]	1.41±0.40[b]	2.57±0.47[ac]
Il10	1.32±1.23	1.26±0.95	0.80±0.61	0.65±0.22
Pnpla2	1.02±0.21	1.03±0.21	0.97±0.16	0.96±0.11
Dgal	1.04±0.32	1.24±0.19	1.03±0.08	1.15±0.12
Cyp4a1	1.01±0.15	1.20±0.32	0.92±0.24	1.32±0.32
Fasn	1.03±0.29	0.32±0.19[a]	0.17±0.12[a]	0.35±0.12[a]
Scd1	1.05±0.34	0.28±0.18[a]	0.22±0.13[a]	0.45±0.40[a]
Plin5	1.01±0.17	2.05±0.17[a]	1.37±0.38[b]	1.70±0.29[a]
Catalase	1.01±0.15	1.14±0.28	1.10±0.29	1.21±0.22
Mn-SOD	1.01±0.12	1.15±0.23	0.95±0.16	1.03±0.19
Gpx1	1.01±0.14	1.52±0.34[a]	1.32±0.33	1.32±0.15*
Tnf-alpha	1.04±0.33	1.20±0.49	0.94±0.41	1.30±0.31*
Cyp1a2	1.04±0.33	1.17±0.31	1.23±0.28	1.26±0.45
Cyp2e1	1.02±0.23	1.49±0.30	1.29±0.28	1.35±0.40

Abbreviations: CTL, control; HFD, high fat diet; DRZ, dimetridazole; APO, apocynin; BW, body weight; DEN, N-diethylnitrosamine.

§: All animals were subjected to two-thirds partial hepatectomy at week 1 after starting DRZ promotion.

Data are shown as the mean ± standard deviation.

[a]: $p<0.05$ vs CTL (Tukey's or the Steel-Dwass test).

[b]: $p<0.05$ vs HFD (Tukey's or the Steel-Dwass test).

[c]: $p<0.05$ vs HFD+DRZ (Tukey's or the Steel-Dwass test).

*: The number of samples is 5

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研究成果の刊行に関する一覧表

書籍

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kijima, A., Ishii, Y., Takasu, S., Matsushita, K., Kuroda, K., Hibi, D., Suzuki, Y., Nohmi, T., Umemura, T.	Chemical structure-related mechanisms underlying in vivo genotoxicity induced by nitrofurantoin and its constituent moieties in gpt delta rats.	Toxicology	331	125–135	2015
Kimura, M., Abe H., Mizukami, S., Tanaka, T., Itahashi, M., Onda, N., Toshinori, Yoshida T., Shibutani, M.	Onset of hepatocarcinogen-specific cell proliferation and cell cycle aberration during the early stage of repeated hepatocarcinogen administration in rats.	J. Appl. Tox.	36(2)	223–237	2016
Kimura, M., Mizukami, S., Watanabe, Y., Hasegawa-Baba, Y., Onda, N., Yoshida, T., Shibutani, M.	Disruption of spindle checkpoint function ahead of facilitation of cell proliferation by repeated administration of hepatocarcinogens in rats.	J. Toxicol. Sci.	40(6)	855–871	2015
Kimura, M., Mizukami, S., Watanabe, Y., Hasegawa-Baba, Y., Onda, N., Yoshida, T., Shibutani, M.	Disruption of spindle checkpoint function in rats following 28 days of repeated administration of renal carcinogens.	J. Toxicol. Sci.	41(1)	91–104	2016

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Chemical structure-related mechanisms underlying *in vivo* genotoxicity induced by nitrofurantoin and its constituent moieties in *gpt* delta rats

Aki Kijima [a], Yuji Ishii [a], Shinji Takasu [a], Kohei Matsushita [a], Ken Kuroda [a], Daisuke Hibi [a], Yuta Suzuki [a], Takehiko Nohmi [b], Takashi Umemura [a,*]

[a] Division of Pathology, National Institute of Health Sciences, Tokyo 158-8501, Japan
[b] Biological Safety Research Center, National Institute of Health Sciences, Tokyo 158-8501, Japan

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ABSTRACT

Nitrofurans are antimicrobial compounds containing a nitro group at the 5-position of the furan ring and an amine or hydrazide side chain derivative. One member of the nitrofurans, nitrofurantoin (NFT), is a renal carcinogen in male rats despite its still controversial genotoxicity. We investigated chemical structure-related modes of action of NFT, and reporter gene mutation assays for NFT and its constituent moieties were performed. NFT, 5-nitro-2-furaldehyde (NFA), or 1-aminohydantoin (AHD) was administered to male F344 *gpt* delta rats by gavage for 4 or 13 weeks at a carcinogenic or the maximum tolerated dose. NFT caused a significant increase in *gpt* mutant frequency (MF) at 13 weeks with G-base substitution mutations. An increase in *gpt* MF was also observed in the NFA-treated group at 13 weeks, but not in the AHD-treated group. 8-Hydroxydeoxyguanosine (8-OHdG) levels in the kidney DNA of NFT-treated rats were significantly increased after 4 weeks. NFT caused accumulation of hyaline droplets indicated by positive immunostaining and western blot analysis for α_{2u}-globulin in the proximal tubules. An additional study, in which female *gpt* delta rats were given NFT at the same dose used for males, was performed to mitigate the effect of α_{2u}-globulin. NFT exerted the same effects on female rat kidneys to the same extent as males in terms of *gpt* MF and 8-OHdG level. Thus, it is highly probable that the structure of the nitro furan plays a key role in NFT-induced genotoxicity and genotoxic mechanisms including oxidative DNA damage are involved in NFT-induced renal carcinogenesis. α_{2u}-globulin-mediated nephropathy may be a prerequisite for NFT-induced renal carcinogenesis in male rats, and additionally NFT could be a latent carcinogen in female rats and other animal species.

1. Introduction

Nitrofurans are compounds containing a nitro group at the 5-position of the furan ring and an amine or hydrazide side chain derivative that are used as human and animal antimicrobial and food additives. Since a number of studies have demonstrated that some nitrofurans have genotoxic and/or carcinogenic potential in particular, the use of nitrofurans including furaltadone, furazolidone, nitrofurazone, and nitrofurantoin (IARC, 1974, 1983, 1990a,b) as food additives or veterinary medicines is prohibited in Japan. The mechanisms underlying the genotoxicity or carcinogenicity of these compounds are still unclear. Nevertheless, new nitrofuran agents with various hydrazide derivatives on the side chain are still being developed (Zorzi et al., 2014; Fleck et al., 2014). Thus, clarifying the underlying mechanisms of this class of chemicals is an urgent matter for assessment of human risk.

The antibacterial activities of nitrofurans are known to involve formation of reactive oxygen species (ROS) and/or reactive intermediates resulting from reduction of the nitro group (Bartel et al., 2009; Boelsterli et al., 2006; Chung et al., 2011), in common with other nitro compounds such as nitro heterocyclic antimicrobials, consequently inducing oxidative modifications of DNA/protein in target bacteria. Likewise, it is thought that these detrimental functions of nitrofurans also affect mammal hosts causing cytotoxicity, genotoxicity, and carcinogenicity (Hiraku et al., 2004; Jin et al., 2011; McCalla, 1983). However, despite nitrofurans possessing similar basic structures, there is variability in the degree of their toxicity together with a variation in target

* Corresponding author. Tel.: +81 3 3700 9819; fax: +81 3 3700 1425.
E-mail address: umemura@nihs.go.jp (T. Umemura).

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organ sites, which indicates that not only the common structure, nitrofuran, but also amine or hydrazide derivatives on the side chain may be responsible for their toxicity. In addition, there might be effects of the side chain structure on the properties of the nitrofurans.

Nitrofurantoin (*N*-(5-nitro-2-furfurylidene)-1-aminohydantoin; NFT) is generated by the condensation of 5-nitro-2-furaldehyde (NFA) and 1-aminohydantoin (AHD) (Fig. 1), and it is used extensively as a prophylactic for urinary tract infections in humans and animals (IARC, 1990b; Wagenlehner et al., 2011; Maaland and Guardabassi, 2011). The National Toxicology Program (1989) has reported on the carcinogenicity of NFT in the kidneys of male F344 rats. However, there are inconsistent results between *in vitro* and *in vivo* genotoxicity tests. Although the rat micronucleus test showed negative results, in the test for detecting DNA strand breaks, many positive results were shown using rat liver, kidney, lung, spleen, and mice bone marrow cells (IARC, 1990b). An *in vivo* mutation assay using the kidneys of Big Blue mice gave a positive result with significant incremental incidence of the G:C-T:A transversion mutation (Quillardet et al., 2006) despite mouse kidney not being the carcinogenic target site of NFT. Thus, although possible participation of genotoxic mechanisms in NFT-induced renal carcinogenesis has been suspected, there is insufficient evidence to clarify the mode of action.

In the present study, to evaluate the chemical structure-related carcinogenic mechanism of NFT, we performed a reporter gene mutation assay with the kidneys of male *gpt* delta rats (Matsushita et al., 2015; Nohmi et al., 2000) administered NFT (parent compound), NFA (a constituent compound of NFT with the nitrofuran group) or AHD (a metabolite of NFT with a hydrazide group). Additionally, the level of 8-hydroxydeoxyguanosine (8-OHdG), one type of oxidized DNA damage (Williams and Jeffrey, 2000), in the kidney DNA was quantitatively measured. An additional study using female *gpt* delta rats was performed to clarify the relationship between oxidative DNA damage and *in vivo* genotoxicity induced by NFT.

2. Materials and methods

2.1. Chemicals and reagents

NFT ($C_8H_6N_4O_5$, MW 238.2, CAS No. 67-20-9), NFA ($C_5H_3NO_4$, MW 141.08, CAS No. 698-63-5) and AHD ($C_3H_5N_3O_2$·HCl, MW 151.55, CAS No. 2827-56-7) were purchased from Sigma–Aldrich Co., Llc. (St. Louis, MO, USA), and were suspended in 0.5 w/v% methyl cellulose 400 cP solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Suspensions of the test chemicals were used at a volume of 10 ml/kg body weight (BW), based on body weight on the day of administration to *gpt* delta rats.

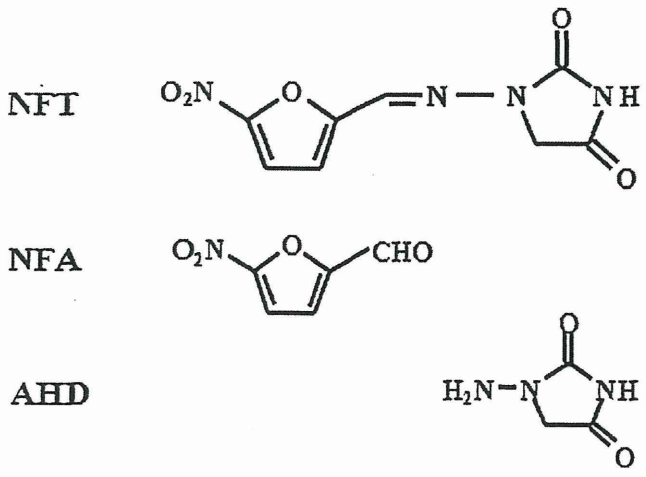

Fig. 1. Chemical structure of NFT, NFA and AHD.

2.2. Animals and housing conditions

Five-week-old F344 *gpt* delta rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). After the animals had been acclimated for one week, housed 2–3 rats in a cage with hardwood chips, and food (CRF-1, solid form; Oriental yeast Co., Ltd., Tokyo, Japan) and distilled water provided *ad libitum*, they were kept under controlled conditions (temperature 23 ± 2 °C, humidity 55 ± 5 %, air changed 12 times per hour, and lighting 12 h light/dark cycle). This study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo, Japan).

2.3. Experimental design

In experiment 1, male *gpt* delta rats were randomized into four groups (vehicle control, NFT-treated, NFA-treated, and AHD-treated groups) of 10 rats, with five rats from each group sacrificed at week 4 and week 13. In experiment 2, female *gpt* delta rats were allocated to two groups (vehicle control and NFT-treated groups) of 5 rats that were sacrificed at week 13. NFT, NFA, and AHD were administered by gavage for five consecutive days, and the control group was administered vehicle alone. For daily doses, NFT at 125 mg/kg BW, which corresponded to the value in renal carcinogenic levels of the dietary administration study (NTP, 1989) were calculated using a conversion value of Joint FAO/WHO Expert Committee on Food Additives (JECFA; IPCS, EHC70). A dose-determination study with NFA and AHD was performed using the same molar concentrations as the NFT dose. NFA and AHD were set to 50 and 80 mg/kg BW as the maximum-tolerated doses, respectively. In experiment 2, NFT was administered to female rats at the same dose as was used for males. At autopsy, all test animals were euthanized with isofluran (Mylan Inc., Tokyo, Japan), and blood samples were collected. Kidneys were collected and their weights were measured. A portion of the kidney tissues were frozen with liquid nitrogen and were stored at −80 °C, for use in the analysis by the *in vivo* mutation assay, western blotting, and 8-OHdG measurement. The remaining kidney tissues were fixed in 10% formalin-buffer and were used in a histopathology and immunostaining examination.

2.4. In vivo *mutation assays*

6-Thioguanine (6-TG) and Spi^- selection were performed as described previously (Nohmi et al., 2000). In brief, genomic DNA was extracted from kidney tissues of the male or female rats, and lambda EG10 DNA (48 kb) was rescued as the lambda phage through *in vitro* packaging. For 6-TG selection, the packaged phages were incubated with *Escherichia coli* YG6020 that expressed Cre recombinase and were converted to plasmids carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and were poured onto agar plates containing chloramphenicol and 6-TG. For determination of the total number of rescued plasmids, 3000-fold diluted phages were infected with YG6020, and the suspension was poured into plates containing chloramphenicol without 6-TG. These plates were incubated at 37 °C, and positive colonies were counted on day 3, and recovered on day 4. The *gpt* mutant frequency (MF) was calculated by dividing the number of *gpt* mutants by the total

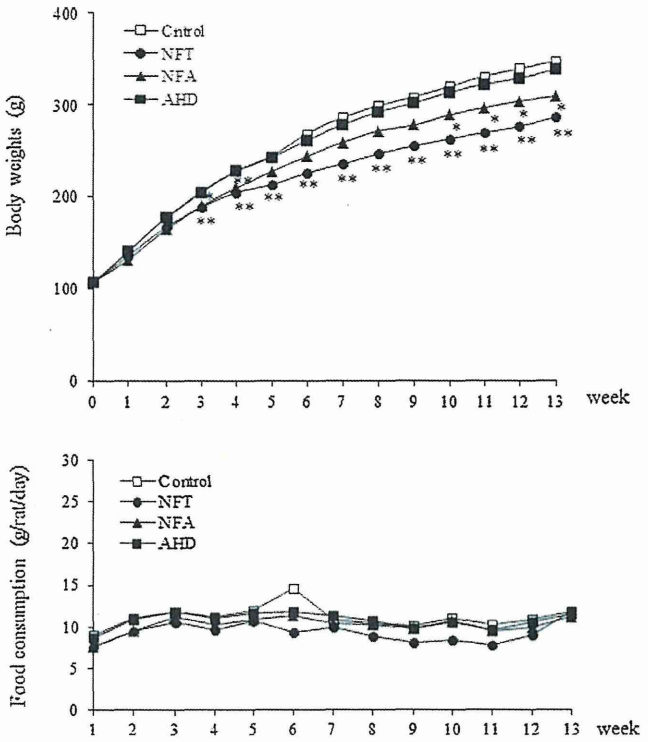

Fig. 2. Growth curves and food consumption for male *gpt* delta rats treated with NFT, NFA or AHD for 13 weeks. *,** Significantly different ($P < 0.05$, 0.01) from the control group by Dunnett's test.

number of rescued phages. In collected positive colonies, the *gpt* mutant spectra were characterized. To characterize the spectrum of the *gpt* mutants, a 739 bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR using the collected positive colonies as a template DNA, as previously described (Matsushita et al., 2015). The amplified DNA was separated by agarose gel electrophoresis, which confirmed the amplification size, and DNA sequences were analyzed at the Dragon Genomics Center of Takara Bio (Mie Japan). For Spi^- selection, packaging phages were incubated with *E.coli* XL-1 Blue MRA (for survival titration) and *E. coli* XL-1 Blue MRA P2 (for mutant selection). Infected cells were mixed with molten soft agar and were poured onto lambda-trypticase agar plates. The plates were incubated at 37 °C for one day. Plaques that appeared were collected and suspended in SM buffer. To confirm the Spi^- phenotype of false-positives, the suspensions were spotted on 3 types of plates (XL-Blue, XL-Blue-P2, and WL95-P2 strains). Samples with clear plaque in all of the plate types were confirmed to be the true Spi^- mutation.

Table 1
Final body and kidney weights of male *gpt* delta rats treated with NFT, NFA, or AHD for 4 or 13 weeks.

	Final body weights (g)	Kidney weights	
		Absolute (g)	Relative (g%)[a]
4 Weeks			
Control	223.4 ± 6.8 [b]	1.49 ± 0.08	0.67 ± 0.04
NFT	202.3 ± 13.4	1.50 ± 0.06	0.74 ± 0.02*
NFA	206.6 ± 19.0	1.43 ± 0.17	0.69 ± 0.02
AHD	227.9 ± 9.1	1.53 ± 0.07	0.67 ± 0.01
13 Weeks			
Control	347.3 ± 21.9	1.87 ± 0.12	0.54 ± 0.01
NFT	285.8 ± 7.7**	1.92 ± 0.09	0.67 ± 0.02*
NFA	308.9 ± 12.2*	1.91 ± 0.07	0.62 ± 0.01*
AHD	338.0 ± 25.9	1.86 ± 0.10	0.55 ± 0.03

*,** Significantly different ($P < 0.05$, 0.01) from the control group by Dunnett's or Steel's test.

[a] Kidney weight-to-body weight ratios (relative weights) are given as g organ weight/g body weight.

[b] Means ± SD.

Table 2
gpt MFs in the kidneys of male *gpt* delta rats treated with NFT, NFA, or AHD for 4 weeks.

Treatment	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
Control	1	10.7	3	0.28	
	2	17.8	3	0.17	
	3	27.5	2	0.07	
	4	15.7	3	0.19	
	5	16.7	2	0.12	0.17 ± 0.08
NFT	11	13.4	9	0.67	
	12	11.5	3	0.26	
	14	23.9	13	0.54	
	15	14.8	9	0.61	0.52 ± 0.18
NFA	21	16.3	3	0.18	
	22	14.2	4	0.28	
	24	15.6	2	0.13	
	25	10.8	9	0.83	0.36 ± 0.32
AHD	31	8.0	2	0.25	
	32	12.2	3	0.25	
	33	29.2	6	0.21	
	34	22.1	6	0.27	
	35	11.9	13	1.09	0.41 ± 0.38

Cm^R, chloramphenicol resistant; 6-TG^R, 6-thioguanine resistant; and MF, mutant frequency.

Table 3
gpt MFs in the kidneys of male *gpt* delta rats treated with NFT, NFA, or AHD for 13 weeks.

Treatment	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
Control	6	4.5	1	0.22	
	7	5.3	1	0.19	
	8	4.1	3	0.73	
	9	25.1	9	0.36	
	10	6.6	3	0.45	0.39 ± 0.22
NFT	17	1.3	5	3.83	
	18	2.4	2	0.84	
	19	4.2	8	1.89	
	20	7.7	10	1.31	1.97 ± 1.32**
NFA	26	4.1	5	1.23	
	27	3.6	2	0.56	
	28	14.6	19	1.30	
	29	3.2	3	0.95	
	30	3.4	3	0.88	0.99 ± 0.30*
AHD	36	3.5	2	0.57	
	37	4.0	1	0.25	
	38	8.2	1	0.12	
	39	4.5	2	0.44	
	40	3.8	3	0.78	0.43 ± 0.26

Cm^R, chloramphenicol resistant; 6-TG^R, 6-thioguanine resistant; and MF, mutant frequency.
*,** Significantly different ($P < 0.05$, 0.01) from the control group by Dunnett's test.

Table 4
Mutation spectra of male *gpt* delta rats treated with NFT, NFA, or AHD for 4 weeks.

	Control		NFT		NFA		AHD	
	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)
Base substitution								
Transversions								
G:C-T:A	3(23.1)	0.04 ± 0.04[a]	8(25.0)	0.13 ± 0.06	2(15.4)	0.03 ± 0.04	5(27.8)	0.07 ± 0.07
G:C-C:G	0	0	10(31.3)	0.16 ± 0.14**	0	0	0	0
A:T-T:A	1(7.7)	0.02 ± 0.04	3(9.4)	0.03 ± 0.08	0	0	0	0
A:T-C:G	0	0	0	0	1(7.7)	0.02 ± 0.05	1(5.6)	0.01 ± 0.02
Transitions								
G:C-A:T	6(46.2)	0.08 ± 0.05	3(9.4)	0.05 ± 0.03	7(53.8)	0.14 ± 0.11	8(44.4)	0.09 ± 0.08
A:T-G:C	1(7.7)	0.01 ± 0.03	3(9.4)	0.05 ± 0.03	1(7.7)	0.02 ± 0.05	2(11.1)	0.04 ± 0.06
Deletion								
Single bp	2(15.4)	0.01 ± 0.03	3(9.4)	0.04 ± 0.05	0	0	1(5.6)	0.01 ± 0.02
Over 2 bp	0	0	0	0	0	0	0	0
Insertion	0	0	1(3.1)	0.01 ± 0.02	0	0	0	0
Complex	0	0	1(3.1)	0.02 ± 0.04	2(15.4)	0.03 ± 0.06	1(5.6)	0.02 ± 0.04
Total	9	0.17	32	0.49	13	0.25	18	0.24

[a] Means ± SD.
** Significantly different ($P < 0.01$) from the control group by Steel's test.

2.5. Measurement of 8-OHdG

Kidney DNA of male or female *gpt* delta rats was extracted and digested as described previously (Umemura et al., 2003). In brief, renal DNA was extracted with a DNA extractor WB kit (Wako Pure Chemical Inc., Osaka, Japan) containing an oxidant NaI solution to dissolve cellular components. For prevention of auto-oxidation, deferoxamine mesylate (Sigma Chemical, MO, USA) was added to the lysis buffer. The collected DNA pellet was digested into deoxynucleosides by treatment with nuclease P1 and alkaline phosphatase. Digested DNA was measured for 8-OHdG (8-OHdG/10^5 dG) levels by high-performance liquid chromatography with an electrochemical detection system (Coulochem II; ESA, Bedford, MA, USA) as previously reported (Umemura et al., 2006).

2.6. Histopathology

Fixed kidney tissues were embedded in paraffin, and after sectioning into 4 μm thick sections, samples were stained with hematoxylin and eosin (H&E) according to a conventional method and were examined histopathologically.

Table 5
Mutation spectra of male *gpt* delta rats treated with NFT, NFA, or AHD for 13 weeks.

	Control		NFT		NFA		AHD	
	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)
Base substitution								
Transversions								
G:C-T:A	3(20.0)	0.05 ± 0.07[a]	7(31.8)	0.65 ± 0.60*	3(20.0)	0.18 ± 0.16	1(11.1)	0.02 ± 0.05
G:C-C:G	0	0	7(31.8)	0.51 ± 0.17*	0	0	0	0
A:T-T:A	2(13.3)	0.02 ± 0.04	1(4.5)	0.06 ± 0.12	2(13.3)	0.10 ± 0.22	0	0
A:T-C:G	1(6.7)	0.01 ± 0.02	0	0	1(6.7)	0.05 ± 0.11	0	0
Transitions								
G:C-A:T	5(33.3)	0.18 ± 0.31	4(18.2)	0.32 ± 0.32	4(26.7)	0.21 ± 0.21	5(55.6)	0.26 ± 0.32
A:T-G:C	2(13.3)	0.02 ± 0.03	0	0	2(13.3)	0.10 ± 0.22	0	0
Deletion								
Single bp	1(6.7)	0.04 ± 0.10	2(9.1)	0.22 ± 0.37	1(6.7)	0.05 ± 0.11	3(33.3)	0.15 ± 0.21
Over 2 bp	0	0	1(4.5)	0.06 ± 0.12	0	0	0	0
Insertion	0	0	0	0	0	0	0	0
Complex	1(6.7)	0.04 ± 0.08	0	0	2(13.3)	0.11 ± 0.16	0	0
Total	15	0.35	22	1.82	15	0.80	9	0.43

[a] Means ± SD.
* Significantly different ($P < 0.05$) from the control group by Steel's test.

Table 6
Spi⁻ MFs in the kidneys of male *gpt* delta rats treated with NFT, NFA, or AHD for 4 weeks.

Treatment	Animal No.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within WL95 (P2)	MF ($\times 10^{-5}$)	Mean ± SD
Control	1	11.1	6	0.54	
	2	12.3	4	0.32	
	3	13.1	12	0.92	
	4	7.7	3	0.39	
	5	6.6	3	0.46	0.53 ± 0.23
NFT	11	18.1	5	0.28	
	12	10.4	4	0.39	
	14	14.4	11	0.76	
	15	6.9	3	0.43	0.46 ± 0.21
NFA	21	8.6	3	0.35	
	22	11.7	7	0.60	
	24	5.6	9	1.61	
	25	5.2	2	0.38	0.74 ± 0.60
AHD	31	11.1	6	0.54	
	32	9.7	2	0.21	
	33	13.3	2	0.15	
	34	10.4	2	0.19	
	35	7.0	11	1.57	0.53 ± 0.60

MF, mutant frequency.

2.7. Immunohistochemical staining for α_{2u}-globulin

Kidney sections of NFT-treated male *gpt* delta rats were immunostained with a polyclonal anti-rat-α_{2u}-globulin antibody. Antigen retrieval was carried out by microwaving for 10 min in pH 6.0 citrate buffer. Sections were incubated with anti-rat-α_{2u}-globulin antibody (1:200 dilution in PBS, R&D Systems, Inc., MN, USA) overnight at 4 °C and then incubated with substrate solution containing an enzyme-labeled secondary antibody for 30 min at room temperature (N-Histofine Simple Stain, Nichirei Biosciences Inc., Tokyo, Japan). Finally, sections were counter-stained with hematoxylin for microscopic examination.

2.8. Western blot analysis for α_{2u}-globulin

Kidney tissues of male and female rats were homogenized in RIPA buffer (50 mmol/l Tris–HCl pH 8.0, 150 mmol/l sodium chloride, 0.5 w/v% sodium deoxycholate, 0.1 w/v% sodium dodecyl sulfate, 1.0 w/v% NP-40; Takara Bio Inc., Shiga, Japan) containing 1% protease inhibitor cocktail (Sigma Chemical Co.). The homogenate was centrifuged for 30 min at 10,000 × *g* at 4 °C, and the supernatant was collected. Protein concentrations were measured with a Multiskan FC (Thermo Fisher Scientific Inc., MA, USA) for the BCA protein assay using advanced protein assay reagent (Cytoskelton Inc. CO, USA). Kidney proteins were diluted with

Table 7
Spi⁻ MFs in the kidneys of male *gpt* delta rats treated with NFT, NFA, or AHD for 13 weeks.

Treatment	Animal No.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within WL95 (P2)	MF ($\times 10^{-5}$)	Mean ± SD
Control	6	5.0	5	1.01	
	7	10.4	3	0.29	
	8	4.4	2	0.45	
	9	56.7	2	0.35	
	10	5.1	4	0.78	0.58 ± 0.31
NFT	17	4.3	3	0.69	
	18	9.7	9	0.93	
	19	5.7	5	0.88	
	20	5.3	9	1.69	1.05 ± 0.44
NFA	26	14.9	16	1.08	
	27	11.1	12	1.08	
	28	14.8	31	2.10	
	29	3.9	3	0.78	
	30	2.8	2	0.72	1.15 ± 0.56
AHD	36	3.7	2	0.54	
	37	6.1	2	0.33	
	38	22.6	81	3.59	
	39	3.1	2	0.65	
	40	4.2	1	0.24	1.07 ± 1.42

MF, mutant frequency.

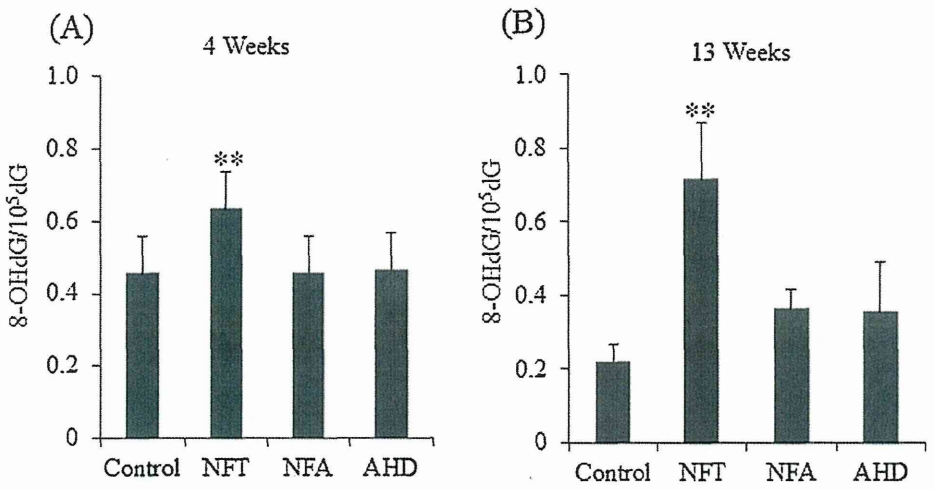

Fig. 3. 8-OHdG levels in the kidneys of male F344 *gpt* delta rats treated with NFT, NFA, or AHD for 4 (A) or 13 (B) weeks. Values are means ± SD for 5 rats, (4 rats; NFT (A, B) and NFA (A)). ** Significantly different ($P < 0.01$) from the control group by Dunnett's test.

sample buffer (BIO-RAD, CA, USA) containing 2-mercaptoethanol, heated for 2 min at 95 °C, and then quenched. Samples containing total proteins of 0.5 μg were electrophoresed on 4–15% sodium dodecyl sulfate-polyacrylamide (SDS) gradient gel (BIO-RAD) and were transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Germany) using a semi-dry blotting system. After blocking with 0.5% casein-TBS-T for 1 h at room temp, the membrane was incubated with anti-rat-α_{2u}-globulin (1:200 dilution in blocking buffer, R&D Systems, Inc. USA) overnight at 4 °C. The membrane was incubated with peroxidase-conjugated secondary antibody, polyclonal-anti-goat IgG (1:2000 dilution in blocking buffer, Dako Japan Inc.) for 1 h at room temp. Recognized protein bands were visualized with ECL-prime reagents (GE Healthcare, NJ, USA), and specific bands were detected with BIO-RAD Molecular Image ChemiDoc™ XRS+ with Image Lab™ Software.

2.9. Statistical analysis

The body and kidney weights, serum biochemistry, 8-OHdG levels, *gpt* and Spi⁻MFs, and *gpt*-mutation spectra are presented as means ± SD. All data were evaluated for consistency with a normal distribution and variance homogeneity using Levene's test or visual examination of a scatter plot. The presence or absence of outliers was confirmed visually from the same scatter plot. The data in experiment 1 were analyzed with Dunnett's multiple comparison test, and when normality or variance homogeneity was an issue, they were analyzed with Steel's test. The data in experiment 2 between controls and the NFT-treated group were analyzed with the Student's *t*-test or Wilcoxon rank sum test.

3. Results

3.1. Experiment 1

3.1.1. General conditions, food consumption, body and kidney weights

Although the cause of death was not able to be determined, deaths of NFT-treated rats occurred with one male rat at both weeks 3 and 5, and one NFA treated rat died at week 3. The change in body weights and food consumption after 13 weeks is shown in Fig. 2. In the NFT or NFA treated groups, small increased rates of body weight gain compared to the control group were observed. Food consumption was similar for all groups. The final mean body weights of male rats that received NFT, NFA, or AHD for 13 weeks were 18%, 11%, or 2.6% lower than that of controls, respectively (Table 1). Relative kidney weights were significantly increased in the NFT and NFA group at week 13 (Table 1).

3.1.2. In vivo *mutation assay of kidneys*

Results of the *gpt* assay with male rats treated with NFT, NFA, or AHD for 4 or 13 weeks are shown in Tables 2 and 3. In the NFT-treated group, the *gpt* MF was 3 times higher than in the control group at week 4. The *gpt* MF in the NFT group at week 13 was 5 times higher than in the control group and was statistically significant. Notably, an increase in *gpt* MF was observed in the NFA-treated group at week 13, but was not observed in the AHD-treated group. Tables 4 and 5 show the results of spectrum analysis of *gpt* mutants observed at week 4 or 13. NFT caused an increase in G-base substitution mutations compared with control; G:C-C:G and G:C-T:A transversion mutations were significant at weeks 4 and 13, respectively. These G-base substitution mutations accounted for

Table 8
Histopathological findings in the kidneys of male *gpt* delta rats treated with NFT, NFA, or AHD for 4 or 13 weeks.

		4 Weeks				13 Weeks			
	Group	Control	NFT	NFA	AHD	Control	NFT	NFA	AHD
	No. of animals	5	4	4	5	5	4	5	5
	Grade								
Hyaline droplet, proximal tubule	±	5	–	1	5	5	–	1	5
	+	–	3	3	–	–	–	4	–
	++	–	1	–	–	–	4	–	–

±, Very slight; +, Slight; ++, Moderate.

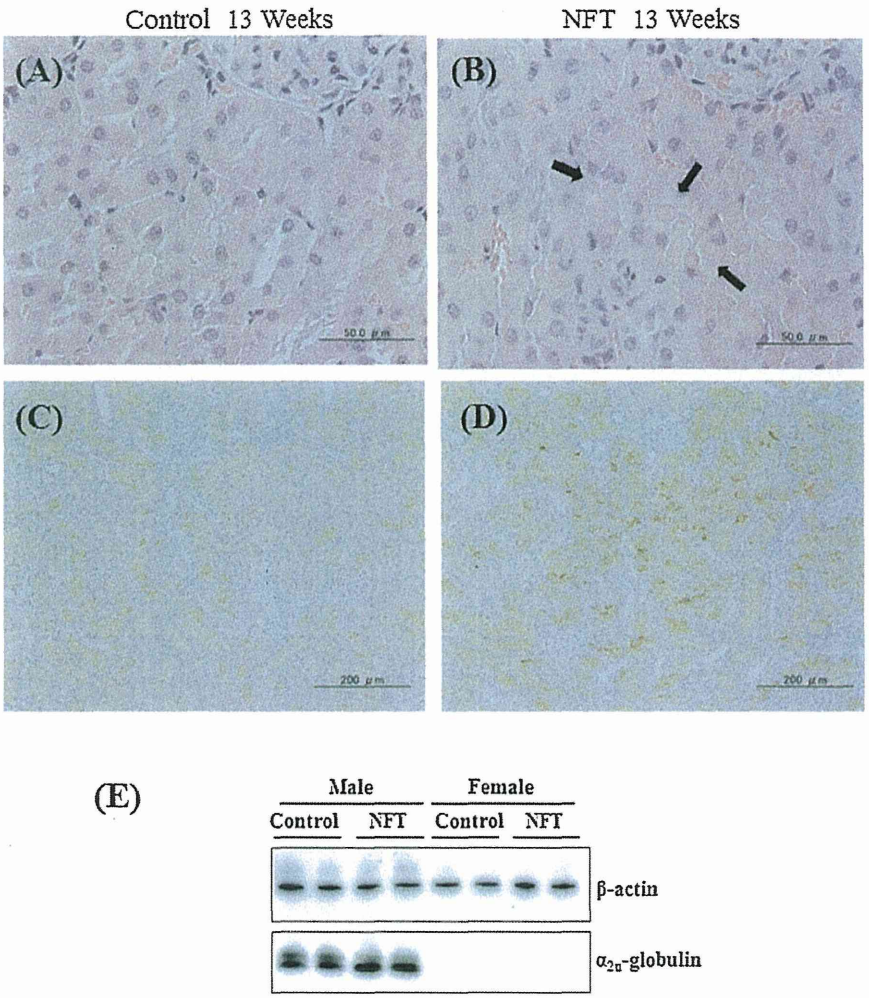

Fig. 4. Histopathological features from H&E staining (A, control; B, NFT-treated), and photomicrographs of immunohistochemical staining a_{2u}-globulin (C, control; D, NFT-treated) of the kidneys of male F344 *gpt* delta rats at week 13. Note (B) hyaline droplets in the proximal tubules (arrow head) were observed. Western blotting analysis for a_{2u}-globulin (E). Note (E) kidney protein samples of the controls and NFT-treated rats at week 13 are shown with samples from each sex. a_{2u}-globulins of 15.5 and 18.7 kDa were detected only in males, and NFT treatment caused an increase in the 15.5 kDa protein.

a higher percentage in the total *gpt* mutants observed in the NFT-treated group, and were 56% and 64% at weeks 4 and 13, respectively. In the Spi$^-$ assay, no significant changes in Spi$^-$ MF were observed in rats treated with NFT, NFA, or AHD compared with controls after administration for 4 (Table 6) and 13 weeks (Table 7).

3.1.3. Measurement of 8-OHdG in kidney DNA

8-OHdG levels in NFT-treated rats were significantly increased after 4 weeks and significantly increased by over 3 fold compared to controls at week 13 (Fig. 3). Neither NFA nor AHD treated rats exhibited a significant change in their 8-OHdG level.

3.1.4. Histopathologic examination of kidneys, including immunostaining of α_{2u}-globulin

The results of histopathological examinations of the kidneys are shown in Table 8. NFT treatment caused an increase in the number of hyaline droplets in the proximal tubules depending on the treatment period (Fig. 4A and B) and showed positive immunostaining for α_{2u}-globulin in male rat kidneys (Fig. 4C and D). Likewise, elevation of the α_{2u}-globulin protein level was confirmed by western blotting analysis, shown in Fig. 4E. α_{2u}-globulin is a major male rat specific urinary protein, which has two molecular weight types: an 18.7 kDa major urinary protein (native type) and a 15.5 kDa (kidney type) that is proteolytically modified from the native type (Wang et al., 2000; Hai and Kizilbash, 2013). The expression of this protein is well known to change in response to drug treatment. Western blotting analysis showed that NFT induced formation of the 15.5 kDa protein.

3.2. Experiment 2

3.2.1. General conditions, food consumption, body and kidney weights

No animal deaths were detected during 13 weeks of NFT administration. In the NFT-treated group, low body weight compared with the control group was noticeable from week 1 (Fig. 5), and an increase in kidney weight compared with controls was also observed (Table 9).

3.2.2. In vivo mutation assay in kidneys

Results of the *gpt* assay for the kidneys of female rats treated with NFT for 13 weeks are shown in the Table 10. With the same tendency as with males, *gpt* MFs in the NFT-treated group were significantly higher, over 5 fold compared with the control group. Increases in G-base substitution mutations, G:C-T:A and G:C-C:G