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Mutation Research 506–507 (2002) 55–63

Fundamental and Molecular Mechanisms of Mutagenesis

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Mutagenicity and carcinogenicity in relation to DNA adduct formation in rats fed leucomalachite green

S.J. Culp [a,*], F.A. Beland [a], R.H. Heflich [a], R.W. Benson [a], L.R. Blankenship [a], P.J. Webb [a], P.W. Mellick [b], R.W. Trotter [b], S.D. Shelton [a], K.J. Greenlees [c], M.G. Manjanatha [a]

[a] *Food and Drug Administration, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079, USA*
[b] *Pathology Associates A Charles River Company, Jefferson, AR 72079, USA*
[c] *Food and Drug Administration, Center for Veterinary Medicine, Rockville, MD 20855, USA*

Received 12 November 2001; received in revised form 12 March 2002; accepted 13 March 2002

Abstract

Leucomalachite green is a persistent and prevalent metabolite of malachite green, a triphenylmethane dye that has been used widely as an antifungal agent in the fish industry. Concern over the use of malachite green is due to the potential for consumer exposure, evidence suggestive of tumor promotion in rodent liver, and suspicion of carcinogenicity based on structure–activity relationships. Our previous study indicated that feeding rodents malachite or leucomalachite green resulted in a dose-related increase in liver DNA adducts, and that, in general, exposure to leucomalachite green caused an increase in the number and severity of changes greater than was observed following exposure to malachite green. To characterize better the genotoxicity of leucomalachite green, female Big Blue® rats were fed leucomalachite green at doses of 0, 9, 27, 91, 272, or 543 ppm for up to 32 weeks. The livers were analyzed for *lacI* mutations at 4, 16, and 32 weeks and DNA adducts at 4 weeks. Using a ^{32}P-postlabeling assay, we observed a dose-related DNA adduct in the livers of rats fed 91, 272, and 543 ppm leucomalachite green. A ~3-fold increase in *lacI* mutant frequency was found in the livers of rats fed 543 ppm leucomalachite green for 16 weeks, but significant increases in mutant frequencies were not found for any of the other doses or time points assayed. We also conducted 2-year tumorigenesis bioassays in female and male F344 rats using 0, 91, 272, and 543 ppm leucomalachite green. Preliminary results indicate an increasing dose trend in lung adenomas in male rats treated with leucomalachite green, but no increase in the incidence of liver tumors in either sex of rat. These results suggest that the DNA adduct formed in the livers of rats fed leucomalachite green has little mutagenic or carcinogenic consequence.
Published by Elsevier Science B.V.

Keywords: Leucomalachite green; Malachite green; *lacI*; Mutagenicity; Carcinogenicity; Transgenic rats

1. Introduction

Leucomalachite green is a reduction product and major metabolite of malachite green, a triphenylmethane dye that has been used widely as an antifungal agent in the fish industry since the 1930s. Although little is known about how malachite green is metabolized by fish or other species, studies show that the dye is reduced to leucomalachite green after entering the fish [1]. This has potential significance for human exposure since leucomalachite green is the most prevalent and persistent product in malachite

* Corresponding author. Tel.: +1-870-543-7941; fax: +1-870-543-7485.
E-mail address: sculp@nctr.fda.gov (S.J. Culp).

0027-5107/02/$ – see front matter. Published by Elsevier Science B.V.
PII: S0027-5107(02)00152-5

green-treated fish. These data have been corroborated by analysis of random samples of trout sold in markets in the UK [2]. In these samples, leucomalachite green (9–96 μg/kg) was detected at levels 30-fold higher than malachite green (0.4–3.4 μg/kg; [3]).

Malachite green is not approved for use in the US, but it is probable that application has continued in some of the approximately 3000 fisheries in the country because of its relatively low cost, availability, and efficacy. Concern over the use of malachite green is due to the potential for significant worker and consumer exposure [4], suggestive evidence of tumor promotion in rodent liver [5], and suspicion of carcinogenicity based on its structural similarity to known carcinogens [6,7]. As a result, both malachite green and leucomalachite green were selected for comprehensive toxicological evaluation by the National Toxicology Program. We have contributed to this effort by conducting short-term feeding studies, 2-year carcinogenicity bioassays, and in vitro and in vivo mutagenicity assays.

Recently, we reported the results of 28-day studies with F344 rats and B6C3F$_1$ mice fed malachite green or leucomalachite green [8]. In general, our data indicated that, in comparison to malachite green, leucomalachite green treatment resulted in a greater number of toxic effects and that the effects were of greater severity. The presence of *N*-demethylated and *N*-oxidized malachite green and leucomalachite green metabolites, including primary arylamines, was detected by high performance liquid chromatography/mass spectrometry in the livers of treated rats. This suggested that both compounds were metabolized in a manner similar to carcinogenic aromatic amines. ^{32}P-postlabeling analyses of liver DNA indicated the formation of a DNA adduct and the concentration of this DNA adduct increased with increasing doses of both compounds. Although DNA adduct formation by malachite and leucomalachite green occurs in vivo, attempts to produce the adduct in vitro have not been successful. Moreover, malachite and leucomalachite green are poor in vitro mutagens [9].

In order to make a more complete evaluation of the mutagenicity of leucomalachite green, we have conducted in vivo mutation assays using the *lacI* transgene, in conjunction with DNA adduct analyses, in the female Big Blue® rat. In Big Blue® rats, the *lacI* gene is integrated into the genome of every cell of the animal and can be retrieved as a reporter gene for mutational analyses [10]. The *lacI* assay has the major advantage of being able to measure mutations (base pair substitutions, frameshifts, and small deletions) in any tissue from which DNA can be isolated. Also, mutant induction in a neutral reporter gene, like *lacI*, can accumulate with time during chronic exposure and, thus, be a sensitive indicator of genotoxic damage [11]. In parallel with these studies, we have been conducting a 2-year tumorigenesis bioassay in female and male F344 rats fed similar doses of leucomalachite green. Preliminary observations from the bioassay are reported here.

2. Materials and methods

2.1. Chemicals

Leucomalachite green (CAS registry number 129-73-7; 98% pure) was purchased from Chemsyn, Lenexa, KS. Impurities detected by atmospheric pressure chemical ionization/mass spectrometry were monodesmethyl leucomalachite green and malachite green. (±)-*Anti*-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide was also purchased from Chemsyn.

2.2. Big Blue® rat short-term feeding study

Female Big Blue® rats (Taconic Laboratories, Germantown, NY; homozygous for *lacI* gene; approximately 6-weeks old, six per dose group per time point) were fed 0, 9, 27, 91, 272, and 543 ppm leucomalachite green for 4, 16, and 32 weeks. Body weights and feed consumption were measured weekly. At the end of the feeding period, the rats were euthanized by exposure to carbon dioxide, and the livers were removed and frozen at −80 °C for use in the *lacI* mutational analyses. An additional 24 female Big Blue® rats (approximately 6-weeks old, four per dose group) were fed the same doses as described above for 4 weeks, and the livers were removed and frozen for DNA adduct analyses.

2.3. The lacI mutant assay

DNA was extracted from the livers, packaged into λ vectors, and plated to assay *lacI* mutant plaques

as described in Manjanatha et al. [12]. The analyses were performed in a "blocked" manner so as to minimize bias from day-to-day variations in the procedure. The plates were incubated overnight at 37 °C and scored for mutant blue plaques. Positive plaques were confirmed by replating. Color control mutants were included to insure the sensitivity of the assays. The packaging and replating were repeated until 33,000–400,000 plaques were scored for each sample. The number of sectored plaques recovered from both control and leucomalachite green-treated groups was extremely low and, therefore, the frequency was not determined (data not shown). Since an increased mutant frequency was not observed in the 91 or 272 ppm dose groups, the lower dose groups were not analyzed.

2.4. ^{32}P-Postlabeling assay

DNA was isolated from the liver by the method reported in Culp and Beland [13]. Approximately 10 μg of DNA was ^{32}P-postlabeled using *n*-butanol enrichment [14]. Adducts were separated by thin layer chromatography performed on 0.1 mm Machery Nagel 300 polyethylene imine cellulose plates (Alltech, Deerfield, IL) using the following solvent directions, D1: 0.9 M sodium phosphate, pH 6.8; D2: 3.6 M lithium formate, 8.5 M urea, pH 3.5; D3: 1.2 M lithium chloride, 0.5 M Tris–HCl, 8 M urea, pH 8.0. A final wash was conducted in D3 with the solvent used in D1. DNA adducts were visualized using a Storm 860 phosphor imaging system (Molecular Dynamics, Sunnyvale, CA). The adduct levels were quantified by comparison to a 10β-(deoxyguanosin-N^2-yl)-7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene standard, obtained by reacting DNA with (±)-*anti*-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide [13].

2.5. Two-year tumorigenicity bioassay

2.5.1. Animal treatments

Female and male rats (F344/N Nctr BR, obtained from the breeding colony at the National Center for Toxicological Research (NCTR); 48 per dose group, per sex) were fed 0, 91, 272, or 543 ppm leucomalachite green for 2 years. The rats were approximately 5 weeks of age at the beginning of treatment and were housed two per cage in polycarbonate cages with micro-isolator bonnets and hardwood chip bedding. Treatment diets and Millipore-filtered tap water were available ad libitum throughout the study. The animal rooms were maintained on a 12 h light–dark cycle.

2.5.2. Necropsy and histopathology

All rodents, including those that died during the experiment, were examined grossly at necropsy. A full histopathological examination was conducted on all animals. The tissues were fixed in 10% neutral buffered formalin, processed, embedded, sectioned, and stained with hematoxylin and eosin for microscopic examination.

2.6. Statistical analysis

The *lacI* mutant frequencies, as a function of dose and time after treatment, were analyzed by a two-way analysis of variance that included the fixed effects of the time after treatment, the dose, and the dose–time interactions. Contrasts were constructed to make the comparisons of interest as well as the effects of each treatment with the control, and the *P* values were adjusted by Holm's modification [15] of the Bonferroni procedure to correct for multiple comparisons. Since the S.D. of the *lacI* mutant frequencies tended to increase with the magnitude of the response, a logarithmic or square root transformation was performed before conducting the analyses.

DNA adduct levels were analyzed by one-way analysis of variance and Dunnett's test. Dose-related trends were analyzed by linear regression analysis. Statistical analysis of non-neoplastic liver lesions was conducted using χ^2 test with Bonferroni corrections applied. *P* values <0.05 were considered significant.

3. Results

3.1. The lacI mutant frequency in liver

Liver *lacI* mutant frequencies were measured in female Big Blue® rats fed leucomalachite green in the diet for up to 32 weeks (Tables 1 and 2; Fig. 1). The spontaneous mutant frequency in control rats was $(18 \pm 3.3$ to $26 \pm 4.3) \times 10^{-6}$. For treated rats, mutant frequencies were at most 2.9-fold greater than

Table 1
Raw data for *lacI* liver mutant frequency (MF) in female Big Blue® rats following exposure to leucomalachite green (LMG) in diet for up to 32 weeks

Week	LMG (ppm)	Animal ID[a]	No. of packaging reactions	PFU screened	Mutant plaques	MF ($\times 10^{-6}$)
4	0	A	4	220,000	7	32
	0	B	5	300,000	7	23
	0	C	3	180,000	1	06
	0	D	4	245,000	7	29
	0	E	4	240,000	4	17
	0	F	5	265,000	5	19
	272	A	1	33,000	1	30
	272	B	2	50,000	1	20
	272	C	1	48,000	1	21
	272	D	2	65,000	1	15
	272	E	2	100,000	1	10
	272	F	1	49,000	1	20
	543	A	4	180,000	2	11
	543	B	4	160,000	0	0
	543	C	4	198,000	5	25
	543	D	3	155,000	2	13
	543	E	5	210,000	9	43
	543	F	4	195,000	5	26
16	0	A	4	266,000	3	1
	0	B	4	267,000	6	22
	0	C	4	292,000	6	21
	0	D	4	217,000	1	5
	0	E	4	295,000	8	27
	0	F	3	253,000	5	20
	91	A	4	240,000	2	8
	91	B	4	210,000	2	10
	91	C	4	200,000	5	25
	91	D	4	200,000	0	0
	91	E	4	200,000	2	10
	91	F	4	275,000	3	11
	272	A	4	285,000	5	18
	272	B	4	235,000	4	17
	272	C	4	242,000	4	17
	272	D	4	235,000	2	9
	272	E	4	255,000	12	47
	272	F	4	245,000	6	25
	543	A	4	226,000	30	133
	543	B	4	238,000	10	42
	543	C	4	260,000	15	58
	543	D	4	243,000	7	29
	543	E	4	222,000	6	27
	543	F	4	204,000	6	29
32	0	A	10	267,000	10	37
	0	B	11	224,000	7	31
	0	C	10	250,000	7	28
	0	D	12	330,000	2	6
	0	E	10	330,000	9	27
	0	F	11	345,000	9	26
	272	A	5	225,000	7	31
	272	B	4	155,000	6	39
	272	C	5	240,000	2	8

Table 1 (*Continued*)

Week	LMG (ppm)	Animal ID[a]	No. of packaging reactions	PFU screened	Mutant plaques	MF ($\times 10^{-6}$)
	272	D	4	183,000	4	22
	272	E	5	255,000	9	35
	272	F	5	255,000	5	20
	543	A	13	185,000	4	22
	543	B	11	400,000	28	70
	543	C	11	285,000	6	22
	543	D	10	310,000	13	42
	543	E	12	165,000	6	36
	543	F	10	350,000	13	37

[a] Six animals (A–F) were used for control and each dose of LMG. The total number of λ packaging reactions used per animal as well as the total number of plaque forming units (PFU) screened per animal are shown. The number of sectored plaques observed is neither listed nor included in the calculation of *lacI* MF.

concurrent control frequencies, $(53 \pm 17) \times 10^{-6}$ at 16 weeks exposure to 543 ppm leucomalachite green versus $(18 \pm 3.3) \times 10^{-6}$ for control rats. This was also the only time–dose combination for which the increase in mutant frequency was significant ($P < 0.05$).

3.2. ^{32}P-Postlabeling DNA analysis

DNA was isolated from the liver of female Big Blue® rats fed leucomalachite green for 4 weeks and DNA adducts were analyzed using the ^{32}P-postlabeling assay. A single adduct (or co-eluting adducts) was observed in the 91, 272, and 543 ppm dose groups (Fig. 2). This adduct co-eluted with an adduct observed from the liver of male F344 rats fed up to 580 ppm leucomalachite green, as well as from male and female F344 rats fed up to 600 ppm malachite green for 4 weeks [8]. No discernable adduct was apparent in the 0, 9, or 27 ppm dose groups (data not shown). DNA adduct levels were significantly higher in the 91, 272, and 543 ppm dose groups of leucomalachite green as compared to rats fed control diet and there was an overall increasing linear dose trend (Fig. 3; $r^2 = 0.91$; $P < 0.001$).

Table 2
Average *lacI* mutation frequency (MF) in the liver of female Big Blue® rats exposed to leucomalachite green (LMG) in diet for up to 32 weeks

Week	LMG (ppm)	*lacI* MF (mean ± S.E.M. ($\times 10^{-6}$))	*lacI* MF (fold-increase)	*lacI* induced MF ($\times 10^{-6}$)[a]
4	0	21 ± 3.8	–	–
	272	19 ± 2.7	–	0
	543	20 ± 6.1	–	0
16	0	18 ± 3.3	–	–
	91	11 ± 3.3	–	0
	272	22 ± 5.4	1.2	4
	543	53 ± 17*	2.9	35
32	0	26 ± 4.3	–	–
	272	26 ± 4.6	1	0
	543	38 ± 7.2	1.5	12

[a] If treatment with LMG produced a MF equal to or less than the age-matched control frequency, the induced *lacI* MF for that treatment is considered 0.

* Significantly different from control ($P < 0.05$).

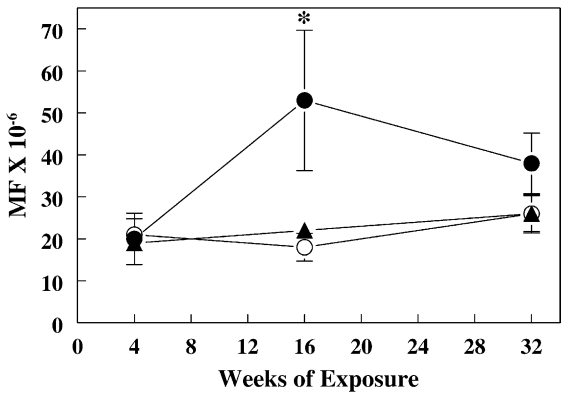

Fig. 1. Average *lacI* mutant frequencies (MFs) measured for up to 32 weeks in the liver of Big Blue® rats treated with 0 (○), 272 (▲), and 543 (●) ppm leucomalachite green. Each point represents the mean ± S.E.M. of six rats. *Significantly different from control ($P < 0.05$).

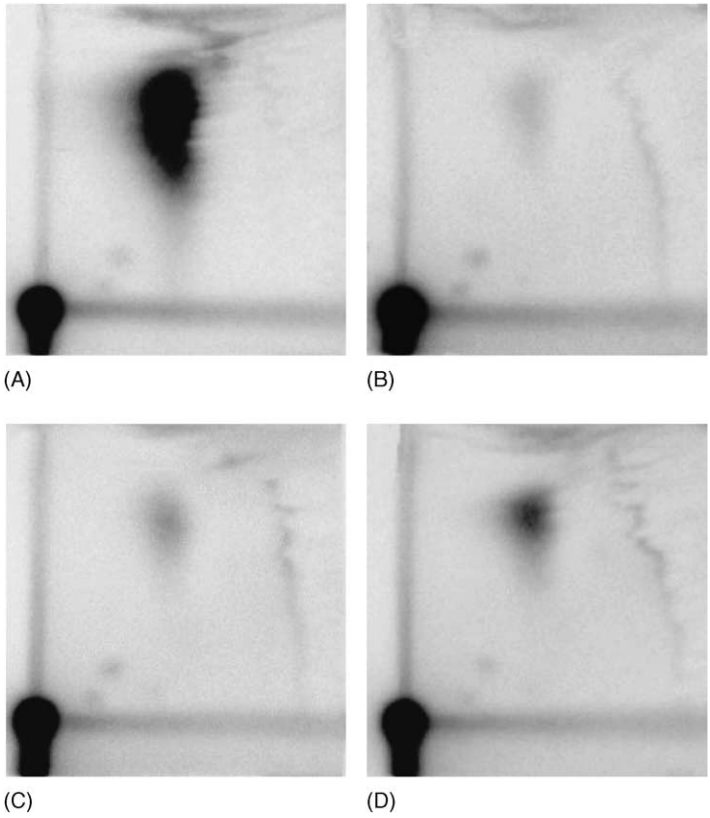

Fig. 2. ^{32}P-Postlabeling images from liver DNA of (A) a male F344 rat fed 580 ppm leucomalachite green for 4 weeks; and female Big Blue® rats fed (B) 91 ppm; (C) 272 ppm; and (D) 543 ppm leucomalachite green for 4 weeks.

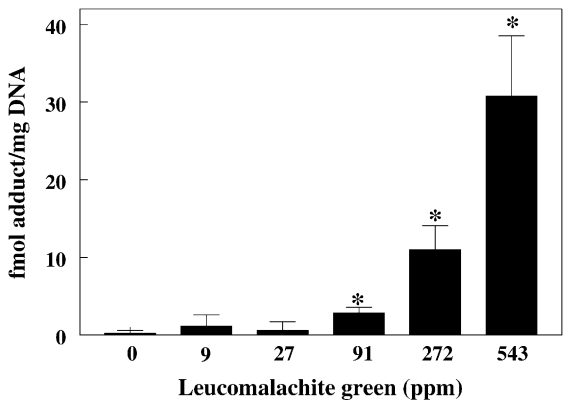

Fig. 3. DNA adduct levels in the livers of female Big Blue® rats fed 0, 9, 27, 91, 272, or 543 ppm leucomalachite green for 4 weeks. Each bar represents the mean ± S.D. of three or four rats. *Significantly different from control ($P < 0.05$).

3.3. Carcinogenicity bioassay of female and male rats fed leucomalachite green for 2 years.

Survival of female rats fed leucomalachite green to the terminal sacrifice at 104 weeks was 69–75% and was not affected by the concentration of leucomalachite green. In male rats, the survival was 48–71% at 104 weeks and was not adversely affected by the concentration of leucomalachite green.

Preliminary pathological examination revealed a linear increase with dose in the incidence of alveolar/bronchiolar adenomas in the lungs of male rats ($P = 0.041$), but not female rats fed leucomalachite green. The incidence in each dose group was 1/48—control, 2/47—91 ppm, 5/48—272 ppm, and 6/47—543 ppm. A marginally significant increase ($P = 0.052$) occurred in alveolar/bronchiolar adenomas

Table 3
Incidence of morphological liver changes in male and female F344 rats fed leucomalachite green for 2 years

Morphological liver changes	Leucomalachite green (ppm)			
	0	91	272	543
Eosinophilic focus				
Males	3/48	14/47*	17/48*	31/47*
Females	4/48	19/48*	22/48*	15/48*
Cystic degeneration				
Males	3/48	17/47*	12/48*	16/47*
Females	3/48	2/48	5/48	3/48
Cytoplasmic vacuolization				
Males	8/48	19/47	8/48	11/47
Females	2/48	4/48	16/48*	19/48*

* Significantly different from control ($P < 0.05$).

in the male rats fed 543 ppm leucomalachite green as compared to the control group.

Non-neoplastic lesions related to leucomalachite green exposure were limited to the liver and consisted of eosinophilic foci, cystic degeneration, and cytoplasmic vacuolization of hepatocytes (Table 3). Eosinophilic foci were characterized by distinct, variably sized foci in which hepatocytes were larger than normal due to increased amounts of eosinophilic cytoplasm. For both males and females, there were significant increases in the incidence of eosinophilic foci in the liver for each dose group compared to the respective control groups. Cystic degeneration in the liver was characterized by a multilocular cystic lesion containing finely granular or flocculent eosinophilic material. The incidence of cystic degeneration in the liver of male rats fed leucomalachite green was significantly increased at each dose group compared to the control group. Hepatocellular cytoplasmic vacuolization was characterized by clear cytoplasmic vacuoles (probably intracellular lipid accumulation) that were of variable size. The incidence of hepatocellular cytoplasmic vacuolization was significantly greater in female rats fed 272 or 543 ppm leucomalachite green compared to the control group.

4. Discussion

In recent studies, we reported the adverse effects that were observed in mice and rats fed leucomalachite green and malachite green for 28 days [8]. In general, the effects were more extensive and severe in rodents exposed to leucomalachite green compared to malachite green. Moreover, both leucomalachite green and malachite green were sequentially *N*-demethylated to secondary and primary aromatic amines in the livers of treated rodents. ^{32}P-Postlabeling analyses of liver DNA from rats and mice exposed to either leucomalachite green or malachite green indicated the formation of a DNA adduct (or co-eluting adducts) that increased with increasing doses. Taken together, these data suggested that the *N*-demethylated metabolites of leucomalachite green and malachite green could undergo metabolic activation in a manner similar to that observed with carcinogenic aromatic amines, i.e. oxidation to metabolites that react with DNA either directly or after esterification [16]. However, the adduct has not been characterized, since existing in vitro mutagenicity assays and metabolic activation systems have been unsuccessful in activating leucomalachite green or malachite green to DNA-damaging species (Culp and Blankenship, unpublished results).

In order to characterize better the mutagenic and carcinogenic potential of leucomalachite green in the liver, we conducted in vivo gene mutation and DNA adduct analyses, concomitantly with a 2-year tumorigenicity bioassay. In the *lacI* gene mutation assay, female Big Blue® rats, a transgenic version of the same sex and strain of rat (F344) used in the 2-year bioassay, were fed 0, 9, 27, 91, 272, and 543 ppm leucomalachite green for 4, 16, and 32 weeks. The three highest doses were the same doses of compound used in the 2-year bioassay and the feeding periods of 4, 16, and 32 weeks were selected based on previous experiments demonstrating the accumulation of gene mutations with time as a result of long-term feeding/treatment protocols [11,17–20]. An approximately three-fold increase in the *lacI* mutant frequency was found in the livers of female Big Blue® rats fed 543 ppm leucomalachite green for 16 weeks compared to the control group; but significant differences were not observed for the other dose or time points analyzed, including the 32-week time point (Tables 1 and 2; Fig. 1). Since *lacI* mutations are not expected to decline upon continued dosing for a long period of time after reaching their maxima, the biological significance of the 16-week spike is not clear [21,22]. The *lacI* assay measures mutations from base pair

substitutions, frameshifts, and small deletions. We are currently evaluating the 16-week mutants observed in this study to determine the nature of the DNA sequence alterations in both control and treated rats. Early indications are that some of these mutants are clonal in origin and involve G:C → A:T transition mutations that are not consistent with mutations induced from bulky arylamine adducts. These preliminary data suggest that an increase in 16-week mutant frequency may be a proliferative response of background mutations as opposed to a chemical effect from the administration of leucomalachite green.

A significant dose-related increase was observed in liver DNA adduct levels in female Big Blue® rats fed 91, 272, and 543 ppm leucomalachite green (Fig. 3). The adduct co-eluted with that observed from the livers of male F344 rats fed up to 580 ppm leucomalachite green for 4 weeks in our earlier study [8]. However, we observed a difference between the transgenic Big Blue® rats and F344 rats in response to liver DNA adduct formation from leucomalachite green administration. In the earlier study using similar doses of leucomalachite green, male F344 rats had seven to nine times higher adduct levels than measured in the female Big Blue® rats in this study. The apparent inconsistency between the DNA adduct levels may be due to the difference in sex or to other genetic factors. Manjanatha et al. [21] observed differences in *Hprt* mutation frequencies between NCTR F344 rats and transgenic Big Blue® rats treated with 7,12-dimethylbenz[*a*]anthracene. They suggested a genetic drift between the F344 lines from Taconic Farms (colony established from NIH stock in 1984) and NCTR (colony derived from NIH stock in 1979) over the last 20 years. Although the liver DNA adduct observed from leucomalachite green treatment has not been identified, our data are consistent with an arylamine adduct bound to the C8 position of guanine. The adduct was observed using *n*-butanol enrichment (Fig. 3), but not the nuclease P1 enhancement (data not shown) variation of the ^{32}P-postlabeling assay. Gupta and Early [23] showed that recovery of dG-C8–arylamine adducts can be optimized using *n*-butanol extraction, while the adducts were almost completely lost by nuclease P1 treatment.

Fernandes et al. [5] have reported that malachite green enhanced the development of hepatic preneoplastic lesions in male Wistar rats induced by diethylnitrosamine and was comparable to phenobarbital in increasing γ-glutamyl-transpeptidase-positive foci. In our 2-year carcinogenicity bioassay of F344 rats fed leucomalachite green, only non-neoplastic lesions (eosinophilic foci, cystic degeneration, and cytoplasmic vacuolization of hepatocytes) were observed in the livers (Table 3). It is possible that while we detected DNA adducts in the livers of rats treated with leucomalachite green, the levels were not sufficiently high to initiate hepatotumorigenesis. Our data do not rule out the possibility that leucomalachite green could be a liver tumor promoter in the presence of an appropriate initiating event.

A suggested carcinogenic response observed in the 2-year feeding bioassay was a linear increase with dose in alveolar/bronchiolar lung adenomas in male F344 rats fed leucomalachite green. In contrast, preliminary results from another 2-year feeding bioassay indicate leucomalachite green induces hepatocarcinogenicity in female B6C3F$_1$ mice (Culp et al., unpublished data). As such, further studies are planned to examine and compare the changes in *lacI* mutant frequency in female Big Blue® mice administered leucomalachite green.

In summary, we have reported preliminary results from a 2-year carcinogenicity bioassay indicating that leucomalachite green increases the incidence of lung adenomas in male F344 rats, but is not hepatocarcinogenic in either male or female rats. These data are consistent with the weak response in *lacI* mutant frequencies in the liver of female Big Blue® rats fed similar doses of leucomalachite green for up to 32 weeks. The DNA adducts formed in the livers of rats fed leucomalachite green do not appear to have mutagenic or carcinogenic consequences.

Acknowledgements

We thank Stephanie Hale, Betty Spadoni, and Kathy Carroll for computer support; Michelle Vanlandingham for assistance with animal care services; James Carson, Andrew Matson, Paul Siitonen, Larry Rushing and Malinda Godwin for diet preparation and dose analyses; and Mary Boudreau, Donna Norton, and Kathy Blocker for assistance with animal sacrifice and pathology. This research was supported, in part, by Interagency Agreement #224-93-0001 between

the Food and Drug Administration/National Center for Toxicological Research and the National Institute for Environmental Health Sciences/National Toxicology Program.

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