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Mutations Induced by the Carcinogenic Pyrrolizidine Alkaloid Riddelliine in the Liver *cII* Gene of Transgenic Big Blue Rats

Nan Mei^{*,†}, Robert H. Heflich[†], Ming W. Chou[‡], and Tao Chen[†]

[†]Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079

[‡]Division of Biochemical Toxicology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079

Abstract

Riddelliine is a naturally occurring pyrrolizidine alkaloid that forms a number of different mononucleotide and dinucleotide adducts in DNA. It is a rodent carcinogen and a potential human hazard via food contamination. To examine the mutagenicity of riddelliine, groups of six female transgenic Big Blue rats were gavaged with 0.1, 0.3, and 1.0 mg riddelliine per kg body weight. The middle and high doses resulted in liver tumors in a previous carcinogenesis bioassay. The animals were treated 5 days a week for 12 weeks and sacrificed 1 day after the last treatment. The liver DNA was isolated for analysis of the mutant frequency (MF) in the transgenic *cII* gene, and the types of mutations were characterized by sequencing the mutants. A significant dose-dependent increase in MF was found, increasing from 30×10^{-6} in the control animals to 47, 55, and 103×10^{-6} in the low, middle, and high dose groups, respectively. Molecular analysis of the mutants indicated that there was a statistically significant difference between the mutational spectra from the riddelliine-treated and the control rats. A G:C $\rightarrow$ T:A transversion (35%) was the major type of mutation in rats treated with riddelliine, whereas a G:C $\rightarrow$ A:T transition (55%) was the predominant mutation in the controls. In addition, mutations from the riddelliine-treated rats included an unusually high frequency (8%) of tandem base substitutions of GG $\rightarrow$ TT and GG $\rightarrow$ AT. These results indicate that riddelliine is a genotoxic carcinogen in rat liver and that the types of mutations induced by riddelliine are consistent with riddelliine adducts involving G:C base pairs.

Introduction

Pyrrolizidine alkaloids are constituents of over 6000 plants. About half of the identified pyrrolizidine alkaloids are genotoxic, and many are tumorigenic (1, 2). Thus, the human health risk posed by exposure to pyrrolizidine alkaloids has been a concern. Riddelliine is a representative genotoxic pyrrolizidine (3–5) that is present in plants growing in the rangelands of the western United States. Human foodstuffs, such as grains, herbs, milk, honey, herbal tea, and herbal medicine, may be contaminated with pyrrolizidine alkaloids including riddelliine (2, 5).

^{*}To whom correspondence should be addressed. Fax: +1-870-543-7682. nmei@nctr.fda.gov.

Riddelliine is completely absorbed within 30 min after gavage dosing to rodents (6) and is metabolically activated to DHP.¹ The reactive metabolite binds to cellular macromolecules such as proteins and DNA and is responsible for the toxicities of riddelliine. DHP-derived DNA adducts are formed by in vitro metabolism of riddelliine in human (7) and rat (8) liver. Using ³²P-postlabeling HPLC analysis of DNA adducts, a linear dose-dependent formation of eight DHP-derived DNA adducts was observed in riddelliine-treated rats (8, 9). Two were enantiomers of DHP-derived 7-deoxyguanosin-*N*²-yl adducts, and the others were DHP-modified dinucleotides.

Because of its genotoxicity and potential for human exposure, riddelliine was tested by the NTP for carcinogenicity in a 2 year bioassay. The results showed that riddelliine causes liver tumors in male and female rats and male mice, mononuclear cell leukemia in male and female rats, and lung neoplasms in female mice (3, 4). Riddelliine is genotoxic both in vitro and in vivo, inducing increases in sister chromatid exchange, chromosomal aberrations, unscheduled DNA synthesis, and micronucleated erythrocyte frequencies (reviewed by Fu et al. in ref 2). However, the mutagenicity of riddelliine in the target tissues for carcinogenesis has not been studied. Also, it is not clear to what extent the various types of DNA adducts formed by riddelliine participate in its genotoxicity.

Transgenic mutation assays provide a unique opportunity for studying the induction of in vivo mutation. The assays permit quantitative measurements of mutant frequencies in all tissues/organs of transgenic rodents and molecular analysis of the induced and spontaneous mutations. The *cII* gene, located on the λ vector of Big Blue rodents and Muta mice, can be used as a reporter of mutagenicity. The *cII* gene has advantages over the *lacZ* or *lacI* transgenic reporter genes on λ because of its relatively small size (about 300 base pairs), because of its positive selection system for *cII* mutations, and because the mutant assay for *cII* mutants is relatively less labor intensive (10). In this study, we evaluated the mutagenicity of riddelliine in the liver *cII* gene of Big Blue rats.

Materials and Methods

Chemical and Animals.

Riddelliine (>97% pure by reversed phase HPLC analysis) was obtained from the NTP and dissolved in 0.9% sodium chloride. Female Big Blue Fisher 344 transgenic rats were obtained from Taconic Laboratories (Germantown, NY) through a purchase from Stratagene (La Jolla, CA). All animal procedures followed the recommendations of the NCTR Institutional Animal Care and Use Committee for the handling, maintenance, treatment, and sacrifice of laboratory animals.

Treatments.

The treatment schedule was based on the preliminary results from the NTP 2 year chronic tumorigenicity bioassay (3). Female, 6 week old Big Blue rats were treated with riddelliine at concentrations of 0.1, 0.3, and 1.0 mg/kg body weight by gavage five times a week for 12

¹Abbreviations: DHP, 6,7-dihydro-1-hydroxymethyl-5*H*-pyrrolizine; MF, mutant frequency; NTP, National Toxicology Program; pfus, plaque-forming units.

weeks. Vehicle control rats were gavaged with 0.9% sodium chloride using the same schedule as for the ridelliine-treated rats. Six rats from each treatment group were sacrificed 1 day after the last treatment. The livers were isolated, frozen quickly in liquid nitrogen, and stored at -80°C .

***cII* Mutation Assay.**

High molecular weight genomic DNA was extracted from rat livers using the RecoverEase DNA Isolation Kit (Stratagene) and stored at 4°C until DNA packaging was performed. The packaging of the phage, plating of the packaged DNA samples, and determination of MF were carried out following the manufacturer's instructions for the λ Select-*cII* Mutation Detection System for Big Blue Rodents (Stratagene). The shuttle vector containing the *cII* target gene was rescued from total genomic DNA with phage packaging extract (Transpack; Stratagene). The plating was performed with the *Escherichia coli* host strain G1250. To determine the total titer of packaged phages, G1250 bacteria were mixed with 1:3000 dilutions of phage, plated on TB1 plates, and incubated overnight at 37°C (nonselective conditions). For mutant selection, the packaged phages were mixed with G1250, plated on TB1 plates, and incubated at 24°C for about 42 h (conditions for *cII*-selection). Under these conditions, phages with wild-type *cII* genes undergo lysogenization and become part of the developing bacterial lawn, whereas phages with mutated *cII* genes undergo lytic growth and give rise to plaques. When incubated at 37°C , phages with wild-type *cII* genes also undergo a lytic cycle, resulting in plaque formation. Assays were repeated until a minimum of 2×10^5 pfus from each sample was examined for mutation. The *cII*MF is defined as the total number of mutant plaques (determined at 24°C) divided by the total number of plaques screened (determined at 37°C).

Sequence Analysis of the *cII* Mutants.

The mutants were sequenced using a modification of the methods of Chen et al. (11). The *cII* mutant plaques were selected at random from different animals and replated at low density to verify the mutant phenotype. Single, well-isolated plaques were selected from these plates and transferred to a microcentrifuge tube containing $100\ \mu\text{L}$ of sterile distilled water. The tube was heated at 100°C for 5 min and centrifuged at $12\ 000g$ for 3 min. The *cII* target DNA for sequencing was amplified by PCR using primers 5'-AAAAAGGGCATCAAATTAACC-3' (upstream) and 5'-CCGAAGTTGAGTATTTTGCTG-3' (downstream). For PCR amplification, $10\ \mu\text{L}$ of the supernatant was added to $10\ \mu\text{L}$ of a PCR Master Mix (Promega, Madison, WI) and the primers. The final concentrations of the reagents were $1 \times$ *Taq* polymerase reaction buffer, $0.2\ \mu\text{mol}$ of each primer, $200\ \mu\text{M}$ each dNTP, $1.5\ \text{mM}$ MgCl_2 , and $0.25\ \text{U}$ of *Taq* DNA polymerase. The PCR reaction was performed using a PCR System 9700 (Applied Biosystems, Foster City, CA), with the following cycling parameters: a 3 min denaturation at 95°C , followed by 35 cycles of 30 s at 95°C , 1 min at 60°C , and 1 min at 72°C , with a final extension of 10 min at 72°C . The PCR products were isolated using a PCR purification kit (Qiagen, Chatsworth, CA). The *cII* mutant DNA was sequenced with a CEQ Dye Terminator Cycle Sequencing Kit and a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). The primer for *cII* mutation sequencing was the upstream primer used for the PCR.

Statistical Analyses.

Analyses were performed using the SigmaStat 2.03 program (SPSS, Chicago, IL). All of the MF data are expressed as the mean $\pm$ SD from six rats per group. Statistical significance was determined by one way ANOVA followed by the Tukey test. Because the variance increased with the magnitude of the MF, the data were log-transformed before conducting the analysis. Mutational spectra were compared using the computer program written by Cariello and colleagues (12) for the Monte Carlo analysis developed by Adams and Skopek (13).

Results

MF in the Liver *cII* Gene from Riddelliine-Treated and Control Rats.

Female Big Blue rats were treated with riddelliine for 12 weeks, and the MFs in the liver *cII* gene were determined (Table 1). DNA from each liver was packaged 2–4 times either to confirm the MF or to obtain a minimum of 2×10^5 pfus for mutant detection. The MFs for the control female Big Blue rats ranged from 18 to 44×10^{-6} , with an average of $30 \pm 10 \times 10^{-6}$. The MFs for the riddelliine-treated rats increased in a linear dose-dependent manner (Figure 1), and a statistically significant difference was observed among the four study groups ($P < 0.001$). The MFs for rats treated with 0.1, 0.3, and 1.0 mg/kg riddelliine were $47 \pm 14 \times 10^{-6}$, $55 \pm 8 \times 10^{-6}$, and $103 \pm 16 \times 10^{-6}$, respectively, and all were significantly increased over the control group ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively). The MF in the 1.0 mg/kg riddelliine-treated group was also significantly higher than those in other treatment groups ($P < 0.01$).

Mutation Spectra in the Liver *cII* Gene from Riddelliine-Treated and Control Rats.

Riddelliine-induced and spontaneous mutations in the liver *cII* gene were evaluated by DNA sequence analysis of 92 mutants isolated from six rats treated with 1.0 mg/kg riddelliine and 63 mutants from six rats in the control group (Table 2). Mutations that were found more than once among the mutants isolated from a single animal were assumed to be siblings and to represent only one independent mutation. Accordingly, a total of 83 and 55 independent mutations were identified from the riddelliine-treated rats and control rats, respectively (Table 3). The overall pattern of mutations in the control and riddelliine-treated rats differed significantly ($P < 0.001$). Among the independent mutations, about 82% from both the riddelliine-treated and control rats were base pair substitutions. A G:C $\rightarrow$ T:A transversion (35%) was the major type of mutation in the riddelliine-treated rats, whereas a G:C $\rightarrow$ A:T transition (55%) was the predominant mutation in the controls. In addition, an unusually high frequency of tandem base substitutions (8%) was observed among the mutations from the riddelliine-treated rats; these included four independent GG $\rightarrow$ TT mutations and three independent GG $\rightarrow$ AT mutations.

Discussion

Many carcinogens exhibit tissue specificity. A major target tissue for riddelliine tumorigenesis is liver, where the compound is metabolized to reactive derivatives. Among the tissues examined, the highest concentration of riddelliine-induced DNA adducts was found in rat liver (14). Also, in the NTP 2 year carcinogenesis studies, riddelliine-induced

neoplasms in the rat were mostly found in the liver. Treating rats by gavage with 1.0 mg riddelliine per kg body weight resulted in a 76–86% incidence of liver hemangiosarcomas and an 8–14% incidence of hepatocellular adenoma, along with an 18–28% incidence of mononuclear cell leukemia. The liver is also a major target tissue for tumor induction by riddelliine in the mouse (3, 4). Hemangiosarcoma is a malignant neoplasm of endothelial cells and occurs in many tissues including liver.

If riddelliine's carcinogenicity operates through a genotoxic mechanism, it would be anticipated that liver is also a main target tissue for riddelliine's mutagenesis. To understand the mechanisms of riddelliine's carcinogenesis, we determined MFs in the liver *cII* gene using Big Blue transgenic rats. After treatment with 0.1–1.0 mg/kg riddelliine 5 days per week for 12 weeks, we observed a linear dose-dependent increase of MF in the liver *cII* gene. The increase in MF was consistent with dose-dependent DHP-derived DNA adduct formation (8). Although mutation induction and adduct formation were presumably involved in the liver hemangiosarcomas, the increase of the tumor incidence is nonlinear, with riddelliine doses of 0.1, 0.3, and 1.0 mg/kg producing tumor incidences of 0, 6, and 76%, respectively (3). It is possible that the kinetics for DNA damage formation/repair, *cII* mutation induction, and tumor formation differed over the range of riddelliine doses that were evaluated. However, in the rats treated with riddelliine, the *cII*/MF data correlate better with the DNA adducts that are presumably responsible for the mutations than the incidence of liver tumors. It is tempting to speculate that these relationships indicate that there are one or more events between riddelliine-induced mutation and liver tumor formation that occur at a disproportionately greater frequency at high doses of riddelliine. Alternatively, mutation and adduct formation in the liver cells that are the specific targets of carcinogenic response (the endothelial cells) may correlate better with riddelliine tumorigenicity than mutation and adducts within liver as a whole. A previous study indicates that DHR-derived DNA adduct levels differ between rat endothelial cells and parenchymal cells (15).

The overall pattern of mutations induced by riddelliine was significantly different from the control rats ($P < 0.001$). About 80% of the independent mutations from both the riddelliine-treated and the control rats were base pair substitutions. In contrast to the G:C → A:T transitions that dominated the mutation spectrum in control rats (55% of all mutations), the main type of mutation induced by riddelliine was G:C → T:A transversion (35%). Riddelliine (DHP) reacts with guanine, adenine, and thymine and has the greatest affinity for guanine (9). Two of eight riddelliine-induced DHP-derived DNA adducts are epimers of DHP-deoxyguanosine-monophosphate (8). These DHP-guanosine adducts are bulky DNA adducts, a type of adduct that commonly results in a G:C → T:A transversion mutation. The G:C → T:A transversion may also cause the initiation of tumors in the liver of rats treated with riddelliine because it has been reported that more than half of the riddelliine-induced hemangiosarcomas have a G → T mutation at K-*ras* codon 12 (16). Interestingly, riddelliine also induced a relatively high frequency of tandem base substitutions (8%), while no such mutations were found in the controls. Although no previous reports have described tandem base substitution in the *cII* gene of transgenic rodents, there have been several reports of these mutations in other genes. For example, two tandem mutations were detected in the *lacI* gene of 1,2-epoxybutene-exposed Big Blue mice (17). Acetaldehyde, which is found in tobacco smoke and automotive exhaust gases (18), and *cis*-diamminedichloroplatinum(II),

which is used in clinical oncology as a chemotherapeutic agent (19), induce GG to TT transversions in the *supF* gene. It is believed that these chemicals form intrastrand cross-links in adjacent guanine bases, and then adenines are incorporated opposite the crosslinked guanines during DNA replication, resulting in GG to TT tandem base substitution (18). In addition, NO₂⁺ produced by peroxyacetyl nitrate (a ubiquitous air pollutant) attacks the exocyclic nitrogen of guanine creating a positively charged reactive intermediate, which when in close proximity to an adjacent guanine may produce a structure similar to a GG adduct. Errors in the replication of these dimers may account for the mutation at two adjacent bases (20). It is unknown which of the eight DHP-derived DNA adducts induced by riddelliine (8, 9) and what mechanism result in these specific types of mutations in present study. However, this unique tandem base substitution may serve as a signature mutation for genetic damage produced by riddelliine.

In conclusion, tumorigenic doses of riddelliine increased the MF in the liver *cH* gene of rats and resulted in a unique spectrum of *cH* mutation. The types of mutations induced by riddelliine suggest that both mononucleotide and dinucleotide DNA adducts involving G:C base pairs are mainly responsible for its mutagenicity.

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Figure 1.

Riddelliine-induced *cI*MF as a function of dose. Big Blue rats were gavaged with 0.1–1.0 mg/kg body weight riddelliine for 12 weeks, and the *cI*MF was determined in the liver 1 day after the last treatment. The induced MF was obtained by subtracting the background MF observed in the vehicle control rats. The data represent the mean $\pm$ SD for each group of six rats.

Table 1.*cII*MFs in Livers of the Control and Riddelliine-Treated Transgenic Big Blue Rats

group	rat ID	total plaques screened ($\times 10^3$)	mutant plaques	MF ($\times 10^{-6}$)	mean $\pm$ SD ($\times 10^{-6}$)
control	I-1F	440	13	30	30 ± 10
	I-2F	458	18	39	
	I-3F	442	10	23	
	I-4F	398	7	18	
	I-5F	315	14	44	
	I-6F	431	11	26	
0.1 mg/kg	II-1F	379	12	32	47 ± 14^a
	II-2F	392	16	41	
	II-3F	531	22	41	
	II-4F	269	18	67	
	II-5F	295	11	37	
	II-6F	245	15	61	
0.3 mg/kg	III-1F	570	33	58	55 ± 8^b
	III-2F	378	20	53	
	III-3F	359	18	50	
	III-4F	393	24	61	
	III-5F	216	14	65	
	III-6F	378	16	42	
1.0 mg/kg	IV-1F	349	38	109	$103 \pm 16^{c,d}$
	IV-2F	399	31	78	
	IV-3F	249	30	121	
	IV-4F	271	29	107	
	IV-5F	577	52	90	
	IV-6F	387	43	111	

^a $P < 0.05$.^b $P < 0.01$.^c $P < 0.001$ (significantly higher than the control group, Tukey test).^dSignificantly higher than the groups treated with 0.1 or 0.3 mg riddelliine per kg body weight ($P < 0.01$, Tukey test).

Table 2.
Mutations in the *cII* Gene of Livers from the Riddelliine-Treated and Control Big Blue Rats

position ^a	mutation ^b	amino acid change	sequence context 5' → 3' ^c		no. of mutations (independent)	amino acid change	mutation ^b	position ^a	sequence context 5' → 3' ^c		no. of mutations (independent)	amino acid change	mutation ^b	position ^a	amino acid change	sequence context 5' → 3' ^c		no. of mutations (independent)	control	treated
(-14) (-13)	GG → TT	N/A	ctaAGGaaa	ctaAGGaaa	1	134-135	GG → TT	Arg → Ile	aagAGGgac	1	1	1	1	1	1	1	1	1	1	1
-13	G → T	N/A	ctaAGGaaa	ctaAGGaaa	3 (3)	135	G → A	Arg → Arg	aagAGGgac	1	1	1	1	1	1	1	1	1	1	1
19	C → G	Arg → Gly	aaaCGCaac	aaaCGCaac	1	135	G → T	Arg → Ser	aagAGGgac	1	1	1	1	1	1	1	1	1	1	1
19	C → T	Arg → Cys	aaaCGCaac	aaaCGCaac	1	136	G → T	Asp → Tyr	aagGACtgg	1	1	1	1	1	1	1	1	1	1	1
24	C → G	Asn → Lys	cgcAAcag	cgcAAcag	1	139	T → G	Trp → Gly	gacTGGatt	1	1	1	1	1	1	1	1	1	1	1
27	G → T	Glu → Asp	aacGAGgct	aacGAGgct	1	145	C → A	Pro → Thr	attCCAAag	1	1	1	1	1	1	1	1	1	1	1
28	G → A	Ala → Thr	gagGCTcta	gagGCTcta	1	145	C → T	Pro → Ser	attCCAAag	1	1	1	1	1	1	1	1	1	1	1
29	C → T	Ala → Val	gagGCTcta	gagGCTcta	3 (2)	152	T → G	Phe → Cys	aagTTCtca	2 (2)	2	2	2	2	2	2	2	2	2	2
30	T → A	Ala → Ala	gagGCTcta	gagGCTcta	1	154	T → C	Ser → Pro	ttcTCAatg	1	1	1	1	1	1	1	1	1	1	1
34	C → T	Arg → stop	ctaCGAatc	ctaCGAatc	4 (3)	163	C → T	Leu → Phe	ctgCTTgct	2 (1)	2	2	2	2	2	2	2	2	2	2
35	G → A	Arg → Gln	ctaCGAatc	ctaCGAatc	1	164	T → C	Leu → Pro	ctgCTTgct	1	1	1	1	1	1	1	1	1	1	1
40	G → A	Glu → Lys	atcGAGagt	atcGAGagt	1	166	G → A	Ala → Thr	cttGCTgtt	1	1	1	1	1	1	1	1	1	1	1
46	G → T	Ala → Ser	agtGCCgtt	agtGCCgtt	1	172	C → A	Leu → Ile	gttCTTgaa	1	1	1	1	1	1	1	1	1	1	1
57/62	+ A	frameshift	aacAAaAatc	aacAAaAatc	1	175	G → C	Glu → Gln	cttGAAatg	1	1	1	1	1	1	1	1	1	1	1
60	A → T	Lys → Asn	aacAAaAatc	aacAAaAatc	1	178/185	+ G	frameshift	gaaTGGGGGGTTCgtt	6 (4)	6	6	6	6	6	6	6	6	6	6
61	A → C	Ile → Leu	aaaATCgca	aaaATCgca	1	178	T → C	Trp → Arg	gaaTGGggg	1	1	1	1	1	1	1	1	1	1	1
64	G → C	Ala → Pro	atcGCAatg	atcGCAatg	1	179	G → T	Trp → Leu	gaaTGGggg	1	1	1	1	1	1	1	1	1	1	1
65	C → T	Ala → Val	atcGCAatg	atcGCAatg	1	179/184	- G	frameshift	gaaTGGGGGGTTCgtt	2 (2)	2	2	2	2	2	2	2	2	2	2
65/68	+ A	frameshift	gcaATCgtt	gcaATCgtt	1	180	G → A	Trp → stop	gaaTGGggg	1	1	1	1	1	1	1	1	1	1	1
73-74	GG → AT	Gly → Ile	cttGGAact	cttGGAact	2 (2)	180-181	GG → AT	Trp-Gly → stop-Trp	gaaTGGGGGGgtc	1	1	1	1	1	1	1	1	1	1	1
73-74	GG → TT	Gly → Leu	cttGGAact	cttGGAact	1	182	G → T	Gly → Val	tggGGGgtc	2 (2)	2	2	2	2	2	2	2	2	2	2
74	G → T	Gly → Val	cttGGAact	cttGGAact	4 (4)	184	G → T	Val → Phe	gggGTCgtt	1	1	1	1	1	1	1	1	1	1	1
76	A → G	Thr → Ala	ggaACTgag	ggaACTgag	1	185	T → G	Val → Gly	gggGTCgtt	1	1	1	1	1	1	1	1	1	1	1
81	G → A	Glu → Glu	actGACaag	actGACaag	1	185, 178/186	T → G, -G	complex	gggGTCgtt	1	1	1	1	1	1	1	1	1	1	1
86	C → A	Thr → Lys	aagACAgcg	aagACAgcg	1	187	G → T	Val → Phe	gtcGTTgac	1	1	1	1	1	1	1	1	1	1	1
86	C → G	Thr → Arg	aagACAgcg	aagACAgcg	1	191	A → G	Asp → Gly	gttGACgac	1	1	1	1	1	1	1	1	1	1	1

position ^a	mutation ^b	amino acid change	sequence context 5' → 3' ^c	no. of mutations (independent)		position ^a	mutation ^b	amino acid change	sequence context 5' → 3' ^c	no. of mutations (independent)	
				control	treated					control	treated
89	C → A	Ala → Glu	acaG <u>CC</u> Ggaa	1		192	C → G	Asp → Glu	gttGA <u>C</u> gac		1
89	C → T	Ala → Val	acaG <u>CC</u> Ggaa	3 (3)	4 (2)	196	G → A	Asp → Asn	gacGACatg	2 (2)	1
90-91	GG → TT	Ala-Glu → Ala-stop	acaG <u>CCG</u> AAgct			203	C → A	Ala → Asp	atgGCTcga		1
91	G → T	Glu → stop	gcgGAAgct	1	1	206	G → A	Arg → Gln	gctCGAttg	1	3 (2)
94	G → A	Ala → Thr	gaaGCTgrg	1	1	208	T → A	Leu → Met	cgaTTGgcg	1	
95	C → T	Ala → Val	gaaGCTgrg		1	209	T → G	Leu → Trp	cgaTTGgcg	1	
98	T → C	Val → Ala	gctGTGggc	1		211	G → A	Ala → Thr	ttgGCGcga	1	
99/101	- G	frameshift	gctGTGGGCGgtt	1		212	C → A	Ala → Glu	ttgGCGcga		1
101	G → A	Gly → Asp	gtgGCGgtt	1		212	C → T	Ala → Val	ttgGCGcga	2 (2)	2 (2)
101	G → T	Gly → Val	gtgGCGgtt			212/214	+ G	frameshift	ttgGCGGGAcaa		1
103	G → A	Val → Ile	ggcGTTgat	4 (3)	3 (2)	213	G → T	Ala → Ala	ttgGCGcga	1	
103	G → T	Val → Phe	ggcGTTgat	1	1	214	C → T	Arg → stop	ggcCGAcaa	9 (5)	3 (2)
113	C → A	Ser → stop	aagTCGcag	1	1	217	C → T	Gln → stop	cgaCAAgtt		1
118	A → T	Ile → Phe	cagATCagc	1	1	220	G → T	Val → Phe	caaGTTgct	1	
122	G → A	Ser → Asn	atcAGCagg	1		222	T → G	Val → Val	caaGTTgct		1
125	G → T	Arg → Met	agcAGGtgg	1	1	224	C → A	Ala → Asp	gttGCTgceg	1	
126	G → T	Arg → Ser	agcAGGtgg	1	1	230	T → G	Ile → Ser	gcgATTctc	1	
127	T → A	Trp → Arg	aggTGGaag	1		232	C → A	Leu → Ile	attCTCacc		2 (2)
128	G → A	Trp → stop	aggTGGaag		1	233	T → C	Leu → Pro	attCTCacc	1	
129	G → T	Trp → Cys	aggTGGaag	1	1	266	G → A	Arg → His	gagCGTctt	1	
131	A → T	Lys → Met	tggAAGagg	1		total				63 (55)	92 (83)
133	A → T	Arg → Trp	aagAGGgac		1						

^aPosition 1 is the first base of the start codon in the *cII* coding sequence.

^bPresented in terms of sequence change on a nontranscribed DNA strand.

^cUppercase indicates the target codon, and target bases are underlined. Abbreviations: -, deletion; +, insertion.

Table 3.

Summary of the Independent Mutations in the Liver *cII* Gene from the Riddelliine-Treated and Control Big Blue Rats

type of mutation	control ^a		riddelliine ^a	
	number	%	number	%
G:C → C:G	2	4	4	5
G:C → A:T	30	55	22	26
G:C → T:A	5	9	29	35
A:T → T:A	3	5	4	5
A:T → C:G	3	5	5	6
A:T → G:C	3	5	4	5
frameshift	8	15	8	10
tandem base substitution	0	0	7	8
complex ^b	1	2	0	0
total	55	100	83	100

^aSpectra of mutations from control and riddelliine-treated rats significantly different [$P < 0.001$; Adams and Skopek test (13)].

^bBase substitution plus frameshift.