NTP REPORT

ON THE

TOXICOLOGY STUDY OF SENNA
(CAS NO. 8013-11-4)

IN C57BL/6NTAC MICE

AND TOXICOLOGY AND CARCINOGENESIS STUDY OF SENNA

IN GENETICALLY MODIFIED C3B6.129F1/Tac-*Trp53*^{tm1Brd} N12 HAPLOINSUFFICIENT MICE

(FEED STUDIES)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

April 2012

NTP GMM 15

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National Institutes of Health
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U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

FOREWORD

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA). Established in 1978, the NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

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SUMMARY

Background
Senna is a plant product used in laxatives. We tested senna in a genetically modified mouse strain that shows tumor responses more rapidly than in standard mouse strains.

Methods
We gave groups of male or female heterozygous F1 $p53^{+/-}$ mice feed that contained senna plant material at concentrations of 100, 300, 1,000, 3,000, or 10,000 parts per million for 40 weeks. Tissues from 22 organs were examined for every animal.

Results
Epithelial hyperplasia of the large intestine was seen in all the mice receiving the top dose of 10,000 ppm (1%) senna in the feed. The incidences of cancer were not significantly increased in any of the tissues examined.

Conclusions
We conclude that senna caused epithelial hyperplasia of the large intestine in male and female F1 $p53^{+/-}$ mice. Senna exposure for 40 weeks did not cause any cancers in this mouse strain.

ABSTRACT

SENNA

CAS No. 8013-11-4

[Senna drawing obtained from Samuelsson (1999)]

Synonyms: Alexandrian senna; Khatoum senna; Tinnevelly senna
Botanical names: *Senna alexandrina* P. Mill.; *Cassia senna* L.; *Cassia acutifolia* Del.; *Cassia angustifolia* Vahl
Trade names: Ex-Lax®, Fletcher's Castoria®, Senexon®, Senna-Gen®, Senna Soft®, Senokot®

Senna is used as a stimulant laxative in the management of constipation resulting from opioid use or when treatment with bulking or osmotic agents has failed. Increased use of senna was expected due to the removal of the stimulant laxatives danthron and phenolphthalein from the market. Senna was nominated for study by the Center for Drug Evaluation and Research, United States Food and Drug Administration (FDA) due to the wide use of laxative preparations, positive genotoxicity *in vitro* for some senna components or metabolites, and unknown carcinogenic potential. Because a 2-year rat study was ongoing by the manufacturer, the FDA requested that the NTP conduct a senna study in the p53$^{+/-}$ mouse. In this study, the potential for carcinogenic effects of senna was studied in the C3B6.129F1/Tac-*Trp53*^{tm1Brd} N12 haploinsufficient (heterozygous F1 p53$^{+/-}$) mouse model as an ongoing goal of the NTP to develop and test model systems for toxicology and carcinogenesis studies, especially those that can provide mechanistic information relative to understanding an agent's mode of action. C57BL/6NTac mice were exposed to senna in feed for

5 weeks; heterozygous F1 p53$^{+/-}$ mice were exposed to senna in feed for 40 weeks. Genetic toxicology studies were conducted in *Salmonella typhimurium*, *Escherichia coli*, and mouse peripheral blood erythrocytes.

5-Week Study in C57BL/6NTac Mice

Groups of five male and five female mice were exposed to 0, 625, 1,250, 2,500, 5,000, or 10,000 ppm senna (equivalent to average daily doses of approximately 115, 245, 490, 975, or 2,075 mg senna/kg body weight to males and 160, 310, 625, 1,190, or 2,570 mg/kg to females) in feed for 5 weeks. All mice survived to the end of the study. Mean body weights of exposed groups were similar to those of the controls. No differences in feed consumption were noted between exposed and control groups. Significantly increased incidences of epithelial hyperplasia of the cecum occurred in males exposed to 10,000 ppm and females exposed to 5,000 or 10,000 ppm; significantly increased incidences of epithelial hyperplasia of the colon occurred in males and females exposed to 5,000 or 10,000 ppm.

40-Week Study in Heterozygous F1 p53$^{+/-}$ Mice

Groups of 25 male and 25 female mice were exposed to 0, 100, 300, 1,000, 3,000, or 10,000 ppm senna (equivalent to average daily doses of approximately 12, 36, 120, 365, or 1,260 mg/kg to males and 14, 42, 140, 435, or 1,520 mg/kg to females) in feed for 40 weeks. Mean body weights of exposed male and female mice were within 10% of those of the controls throughout the study. Feed consumption by exposed mice was generally similar to that by the controls.

Significant increases in the incidences of epithelial hyperplasia of the colon and cecum occurred in 10,000 ppm males and females, and the incidence of epithelial hyperplasia of the colon was significantly increased in 3,000 ppm females.

Genetic Toxicology

Four different samples of senna, including three samples of the same lot that was used in the 40-week study were tested for mutagenicity in bacterial test systems. In two samples, no evidence of mutagenicity was seen in several strains of *S. typhimurium* and *E. coli*, with or without exogenous metabolic activation. In the other two samples, mutagenic activity was seen in *S. typhimurium* strains TA98 and TA100, with variable requirements for exogenous metabolic activation.

In addition to senna, the NTP also tested rhein (a component of senna) for mutagenicity in *S. typhimurium* strains TA98 and TA100; dose-related increases in mutant colonies were seen with both strains in the presence of rat or hamster liver S9, at lower concentrations than were required for the positive responses seen with senna samples. Another component of senna, chrysophanic acid, was tested for mutagenicity in TA98, TA100, and TA1535; weak and inconsistent responses were seen in TA100 with rat and hamster liver S9. Sennosides A and B were also tested for mutagenicity in bacterial test systems; neither compound was mutagenic, with or without S9 metabolic activation.

In vivo, no increases in the frequencies of micronucleated erythrocytes were seen in male mice exposed for 40 weeks to senna via dosed feed. No significant changes in the percentage of reticulocytes among erythrocytes were observed in male mice, suggesting that exposure to senna did not induce bone marrow toxicity.

Conclusions

Under the conditions of this 40-week feed study, there was *no evidence of carcinogenic activity** of senna in male or female C3B6.129F1/Tac-*Trp53*^{tm1Brd} N12 haplo-insufficient mice exposed to 100, 300, 1,000, 3,000, or 10,000 ppm.

Senna induced epithelial hyperplasia of the large intestine (colon and cecum) in male and female mice.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 8. A summary of the Technical Reports Peer Review Panel comments and the public discussion on this Report appears on page 10.

Summary of the 40-Week Carcinogenesis and Genetic Toxicology Studies of Senna

	Male Heterozygous F1 p53$^{+/-}$ Mice	Female Heterozygous F1 p53$^{+/-}$ Mice
Concentrations in feed	0, 100, 300, 1,000, 3,000, or 10,000 ppm	0, 100, 300, 1,000, 3,000, or 10,000 ppm
Body weights	Exposed groups similar to the control group	Exposed groups similar to the control group
Survival rates	25/25, 24/25, 25/25, 25/25, 23/25, 24/25	23/25, 23/25, 23/25, 22/25, 22/25, 24/25
Nonneoplastic effects	Large intestine (cecum): epithelium, hyperplasia (0/25, 0/25, 0/25, 0/25, 0/23, 22/25) Large intestine (colon): epithelium, hyperplasia (0/25, 0/25, 0/25, 0/25, 3/24, 25/25)	Large intestine (cecum): epithelium, hyperplasia (0/25, 0/25, 0/25, 0/25, 0/25, 19/25) Large intestine (colon): epithelium, hyperplasia (0/25, 0/25, 0/25, 0/25, 7/25, 25/25)
Neoplastic effects	None	None
Level of evidence of carcinogenic activity	No evidence	No evidence
Genetic toxicology		
Bacterial gene mutations:		
Senna	Positive in sample one in *S. typhimurium* strain TA98 with S9 and weakly positive in TA100 with S9, negative in TA98 and TA100 without S9; negative in sample two in strains TA97, TA98, TA100, and TA1535 with and without S9; negative in sample three in TA98, TA100, and *E. coli* with and without S9; positive in sample four in strain TA98 with and without S9, and in strain TA100 with S9, negative in TA100 without S9 and in *E. coli* with and without S9.	
Rhein	Positive in TA98 and TA100 with S9, negative without S9	
Chrysophanic acid	Weakly positive in TA100 with S9, negative without S9; negative in TA98 and TA1535 with and without S9	
Sennosides A and B	Negative in TA98, TA100, and *E coli.* with and without S9	
Micronucleated erythrocytes		
Mouse peripheral blood *in vivo*:	Negative	

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of evidence observed in each experiment: two categories for positive results **(clear evidence and some evidence)**; one category for uncertain findings **(equivocal evidence)**; one category for no observable effects **(no evidence)**; and one category for experiments that cannot be evaluated because of major flaws **(inadequate study)**. These categories of interpretative conclusions were first adopted in June 1983 and then revised on March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

For studies showing multiple chemical-related neoplastic effects that if considered individually would be assigned to different levels of evidence categories, the following convention has been adopted to convey completely the study results. In a study with clear evidence of carcinogenic activity at some tissue sites, other responses that alone might be deemed some evidence are indicated as “were also related” to chemical exposure. In studies with clear or some evidence of carcinogenic activity, other responses that alone might be termed equivocal evidence are indicated as “may have been” related to chemical exposure.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

NATIONAL TOXICOLOGY PROGRAM TECHNICAL REPORTS PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the draft NTP Report on senna on April 5, 2011, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, panel members have five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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SUMMARY OF PEER REVIEW PANEL COMMENTS

On April 5, 2011, the draft Report on the toxicology and carcinogenesis studies of senna received public review by the National Toxicology Program's Peer Review Panel. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. I. Surh, NIEHS, introduced the study of senna by describing how it is derived, the nomination of senna for study by the FDA, the selection of the p53 heterozygous mouse model, and the major components of senna. She also illustrated the metabolism of sennosides A and B, the main ingredients for laxative action in senna. The proposed conclusions were:

Under the conditions of this 40-week feed study, there was *no evidence of carcinogenic activity* of senna in male or female C3B6.129F1/Tac-*Trp53*^{tm1Brd} N12 haploinsufficient mice exposed to 100, 300, 1,000, 3,000, or 10,000 ppm. Senna induced epithelial hyperplasia of the large intestine (colon and cecum) in male and female mice.

Dr. Klaunig, the first primary reviewer, had no criticisms of the scientific performance of the study and felt that the report was well written. He was particularly pleased with the discussion of the rationale for the construction of the test mice and would have liked a clarification of the rationale for the 5-week study. Dr. Klaunig noted the mention of a reduction in heart weight in the 5-week study but not in the chronic study and wished to see some comment on that issue. He also wondered why the 5-week study had been conducted on wild-type mice and not the transgenics.

Dr. Rogers, the second primary reviewer, agreed with Dr. Klaunig about the quality of the study and draft report, but felt that the choice of the p53 haploinsufficient mouse model may have been incorrect. He recommended that in the future if a substance is to be tested that presents with a lower bowel phenotype, models that would be prone to developing inflammatory bowel disease or bowel cancer should be considered. He said the p53 haplo-insufficiency in the chosen model might have actually had a protective effect.

Dr. Barlow, the third primary reviewer, questioned whether the doses were set high enough in the studies to truly test the compound's carcinogenic potential. He also noted the mention in the report of reduction in heart weight in the 5-week study, but felt that since it was biologically meaningless, it should be removed from the report, rather than discussed further.

Dr. Miller expressed "a great deal of concern about the choice of the p53 model," in that it is known to be unsusceptible to lower GI tumors, and there are many other choices of models that would have been more susceptible to such tumors. Thus, he said, a mouse was selected for a study of colon cancer that was unlikely to get colon cancer, constituting what he characterized as a "fatal flaw" in terms of valid conclusions.

Dr. J.R. Bucher, NIEHS, replied that the p53 model is one of the most evaluated of mouse models, and is accepted by the FDA for genotoxicity and carcin-ogenicity studies. He cited studies involving another laxative using that model as a precedent for the choice of models in the senna studies. Also, he said, the p53 model was not chosen based on a presumption of colon cancer.

Dr. Miller asked if any positive controls with known carcinogens had been employed. Dr. J.E. French, NIEHS, responded that studies in p53 haploinsufficient mouse models had shown induction of colorectal tumors by azoxymethane, which has been described in publication. Other panel members expressed similar concerns about the p53 model and lack of positive controls.

Dr. Surh said the 5-week study had been conducted to set doses for the 40-week study. She added that it had been felt that the 10,000 ppm dose was a sufficient challenge in the mouse model used.

Dr. Klaunig moved and Dr. Barlow seconded that the conclusions be accepted as written. The motion was approved with five yes votes and two no votes. Dr. Miller voted against the motion, citing his concerns about the choice of mouse model. Dr. Heiger-Bernays also voted no, based on the appropriateness of the model, as well as feeling that the study should have lasted longer. Following the vote, Dr. Bucher added that NTP had had similar discussions about the mouse model, and had been fairly concerned about the epithelial hyperplasia and the appearance of the colon in the test animals. He asked for the panel's impression of whether further studies of senna would be in the best interest of the NTP.

Dr. Rogers endorsed further studies with animals with a pro-inflammatory bowel phenotype. Dr. Klaunig men-tioned that there had been a 2-year rat study of senna, and asked if anyone was familiar with the results. Dr. Surh replied that the rat study had shown no increase in tumors.

INTRODUCTION

SENNA

CAS No. 8013-11-4

[Senna drawing obtained from Samuelsson (1999)]

Synonyms: Alexandrian senna; Khatoum senna; Tinnevelly senna
Botanical names: *Senna alexandrina* P. Mill.; *Cassia senna* L.; *Cassia acutifolia* Del.; *Cassia angustifolia* Vahl
Trade names: Ex-Lax®, Fletcher's Castoria®, Senexon®, Senna-Gen®, Senna Soft®, Senokot®

CHEMICAL AND PHYSICAL PROPERTIES

Senna is a pod (fruit) or leaf of *Senna alexandrina* P. Mill. (Leguminosae). The plant is a shrub usually 0.7 to 1 m tall, native to Africa, India, and Asia and cultivated in Sudan, China, India, and Pakistan (Blumenthal, 2000; Cupp, 2000). The plants have compound pinnate leaves with three to eight pairs of leaflets per leaf. Lanceolate and pale-green leaflets are 1.5 to 5 cm long and 5 to 15 mm wide. The pale-green pods turn greenish brown during maturity and dark brown after drying. The pods contain five to 10 obovate and green to pale brown seeds (Franz, 1993; WHO, 1999; Blumenthal, 2000; Cupp, 2000; Srivastava *et al.*, 2006).

Fruits and leaves of these plants contain dianthrone O-glycosides (sennoside A, sennoside A1, sennoside B, sennoside C, sennoside D, sennoside D1, sennidin-A-monoglucoside, sennidin-A1-monoglucoside, and sennidin-B-monoglucoside), dianthrones (sennidin A

and sennidin B), 1,8-dihydroxy anthraquinones (rhein, aloe-emodin, emodin, and chrysophanol), aloe-emodin diglucoside, rhein glucoside, naphthalene derivatives, flavonoids (kaempferol, isorhamnetin, quercetin diglucoside, kaempferol diglucoside, and isorhamnetin diglucoside), vicenin-2, β-sitosterol, salicylic acid, oxalate, resin, saponins, polyol, sugars, and polysaccharide hydrocolloids (Figure 1; Appendix D) (Lemli *et al*., 1981; Franz, 1993; WHO, 1999; Blumenthal, 2000; Terreaux *et al*., 2002).

Dianthrone *O*-glycosides are major proactive constituents and make up 1.3% to 3.9% (w/w) of senna pods or leaves (Grimminger and Witthohn, 1993; Shah *et al*., 2000). Sennosides A and B are stereoisomers. The structure of rhein is similar to danthron, the laxative withdrawn from the market due to possible carcinogenic activity in humans (FDA, 1999; NTP, 2004).

PRODUCTION, USE, AND HUMAN EXPOSURE

Pods and leaves are harvested three times after sowing and dried in shade (Patra *et al*., 2005). Commercial senna comes from Egypt, Sudan, and India (Blumenthal, 2000). From April 2004 through March 2005, India exported 719,570 kg of senna leaf and pod to the United States (ITC, 2006). From April through September of 2006, India exported 319,900 kg of senna leaf and pod to the United States (ITC, 2007).

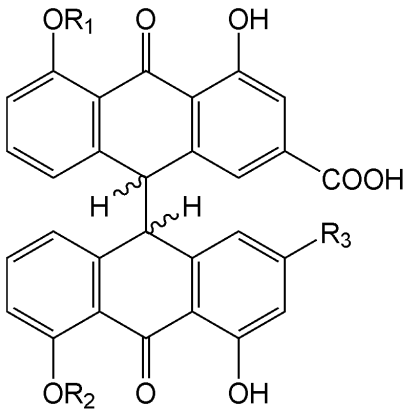
Senna is available in over-the-counter laxatives and is classified as a stimulant laxative (Leng-Peschlow, 1992; 21 CFR § 310.545; Rao, 2009). In the United States, senna is available as a tablet (sennosides), fluid extract, leaf, pod, oral solution, or tea (Cupp, 2000). Senna is used clinically for management of constipation resulting from opioid use or when treatment with bulking or osmotic agents has failed (Agra *et al*., 1998; Foxx-Orenstein *et al*., 2008; Bouras and Tangalos, 2009). In 1999, due to findings of carcinogenic activity in experimental animal models, the FDA reclassified the stimulant laxatives phenolphthalein and danthron as category II (21 CFR § 310.545) and declared them unsafe for use as over-the-counter laxatives (FDA, 1999). Therefore, it was expected that use of senna as a laxative would increase.

As a laxative, the adult daily dose of senna is 0.5 to 2 g, or 12 to 60 mg of sennosides as a single dose at bedtime. The recommended daily dose is 8.8 to 26.4 mg sennoside in children aged 6 to 12 years and 4.4 to 13.2 mg in children aged 2 to 6 years (*Martindale*, 1999; *PDR for Herbal Medicines*, 2007; Novartis, 2010).

In the United States, 2% to 28% of the population suffers from constipation (Higgins and Johanson, 2004). An estimated 7.95 million ambulatory care visits annually (0.18% of the total annual ambulatory care visits) during the period from 2001 to 2004 were for treatment of constipation with 4.6% of patients receiving prescriptions for stimulant laxatives (Shah *et al*., 2008). The estimated United States population using senna in 2002 was 361,000 (0.9%) (Kennedy, 2005). In the United Kingdom, senna-based products accounted for 23% of laxative use (Heaton and Cripps, 1993). A study of 1,012 patients in Seattle with functional bowel disorders found that 2.4% of those with irritable bowel syndrome and 8.2% of those with functional constipation used senna (van Tilburg *et al*., 2008). The prevalence of constipation in children is 0.7% to 29.6% (Van den Berg *et al*., 2006). In interviews with 107 caregivers of African-American children under 2 years of age, seen in a pediatric clinic, 4.7% reported using senna extract to treat colic (Smitherman *et al*., 2005). Women have approximately a twofold higher prevalence of constipation than men (Higgins and Johanson, 2004). During pregnancy, up to 40% of women have constipation (Cullen and O'Donoghue, 2007). In a study of 3,354 women in the Quebec Pregnancy Registry, 0.4% of pregnant women reported taking senna (Moussally *et al*., 2009). Adults over age 65 may have a higher risk of constipation (Higgins and Johanson, 2004); this population uses stimulant laxatives more often than bulk laxatives or stool softeners (Ruby *et al*., 2003). In a survey of 338 geriatric clinic patients aged 65 or older, 1.8% reported using senna tea (Cherniak *et al*., 2008). A high dose of senna (1 mg/kg, up to 158 mg, of sennosides) is used for cleansing the bowel in preparation for a colonoscopy or surgery (Kleibeuker *et al*., 1995; *Martindale*, 1999; Occhipinti and Di Palma, 2009). Senna's use as a laxative is also listed by the International Association for Hospice and Palliative Care as one of the essential medicines for palliative care (De Lima and Doyle, 2007). Extract of senna leaf is also listed in the European Commission Database (2010) as a skin conditioner.

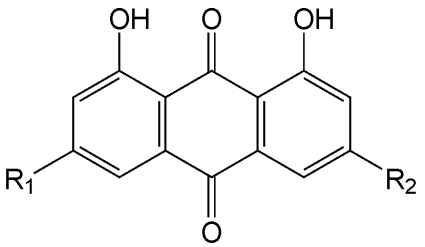
REGULATORY STATUS

Senna is considered a dietary supplement as specified by the Dietary Supplement Health and Education Act (DSHEA) of 1994 and the DSHEA places dietary supplements in a special category under the general umbrella of "foods" (FDA, 1994). The FDA lists Alexandrian senna (*Cassia acutifolia* Delile) as a natural flavoring substance for use in food (21 CFR § 172.510). As of November 1993, senna is listed as an over-the-counter category III (safety and effectiveness



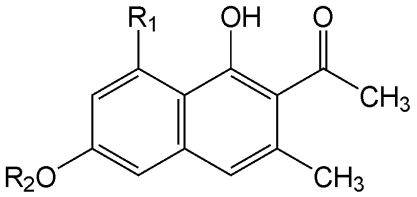
Dianthrones

	R_1	R_2	R_3
Sennosides A & B	Glu	Glu	COOH
Sennosides C & D	Glu	Glu	CH_2OH
Sennidins A & B	H	H	COOH



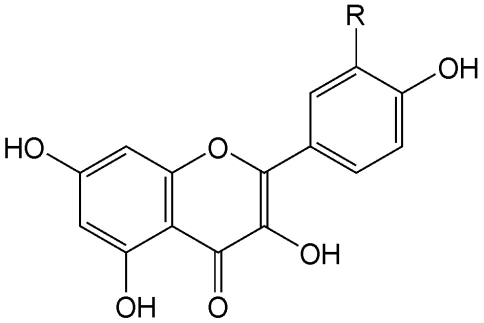
Anthraquinones

	R_1	R_2
Aloe-emodin	H	CH_2OH
Chrysophanol	H	CH_3
Emodin	CH_3	OH
Rhein	H	COOH



Naphthalene Derivatives

	R_1	R_2
6-Hydroxymusizin	CH_3	H
Tinnevellin	OH	H
Torachrysone	OH	CH_3



Flavonoids

	R
Isorhamnetin	OCH_3
Kaempferol	H
Quercetin	OH

FIGURE 1
Primary Structures of Selected Components of Senna
Some are present as glycosides (Franz, 1993; Appendix D). Glu=glucose

are not yet established) digestive aid (21 CFR § 310.545; 21 CFR Part 334) Senna is designated pregnancy-category C because no animal reproduction studies and no adequate and well-controlled reproduction studies in humans are available (Tillett *et al*., 2003; Prather, 2004).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

The pharmacological activity of senna is associated with sennosides A and B, the most abundant anthranoids and the precursors of the active metabolite, rhein anthrone (also known as rhein-9-anthrone) (Breimer and Baars, 1976; Sasaki *et al*., 1979; Lemli and Lemmens, 1980, Hietala *et al*., 1987; de Witte, 1993; Franz, 1993; Figure 2). The *in vivo* fate of the sennosides and the other senna anthranoids are described here. Sennosides are not readily absorbed from the mammalian gut; therefore, the activity depends on formation of rhein anthrone following deconjugation and reduction (Figure 2) by microflora in the large intestine (Breimer and Baars, 1976; Sasaki *et al*., 1979; Lemli and Lemmens, 1980; Dreessen *et al*., 1981). Lemli and Lemmens (1980) postulated that rhein anthrone arises through formation of a free radical following reduction of sennidin in the gastrointestinal tract. The systemic bioavailability of rhein anthrone is low, putatively due to limited absorption associated with binding to gut contents and rapid oxidation to rhein and sennidin once it is absorbed (Lemli and Lemmens, 1980; Grimminger and Leng-Peschlow, 1988; de Witte, 1993). Lemli and Lemmens (1980) recovered less than 4% of an oral dose Small amounts of free anthraquinones and their glycosides are present in senna (Franz, 1993; Newall *et al*., 1996; Figure 1; Appendix D), including rhein. Absorption of rhein from the rat gut appears to be greater than absorption of rhein anthrone (de Witte and Lemli, 1988). The cumulative urinary excretion of a single dose of ^{14}C-labeled rhein or ^{14}C-labeled rhein anthrone over 5 days was 37.1% and 2.8%, respectively. Rhein was primarily excreted as glucuronide and/or sulfate conjugates. Most of the rhein anthrone-derived ^{14}C excreted in urine was recovered as rhein following oxidative hydrolysis and extraction of the urine. In a separate study, rhein-derived ^{14}C was highest in the tissues of the gastrointestinal tract in gavaged rats (Lang, 1988). Other tissues contained low levels of radioactivity 7 days following dosing, probably due to protein binding in the blood. The total absorbed dose was estimated to be 50% and was excreted in urine, primarily as conjugates. In a study conducted by Dahms *et al.* (1997), specific metabolites, mostly glucuronide and sulfate conjugates, were identified in the urine of rats, rabbits, and dogs receiving oral doses of ^{14}C-labeled rhein. Qualitative and quantitative differences in rhein metabolism were observed between species. For instance, rabbit urine contained a glucuronide conjugate of a potentially reactive quinoid metabolite, not evident in the urine of the other species.

The kinetics of anthraquinones were investigated following oral administration of senna-containing products to rats (Mengs *et al*., 2004; Mitchell *et al*., 2006). Concentrations of rhein and aloe-emodin were determined in the blood of rats at various time points on days 90 and 91 of a 13-week gavage study (100 to 1,500 mg/kg) of powdered Tinnevelly senna fruit (Mengs *et al*., 2004). The concentrations in plasma were proportional to dose from 100 to 750 mg/kg, and were generally higher in females than in males. Chrysophanol was detected in some plasma samples. Blood was sampled at 6, 12, and 24 months in a 2-year oral carcinogenicity study in rats receiving powdered Tinnevelly senna fruit (25 to 300 mg/kg) (Mitchell *et al*., 2006). In the study, emodin and chrysophanol were generally not detected and aloe-emodin was only detected in the plasma of the high-dose group. Concentrations of rhein were higher in females at 6 and 12 months, but were similar to males at 24 months. In a radiolabeled study of aloe-emodin in gavaged rats, the ^{14}C was absorbed, distributed to all assayed tissues, and 30% of the total dose was excreted in urine as rhein, an unidentified metabolite, and their conjugates (Lang, 1993). The remainder of the dose was excreted in feces. The biotransformation of emodin and chrysophanol were investigated in induced liver microsomes from male and female rats (Mueller *et al*., 1998). Emodin was metabolized to omega-hydroxyemodin and 2-hydroxyemodin; whereas chrysophanol was metabolized to emodin.

Humans

Kobashi *et al*. (1980) demonstrated that sennosides could be converted to rhein anthrone by specific cultured bacteria strains from the human intestine. Further, Hattori *et al*. (1993) demonstrated cleavage of the *O*-glucosyl bond of sennoside B, reduction of sennidin B, and accumulation of rhein anthrone in a coculture of two bacteria strains isolated from human feces.

Concentrations of rhein and aloe-emodin were determined over time in plasma of human volunteers receiving four daily therapeutic doses of either of two senna-containing laxatives (Krumbiegel and Schulz, 1993). No aloe-emodin was detected in any samples. Two peak concentrations of rhein were observed in plasma following each dose, one at 3 to 5 hours and the

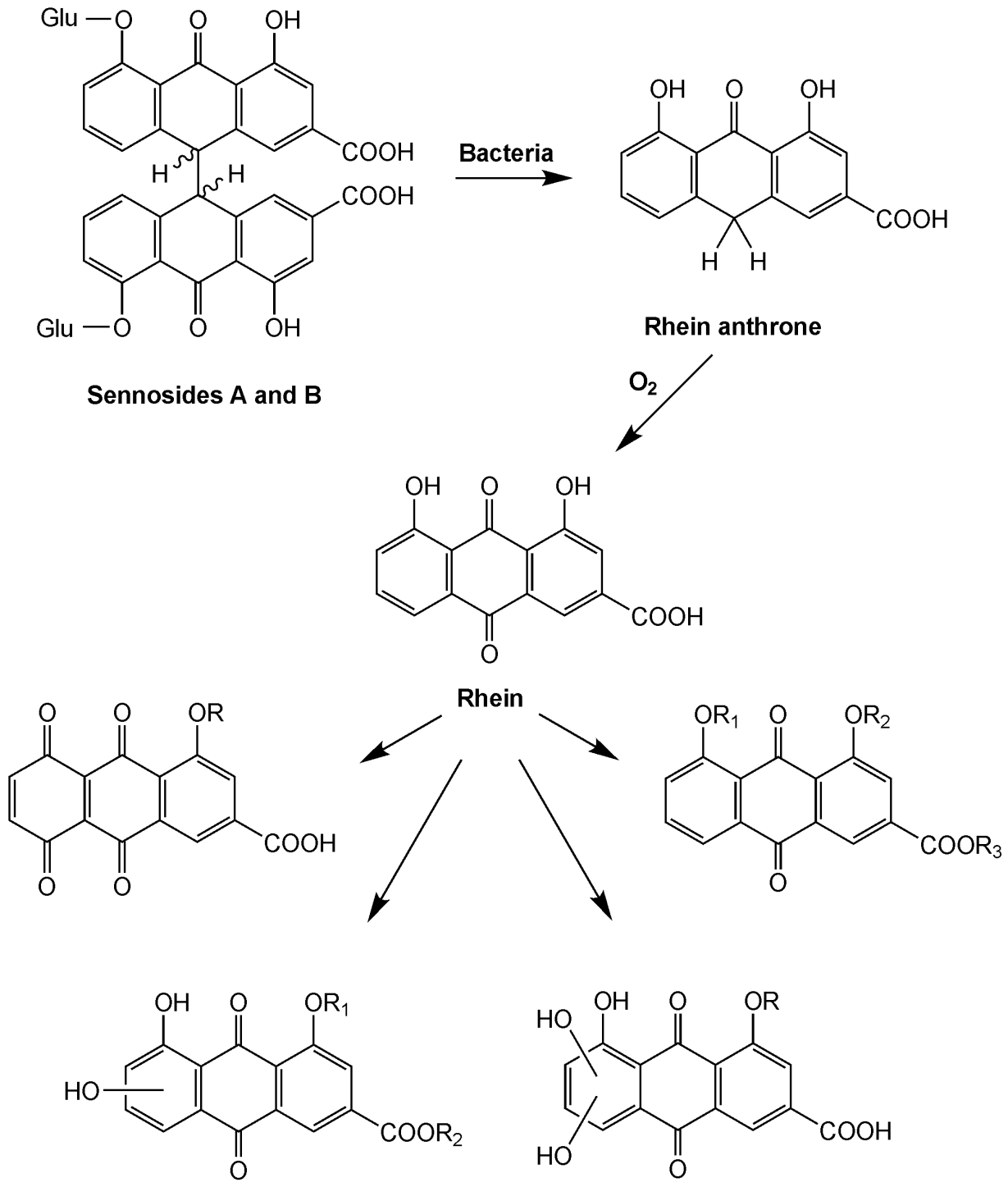


FIGURE 2
Metabolism of Sennosides A and B in Mammals
(Adapted from Lemli and Lemmens, 1980 and Dahms *et al*., 1997. R, R_1, and R_2 are H, glucuronic acid, or sulfate; Glu=glucose)

other at 10 to 11 hours. The authors postulated that the first peak arose from the presence of free or glycosylated rhein in the products and the second peak represented rhein derived from sennosides. In addition to work in animals, Dahms *et al*. (1997) investigated the metabolism of rhein in human volunteers receiving an oral dose of ^{14}C-labeled diacetylrhein. Diacetylrhein was converted to rhein by gut microflora. Some rhein-derived glucuronide and sulfate conjugates excreted in the urine of humans were common to the urine of rats, rabbits, and dogs receiving oral doses of ^{14}C rhein. However, potentially reactive metabolites (i.e., quinoids and diglucuronides) observed in some animals were not present in human urine, and the radioactivity in human serum was highly extractable over time indicating little potential for protein binding.

PHARMACOLOGY

The glucose in sennosides gives characteristics of a prodrug for laxative effect. Glucose makes the molecule hydrophilic. Therefore, absorption of sennosides is prevented before they reach the target organ (the large intestine). Sennosides precipitate at low pH levels in the stomach further inhibiting absorption (de Witte, 1993; van Gorkom *et al*., 1999). Due to their β-glycosidic bond, sennosides are not hydrolyzed in the stomach or in the small intestine. Normal microflora in the large intestine are important for the laxative effect of senna. For example, oral administration of sennoside A produced a laxative effect in conventional male and female C3H mice but not in germ-free male and female C3H mice (Dreessen *et al*., 1981). Sennosides A and B are metabolized by bacteria in the large intestine to rhein-9-anthrone, the active form for laxative effect (Dreessen *et al*., 1981; Grimminger and Leng-Peschlow, 1988). Sennidins A and B, rhein-9-anthrone, and small amounts of rhein were produced from sennosides A and B incubated with cecal extracts from conventional female Fischer rats, whereas incubation with cecal extracts from germ-free female Fischer rats produced no metabolites (Dreessen *et al*., 1981). Further, incubation of sennidins A and B with cecal extracts from conventional female Fischer rats produced rhein-9-anthrone and small amounts of rhein; however, no rhein-9-anthrone was detected upon incubation of sennidins with cecal extracts from germ-free female Fischer rats. Rhein-9-anthrone was relatively stable in the rat large intestine in the presence of bacteria but oxidized readily in germ-free rats and in buffers (Dreessen *et al*., 1981; Grimminger and Leng-Peschlow, 1988). In female albino mice, the ED_{50}s of intracecal administration of rhein anthrone, rhein, and aloe-emodin were 11.4, 91.0, and 246.3 µmol/kg, respectively (Yagi and Yamauchi, 1999). Aloe-emodin anthrone has a weaker laxative effect than rhein anthrone. However, intracecal administration of an equimolar mixture of aloe-emodin anthrone and rhein anthrone produced a synergistic laxative effect in female albino mice (Yagi *et al*., 1997).

A laxative effect is driven by increasing peristalsis and reduced absorption of water and electrolytes (Leng-Peschlow, 1986). In female Wistar rats, oral administration of 50 mg/kg sennosides reduced large intestinal transit time. Intracecal administration of equimolar doses of sennosides A+B, sennidins A+B, and rhein-9-anthrone produced similar responses in reduced large intestine transit time and increased soft feces (Leng-Peschlow, 1988). Application of rhein anthrone on mucosa of isolated guinea pig ileum dose-dependently increased parameters of peristaltic reflex (longitudinal muscle tension, intraluminal pressure, and volume displacement) (Nijs *et al*., 1993). In isolated large intestine from male Wistar rats, application of rhein (1 mM) in the lumen increased contractility in the colon, the number of migrating contractions, and fluid flow (Rumsey *et al*., 1993). In humans, intraluminal introduction of senna, which was preincubated with feces or *Escherichia coli*, produced peristalsis within 1 hour. Intraluminal application of rhein anthrone also produced peristalsis within 1 hour (Hardcastle and Wilkins, 1970). Oral administration of sennosides in rats *in vivo*, as well as application of rhein on the mucosal side of isolated rat colon *in vitro*, decreased absorption of water and sodium; enhanced secretion of water, sodium, and potassium; and reduced Na^+, K^+-ATPase activity (Leng-Peschlow, 1986, 1989, 1993; Wanitschke and Karbach, 1988). In addition, in humans, perfusion with rhein reversed absorption of water and sodium into secretion in the jejunum and colon, increased chloride secretion in the jejunum, reduced chloride absorption in the colon, and enhanced potassium secretion in the jejunum and colon (Ewe, 1980).

TOXICITY

Experimental Animals

Senna

The LD_{50} of senna has not been reported in the literature. Administration of senna induced soft feces and diarrhea, increased water consumption, and reduced body weight gain in rats. Male Wistar rats fed a diet containing 10% senna for 3 or 6 weeks exhibited diarrhea, decreased food intake, and decreased body weight gain compared to controls (Al-Yahya *et al*., 2002). Oral administration of senna (750 mg/kg or greater) to Sprague-Dawley rats for 13 weeks increased

soft feces and water consumption in males and females and reduced body weight gain in males (Mengs *et al*., 2004).

Male Wistar rats fed a diet containing 10% senna for 3 or 6 weeks had an increased serum index of liver toxicity (alanine aminotransferase and aspartate aminotransferase) and increased urea in serum along with slight degenerative changes in the liver, kidney, and intestines but decreased calcium levels in serum and decreased white blood cell counts compared to controls (Al-Yahya *et al*., 2002).

Administration of up to 1,500 mg/kg senna for 13 weeks decreased sodium in urine and increased kidney weights in 1,500 mg/kg male and 750 and 1,500 mg/kg female Sprague-Dawley rats compared to controls (Mengs *et al*., 2004). Minimal to slight hyperplastic changes in the mucosa of the large intestine in rats receiving more than 100 mg/kg of senna and minimal to slight hyperplastic epithelium of the forestomach in rats receiving 1,500 mg/kg were observed. These hyperplastic changes were reversible. Hyperplastic changes were not observed in animals 8 weeks after the 13 weeks of senna administration ended. Several studies in the literature have examined the relationship between the use of senna and damage in the enteric nervous system of the colon. For example, a study by Smith (1968) observed damage to intestinal nerves of mice given senna syrup. However, other rodent studies failed to show damage in the enteric nervous system of the colon after ingestion of senna or sennosides (Dufour and Gendre, 1984; Rudolph and Mengs, 1988; Mengs *et al*., 2004; Mitchell *et al*., 2006).

Senna Extract

In mice, the estimated LD_{50} of senna extracts (calcium sennosides A+B, 20%) administered by gavage was greater than 2.5 g/kg (Marvola *et al*., 1981). Administration of senna extract also showed a laxative effect and reduced body weight gain in rats. Wistar rats administered 100 mg/kg of senna extract (50% sennoside B) by mouth for 13 weeks had reduced body weight gain and increased water content in feces compared to controls (Mascolo *et al*., 1999).

Sennosides

For sennosides administered by gavage, the estimated LD_{50} was greater than 5 g/kg for mice and was greater than 3.5 g/kg for rats (Marvola *et al*., 1981; Mengs, 1988). In rodents, administration of sennosides produced laxative effects and diarrhea, increased water consumption, and decreased body weight gain compared to controls. In male and female Wistar rats, administration of 25 mg/kg sennosides produced a laxative effect and administration of 100 mg/kg for 6 months induced diarrhea and decreased body weight gain by approximately 50% compared to controls. Single administration of sennosides (2 to 7.5 g/kg) to male and female Wistar rats produced diarrhea, sedation, hunched posture, piloerection, and death (Mengs, 1988). Male Sprague-Dawley rats fed a diet containing 0.2% sennosides for 56 days had diarrhea, reduced body weight gain, and decreased survival compared to controls (Mereto *et al*., 1996). In NMRI mice, oral administration of 9.35 mg/kg of sennosides induced a laxative effect and 2.5 g/kg of sennosides induced diarrhea (Dufour and Gendre, 1984; Mengs, 1988). A mild laxative effect was induced in male NMRI mice fed a diet containing 0.03% sennosides (86% sennosides) for 20 weeks (Siegers *et al*., 1993a).

Mild kidney effects of sennosides have been observed. In male and female Wistar rats treated with 2 to 20 mg/kg sennosides for 4 weeks, no changes in hematological, biochemical, or urinary parameters were observed (Mengs, 1988). However, in 20 mg/kg rats, mean kidney weights were higher than that of the control group and small sudanophilic globules within the convoluted tubules of the kidney were observed. In male Wistar rats administered 25 or 100 mg/kg sennosides for 6 months, no hematological or urinary changes were observed, but increased kidney weights as well as dose-related basophilia of convoluted renal tubules were observed.

In male F344 rats fed sennoside A (0.006% to 0.05%) for 7 days, cell proliferation in the colorectum was increased and inflammatory changes in the large intestine were observed (Toyoda *et al*., 1994). However, in female Wistar rats, administration of 30 mg/kg sennosides for 12 weeks did not affect cell proliferation in the large intestine (Geboes *et al*., 1993). Administration of 50 mg/kg sennosides did not affect lactic acid dehydrogenase release into the colon lumen of female Wistar rats (Leng-Peschlow, 1993). In male Wistar rats, administration of sennosides (10 or 40 mg/kg) for 23 weeks did not affect the duration or frequency of the long-spike burst in the large intestine *in vivo* (Fioramonti *et al*., 1993).

Emodin

Toxicity studies of emodin in feed were conducted in male and female F344/N rats and B6C3F1 mice (NTP, 2001). Rats were administered up to 50,000 ppm emodin in feed for 16 days. Males and females exposed to 5,500 ppm or greater had reduced body weights compared to controls. Males and females exposed to 17,000 ppm or greater had reduced feed consumption and microscopic kidney lesions. Rats were administered up to 5,000 ppm emodin for 14 weeks. Males exposed to 2,500 ppm or greater and females

exposed to 1,250 ppm or greater had reduced body weights compared to controls. Mice were fed diets containing up to 50,000 ppm emodin for 16 days. All mice exposed to 50,000 ppm died before the end of the study. Males and females exposed to 17,000 ppm lost weight during the study. Females receiving 5,500 ppm had greater feed consumption than controls. Males and females exposed to 17,000 ppm had microscopic lesions in the gallbladder and kidney. Mice were fed diets containing up to 5,000 ppm emodin for 14 weeks. Males exposed to 2,500 ppm or greater had reduced body weights compared to controls. In males and females exposed to 1,250 ppm or greater, incidences and severities of nephropathy were increased.

Humans

Senna

Administration of senna can cause adverse effects such as abdominal cramps and diarrhea in humans (Langmeade and Rampton, 2001).

Three cases of hepatitis were associated with excessive use of senna (Beuers *et al*., 1991; Seybold *et al*., 2004; Vanderperren *et al*., 2005). In two cases, the causal relationship between senna and hepatitis was demonstrated by a change in serum liver test indexes after reexposure of the patients to senna (Beuers *et al*., 1991; Seybold *et al*., 2004).

Allergic reactions were induced by senna exposure. A 21-year-old man developed IgE-mediated asthma and rhinoconjunctivitis 5 months after he started handling senna at a hair-dye manufacturer (Helin and Mäkinen-Kiljunen, 1996). The patient's IgE was specific to protein in crude senna. The patient became asymptomatic upon changing jobs within the factory. At an Australian pharmaceutical manufacturer of laxatives, 15.3% of workers were sensitized to senna (Marks *et al*., 1991). Incidences of allergic symptoms (upper respiratory tract, eye, and skin) except asthma were higher in workers than in a reference population. In children wearing diapers, unintentional ingestion of senna-containing laxatives was related to severe diaper rash, blisters, and skin sloughing (Spiller *et al.,* 2003).

Sennosides

Stomachache was experienced in 20% of people who took sennosides (Steffen *et al*., 2006). Treatment with sennosides also caused changes in the colon in humans. Treatment with sennosides A and B (2 mg/kg, maximum 150 mg) substantially increased colonic epithelial cell proliferation compared to untreated controls (Kleibeuker *et al*., 1995; van Gorkom *et al*., 2000). At 6 hours after treatment with sennosides A and B (2 mg/kg, maximum 150 mg), more apoptotic bodies in the superficial lamina propria and more intense p53 staining in colonic epithelial crypt cell nuclei were observed in colon biopsies than in untreated controls (van Gorkom *et al*., 2001).

Like senna, sennosides caused skin irritation in chronic users. A 70-year-old woman who used sennosides for a year developed recurrent pruritic erythema; a patch test showed sennosides to be the causative agents (Sugita *et al.*, 2006). A 55-year-old woman who had taken sennosides for 20 years had suffered from pruritic scaly erythematous plaques for 2 years (Fujita *et al*., 2004). Withdrawal of sennosides improved her symptoms and similar lesions emerged upon reexposure to sennosides.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Experimental Animals

Sennosides

Detectable levels of rhein were found in milk after administration of sennosides to monkeys (Cameron *et al*., 1988). Female Wistar rats were administered diarrhea-inducing oral doses of sennosides (50 to 100 mg/kg) from gestation days 6 to 16; body weight gain was reduced but the numbers of implantation sites, resorption sites, fetuses, and fetal malformations and fetal weight were not affected (Mengs, 1986). White Russian rabbits were administered oral doses of 2, 10, or 20 mg/kg sennosides from gestation days 6 to 18; there was no effect on the numbers of resorption sites, fetuses, or fetal malformations or on fetal weight but body weight gain was decreased in the 20 mg/kg group compared to controls (Mengs, 1986). Female Wistar rats were administered a laxative dose of sennosides (20 mg/kg) from the last third of gestation to the fifth week postpartum; there were no effects on the weight gain in dams, gestation length, litter size, pup weight at birth, or pup weight or survival at 5 weeks (Mengs, 1986). Mengs (1986) also tested the effect of sennosides on fertility in rats. Male rats were treated with 2, 10, or 20 mg/kg sennosides for 10 weeks before mating and female rats were treated with 2, 10, or 20 mg/kg sennosides from 2 weeks before mating to the end of lactation. There were no effects on gestation length, the numbers of implantation or resorption sites, litter size, pup weight at birth, or pup weight or survival at 4 weeks. Pregnant ewes received intracolonic administration of sennosides (60 mg/kg) one to three times at intervals of 7 to 10 days between 75 and 125 days of pregnancy; a laxative effect and reduced uterine motility resulted, but there was no effect on the pregnancy or the fetus (Garcia-Villar, 1988).

Emodin
In female F344/N rats exposed to 1,250 or 5,000 ppm emodin for 14 weeks, significant increases in estrous cycle lengths were observed (NTP, 2001).

Developmental toxicity of emodin was evaluated in Sprague-Dawley rats and Swiss albino mice (Jahnke *et al*., 2004). In rats administered up to 1,700 ppm emodin in feed from gestation day 6 to gestation day 20, negative trends in maternal weight gain and feed consumption were observed. However, there were no effects on prenatal mortality, average live litter size, or incidences of fetal malformations or variations. In mice administered up to 6,000 ppm emodin in feed from gestation day 6 to gestation day 17, decreased maternal body weight gain and increased maternal water consumption were observed in the 6,000 ppm group. The only prenatal toxicity was reduced fetal body weight in the 6,000 ppm group. However, there were no effects on incidences of fetal malformations or variations in mice.

Humans

Senna
The FDA lists senna in pregnancy category C (safety in human pregnancy has not been determined) because there are no adequate reproduction studies available (Tillet *et al*., 2003; Prather, 2004). Senna is still used for constipation during pregnancy (Moussally *et al*., 2009). A study of the Hungarian Case Control Surveillance of Congenital Abnormalities database showed that treatment with senna during pregnancy did not increase the risk of congenital abnormalities, and increasing the length of senna exposure during pregnancy did not increase the risk of congenital abnormalities (Ács *et al*., 2009). Rhein, a metabolite of senna, has been excreted in milk of lactating mothers (Faber and Strenge-Hesse, 1988). However, there are no studies reported in the literature on any developmental effects of senna.

CARCINOGENICITY

Experimental Animals

Senna
Mitchell *et al*. (2006) studied the carcinogenicity of senna in male and female Sprague-Dawley rats. Rats were administered 25, 100, or 300 mg senna/kg body weight by gavage for 2 years (60 rats per sex per group). Body weights were slightly reduced in 300 mg/kg females. Feed consumption was not affected by senna administration. In 300 mg/kg groups, mucoid feces and darker urine were observed. During the first 65 weeks, increased water consumption was observed in the 300 mg/kg groups. There were changes in electrolytes in serum (increased potassium and chloride) and urine (decreased sodium, potassium, and chloride) in the 300 mg/kg groups. At the end of the study, complete histopathology examinations of control and high-dose groups and histopathology examinations of the intestinal tract, adrenal gland, liver, kidney, brain, and gross lesions in the low- and mid-dose groups were conducted. There were no treatment-related increases in the incidences of neoplasms. However, dose-dependent epithelial hyperplasia of the large intestine and pigment deposition in the kidney were observed.

Senna Extract
Administration of 30 or 60 mg/kg senna pod extract (50% sennoside B) for 110 weeks increased fecal water content, reduced weight gain, and produced no aberrant crypt foci or tumors in the colon of male Wistar rats (Borelli *et al*., 2005). Sprague-Dawley rats were administered 5, 15, or 25 mg/kg senna extract (35.7% sennosides) in drinking water for 2 years; compared to controls, a laxative effect was induced at 25 mg/kg, reduced body weight gains occurred at 25 mg/kg, and reduced water intake occurred at 15 and 25 mg/kg, but survival and neoplasm incidences were not affected (Lydén-Sokolowski *et al*., 1993).

The effect of senna extract on azoxymethane-treated rats is controversial. Mascolo *et al*. (1999) showed that while a laxative-producing dose of senna pod extract (10 mg/kg) administered to male Wistar rats for 13 to 28 weeks did not promote the formation of tumors induced by azoxymethane, a diarrhea-producing dose (100 mg/kg) administered for 13 weeks increased the incidence and multiplicity of tumors induced by azoxymethane. However, oral administration of 30 or 60 mg/kg senna pod extract for 2 years to male Wistar rats treated with the initiating agent azoxymethane (7.5 mg/kg, intraperitoneal) decreased the formation of aberrant crypt foci and colon tumors compared to animals that received azoxymethane alone (Borrelli *et al*., 2005).

Sennosides
A diet containing 0.03% sennoside (86% sennosides) fed to male NMRI mice for 20 weeks did not induce colorectal tumors; the same diet for 20 weeks with concurrent administration of dimethylhydrazine (20 mg/kg, subcutaneous) for 10 weeks did not promote formation of colorectal tumors (Siegers *et al*., 1993a).

The numbers and multiplicities of aberrant crypt foci were not affected in dimethylhydrazine-treated male Sprague-Dawley rats fed a 0.1% sennoside-containing diet; rats fed a diarrhea-inducing level of the sennoside-containing diet (0.2%) exhibited a significantly

increased number of crypts/foci compared to controls (Mereto *et al*., 1996).

Aloe-Emodin
Dermal application of aloe-emodin (10 µg/mL) before or after UVB (15 kJ/m^2) radiation on C3H/HeNCr (MTV$^-$) mice did not significantly change the total number of neoplasms or the latency of neoplasm development (Strickland *et al*., 2000).

Emodin
Male and female F344/N rats were exposed to up to 2,500 ppm emodin in feed for 2 years (NTP, 2001). Three of 50 females in the 2,500 ppm group had Zymbal's gland carcinomas. In these studies, male B6C3F1 mice were exposed to up to 625 ppm emodin and female mice were exposed to up to 1,250 ppm emodin for 2 years. The incidences of nephropathy were significantly increased in all exposed groups of females; the incidences of renal tubule pigmentation were significantly increased in all exposed groups and the severity of the lesion increased with increasing exposure concentration.

Humans
Studies in the literature suggest that there may be an association between laxative use and colon cancer in humans (Siegers *et al*., 1993b; Satia *et al*., 2009). For example, findings from an epidemiology study revealed that more patients with gastrointestinal cancer were senna users than patients without cancer and patients without gastrointestinal disease (Boyd and Doll, 1954). However, the relationship between senna use and colon cancer has not been clearly demonstrated.

GENETIC TOXICITY
The genetic toxicity of senna products was reviewed in detail by Brusick and Mengs (1997). These authors concluded that most studies of the genetic toxicity of senna products gave negative results, but results from some studies indicated that certain components of senna products, particularly emodin and aloe-emodin, are genotoxic. However, these authors suggested that an overall assessment of the genotoxicity profile of senna, its constituents, and its metabolites, in light of other data from animal and human metabolism or kinetic studies, human clinical trials, and rodent carcinogenicity studies, did not support an increased risk for genotoxicity of senna laxatives in humans when these products were consumed under prescribed-use conditions.

Senna Aqueous Extract
Senna extract (aqueous) did not induce *trp*$^+$ revertants in *Escherichia coli* (Silva *et al*., 2008). There are contradictory results in the literature concerning the ability of senna to induce mutations in *Salmonella typhimurium*. Al-Dakan *et al.* (1995) reported a negative response in TA98, whereas Heidemann *et al*. (1993) showed that senna extract induced *his*$^+$ revertants in TA98, with and without liver microsomes (S9), and in TA1537 in the absence of S9. Sandnes *et al*. (1992) reported that extracts of senna folium and senna fructus induced significant dose-related increases in mutations in *S. typhimurium* strains TA97a, TA98, TA100, and TA102 in the presence of S9; in the absence of S9, mutagenicity was observed in TA97a and TA102. The extract also induced structural chromosomal changes in cultured Chinese hamster ovary (CHO) cells in the presence and absence of S9 (Heidemann *et al*., 1993). In Chinese hamster lung V79 cells, senna did not induce mutations at the *hgprt* locus in the presence of S9; in its absence, the results were equivocal (Heidemann *et al*., 1993). *In vivo*, senna aqueous extract (2,000 mg/kg, single oral administration) did not induce micronuclei in bone marrow polychromatic erythrocytes of male or female NMRI mice sampled 24 and 48 hours after treatment (Mengs *et al*., 1999).

Sennosides A and B
The sennosides were generally negative in most genotoxicity tests reported. Sennosides (up to 5,000 µg/plate) did not induce *his*$^+$ revertants in *S. typhimurium* strains TA97, TA97a, TA98, TA100, TA1537, or TA1538, or in *E. coli,* with or without S9 (Mengs, 1988; Sandnes *et al*., 1992; Heidemann *et al*., 1993). However, a positive response was reported in *S. typhimurium* strain TA102, with and without S9 (Sandnes *et al*., 1992). No mutations were observed at the *tk* locus in mouse lymphoma cells treated with sennosides (up to 3,000 µg/plate), nor did these glycosides induce chromosomal aberrations in CHO cells exposed to up to 5,000 µg/mL without S9 or 4,000 µg/mL with S9 (Mengs, 1988; Heidemann *et al*., 1993).

In mice treated with sennosides at doses up to 2,500 mg/kg per day, there were no increases in the frequencies of micronucleated polychromatic erythrocytes and no effect on the ratio of polychromatic to normochromatic erythrocytes (Mengs, 1988, Heidemann *et al*., 1993). However, a slight elevation in structural chromosomal aberrations was observed in bone marrow cells of mice treated with sennoside B (Mukhopadhyay *et al*., 1998).

Aloe-emodin
Aloe-emodin was mutagenic in *S. typhimurium* strains TA97a, TA98, TA100, TA1537 and TA1538, but not in TA102, with and without S9 (Brown and Dietrich,

1979; Westendorf *et al*., 1990; Kawasaki *et al*., 1992; Sandnes *et al*., 1992; Heidemann *et al*., 1993; Nesslany *et al*., 2009). *In vitro*, aloe-emodin induced $tk^{-/-}$ mutants in mouse lymphoma L5178Y cells and human TK6 lymphoblastoid cells, DNA damage as measured by the comet assay, and micronuclei in mouse lymphoma L5178Y cells (Müller *et al*., 1996; Mueller *et al*., 1998; Nesslany *et al*., 2009). DNA damage, measured by the comet assay, was also reported in H460 human lung carcinoma cells exposed to aloe-emodin (Lee *et al*., 2006). Aloe-emodin induced unscheduled DNA synthesis in male Wistar rat hepatocytes as well as transformed foci in C3H/M2 mouse fibroblasts (Westendorf *et al*., 1990). The literature is contradictory concerning the ability of aloe-emodin to induce *hgprt* mutations in Chinese hamster lung V79 cells (Westendorf *et al*., 1990; Heidemann *et al*., 1993, 1996). Aloe-emodin induced chromosomal aberrations in CHO cells (Heidemann *et al*., 1993, 1996). In mice, aloe-emodin induced DNA damage in kidney and colon cells (Nesslany *et al*., 2009) but did not induce unscheduled DNA synthesis or chromosomal aberrations in Wistar rats nor micronuclei in polychromatic erythrocytes of male and female mice (Heidemann *et al*., 1993, 1996).

Chrysophanol

No increase in the frequency of cells with chromosomal aberrations was observed in cultured Chinese hamster ovary cells exposed to chrysophanol, with or without S9 (Mengs *et al*., 2001).

Rhein (1,8-dihydroxy-3-carboxyl anthraquinone)

Rhein was not found to be mutagenic in *S. typhimurium* strains TA97a, TA98, TA100, TA1535, or TA1538, with or without S9, or in TA1537 in the absence of S9. However, there are contradictory results with rhein in TA1537 in the presence of S9 and in TA102 with or without S9 (Westendorf *et al*., 1990; Sandnes *et al*., 1992; Heidemann *et al*., 1993; Makena and Chung, 2007). Rhein failed to induce $tk^{-/-}$ mutations in mouse L5178Y lymphoma cells, *hgprt* mutations in Chinese hamster lung V79 cells, chromosomal aberrations in CHO cells, unscheduled DNA synthesis in male Wistar rat hepatocytes, or transformed foci in C3H/M2 fibroblasts (Westendorf *et al*., 1990; Sandnes *et al*., 1992; Heidemann *et al*., 1993).

Rhein (1,500 mg/kg single oral administration) also failed to induce micronuclei (polychromatic erythrocytes) in bone marrow cells of male NMRI mice sampled 24, 48, or 72 hours after treatment (Heidemann *et al*., 1993; Mengs and Heidemann, 1993). However, a single oral administration of rhein (2 mg/kg) was reported to induce a small, dose-related increase in the frequency of chromosomally aberrant cells in bone marrow of male Swiss mice (Mukhopadhyay *et al*., 1998).

BACKGROUND ON THE GENETICALLY MODIFIED MOUSE (GMM) MODEL USED IN THE SENNA STUDY

The p53 tumor suppressor gene suppresses cancer in both humans and mice. The p53 protein is critical to cell cycle control, DNA repair and apoptosis, etc., and is often mutated or lost in human and rodent cancers. The haploinsufficient *Trp53* tumor suppressor gene mouse model heterozygous for wildtype and null (+/−) *Trp53* alleles (Donehower *et al.*, 1992, 1995) was used in these studies. In this model, a *Trp53* null mutation was introduced by homologous recombination in AB1 murine embryonic stem cells which were derived from a black agouti 129Sv inbred mouse. By targeted insertion of a *pol*II neo cassette, an engineered null mutation was induced as a result of the deletion of a 450-base pair gene fragment from the *Trp53* gene that included 106 nucleotides of exon 5 and approximately 350 nucleotides of intron 4 that eliminated both mRNA and p53 protein expression from this allele. This Trp53 protein haploinsufficient mouse model has been extensively tested as a short-term cancer bioassay mouse model (Tennant *et al*., 1995; Dunnick *et al*., 1997; French *et al*., 2001a,b; Pritchard *et al*., 2003; French, 2004) based upon the observation that mice with only a single wildtype *Trp*53 allele show a significant decrease in the time required for genotoxic carcinogen-induced tumors to develop. These tumors are often associated with either a mutation and/or a loss of heterozygosity of the remaining wildtype *Trp53* allele. Few to no sporadic tumors occur in concurrent or historical study control groups in this GMM model, which allows tests to be conducted with fewer animals and direct analysis of the target wildtype *Trp53* allele to test for genotoxicity *in vivo* as a mode of action.

For these studies, an outcross between C3H/HeNTac (C3) female mice homozygous for the wildtype (+) *Trp53* allele and the C57BL/6.129Sv-$Trp53^{tm1Brd}$ N12 congenic (abbreviated B6.129-$Trp53^{tm1Brd}$) N12 backcross generation males homozygous for the *Trp53* null (−) allele produced C3B6.129F1/Tac-$Trp53^{tm1Brd}$ N12 progeny heterozygous for a *Trp53* wildtype (+) and null allele (−) inbred mouse progeny [hereafter referred to in the abbreviated form as the heterozygous F1 $p53^{+/-}$ mouse, Taconic Laboratory Animals and Services (Germantown, NY)]. The heterozygous F1 $p53^{+/-}$ mouse was selected for the 40-week study of senna because the B6.129-$Trp53^{tm1Brd}$ (N5) haploinsufficient male and female mice (backcrossed to C57BL/6, subline

unspecified, for two generations and then to C57BL/6NTac females for an additional three generations) were not sufficiently inbred. The N5 generation of this line retained both C57BL/6 and 129Sv strain allele heterozygosity at both the *Trp53* locus and the flanking region on chromosome 11 and at unknown loci throughout the genome of this line. This residual heterozygosity in the B6.129-*Trp53*^{tm1Brd} N5 backcross generation mice was one covariate that may have been responsible for large variations in the *p*-cresidine induced urinary bladder tumors (0% to 80%, 10 of 11 studies were positive) in males, which was used as a positive control genotoxic carcinogen in the ILSI/HESI Alternatives to Carcinogenicity Testing initiative (Storer *et al*., 2001). Therefore, additional inbreeding to the N12 generation was anticipated to decrease the variance in tumor incidence and stabilize the penetrance of tumor phenotypes in NTP studies.

The majority of B6.129-*Trp53*^{tm1Brd} homozygous null females die *in utero* and only a few are born alive and most die early. Thus, the B6.129-*Trp53*^{tm1Brd} N12 line is maintained by intercross of the B6.129-*Trp53*^{tm1Brd} female heterozygote with the B6.129-*Trp53*^{tm1Brd} homozygous null male to produce a 1:2 population of homozygous null males and heterozygous null males and females. Therefore it is necessary to select the B6.129-*Trp53*^{tm1Brd} homozygous null male as the carrier of the null allele. However, the selection of the C3H/HeNTac female as the wildtype *Trp53* allele carrier provides 1) increased fecundity and maternal instincts, 2) increased hybrid vigor of an F1 outcross that increases the number of progeny, 3) the advantage of expanding the pattern of tumor susceptibility associated with this genetic background, and 4) a genetic background similar to the B6C3F1 mouse used in NTP studies (NTP, 2010). Together, these factors provided a rational basis for selection of this GMM test model. In addition, the NTP study to be reported on 3′-azido-3′-deoxythymidine (AZT) and 3′-azido-3′-deoxythymidine/lamivudine (AZT/3TC), also used the C3B6.129F1/Tac-*Trp53*^{tm1Brd} N12 haploinsufficient GMM model (NTP, 2012), and the background rate for spontaneous tumors in the control group C3B6.129F1-*Trp53*^{tm1Brd} haploinsufficient mice in all three studies (AZT, AZT/3TC, and senna) was not statistically different from the background rates for spontaneous tumors observed in control B6.129-*Trp53*^{tm1Brd} (N5) haploinsufficient mice used in previous NTP GMM studies (NTP, 2005a,b; 2007a,b,c,d,e; 2008).

STUDY RATIONALE

Senna was nominated for study by the Center for Drug Evaluation and Research, United States Food and Drug Administration (FDA) due to the wide use of laxative preparations, positive genotoxicity *in vitro* for some senna components or metabolites, and unknown carcinogenic potential. Increased use of senna was expected due to the removal of danthron and phenolphthalein from the market. Because the 2-year rat study was ongoing by the manufacturer (Mitchell *et al*., 2006), the FDA requested that the NTP conduct a senna study in the p53$^{+/-}$ mouse.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF SENNA

Senna was obtained from Madaus AG (Köln, Germany) in one lot (1999000). Identity and purity analyses were conducted by the analytical chemistry laboratory at Research Triangle Institute (Research Triangle Park, NC) and the study laboratory at Battelle Columbus Operations (Columbus, OH) (Appendix D). Reports on analyses performed in support of the senna studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a brown, powdered raw plant material, was identified as senna using two similar high-performance liquid chromatography with ultraviolet detection (HPLC/UV) analytical systems. The bulk test material was characterized as a multi-component natural product with a complex mixture of constituents that were confirmed by identifying as many components as possible in methanol:water (50:50) extracts of the raw material. Chromatographic comparisons were made to purchased reference standard solutions of sennosides A and B, sennidins A and B, aloe-emodin, emodin, rhein, aloe-emodin-8-glucoside, chrysophanic acid, and physcione in the same solvent. In a parallel study conducted by the analytical chemistry laboratory, nine additional reference standards that assisted in identifying constituents in lot 1999000 were isolated and identified in methanol:water (50:50) extracts of a lot of senna obtained from Frontier Natural Products Co-op (Norway, IA). Forty-seven components with a peak area of at least 0.1% of the total peak area were observed in lot 1999000, accounting for 99.6% of the total peak area for the test material extract. Of these 47, 18 were considered major components (≥ 1% each). By comparison to the chromatographic profiles of the purchased and isolated reference standards, peaks were confirmed in lot 1999000 for sennosides A and B, sennidins A and B, rhein, aloe-emodin, quercetin 3-gentiobioside, kaempferol 3-gentiobioside, torachrysone-8-O-ß-D-glucopyranoside, quercetin, and torachrysone. Weight percentages of four postulated active components in the test article were estimated to be: 0.7% sennoside A, 1.3% sennoside B, 0.06% sennidin A, and 0.03% sennidin B. The identities of the marker compounds sennoside A and sennoside B in lot 1999000 were confirmed using resolution standard solutions of these chemicals in methanol:water (50:50).

The water content of lot 1999000 was determined by weight loss on drying and by Karl Fischer titration. The bulk purity of the test material was determined using broad gradient HPLC/UV with monitoring at 210 and 270 nm. Broad screening assays for residual volatiles were conducted on dimethylsulfoxide extracts of the test material using gas chromatography (GC) with flame ionization detection. Nutritional testing, trace metals screening, nitrosamines assays, and pesticide screening were performed on the bulk material using standard methods.

For lot 1999000, moisture content was determined to be 6.0% (w/w) by weight loss on drying and 5.8% by weight by Karl Fischer titration. Broad gradient HPLC/UV analyses with monitoring at 210 or 270 nm each detected 13 major (≥1% of the total peak areas) components accounting for 91% of the total peak areas and 25 minor (<1%) components. GC for residual volatiles detected methanol and isopropanol, both at levels below their estimated limits of quantitation (0.1% and 0.09% by weight, respectively), and a single unknown. Organochlorine and organophophorus pesticide levels were below the detection limits of 200 and 50 ppb, respectively. Mercury, thallium, and cadmium levels were less than the limits of detection, which were respectively 25, 20, and 500 ppb. Lead and selenium were present at 299 and 690 ppb, respectively. Nitrosamines were not present above the detection limit of 1.0 ppb. Results of the nutritional and contaminant tests were deemed acceptable for use in these studies.

Lot 1999000 was sterilized by exposure to cobalt-60. The irradiation procedure was performed by Neutron Products, Inc. (Dickerson, MD). Comparison of the irradiated material with the nonirradiated material indicated that all 10 major peaks present in the nonirradiated material were present in the irradiated material at a relative concentration of ≥ 90.7%. Examination of the chromatograms before and after indicated that no new significant peaks appeared after irradiation.

To ensure stability, the bulk chemical was stored at approximately 5° C in a sealed plastic bag inside a 6-gallon metal pail (5-week study) or in amber glass bottles (40-week study). All transferring and handling of the study material was done under yellow lights. Periodic reanalyses of the bulk chemical were performed during the 5- and 40-week studies using HPLC/UV, and no degradation of the test material was detected.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared once by mixing senna with feed (Table D2). Using HPLC/UV, homogeneity and stability studies of the 300 and 10,000 ppm dose formulations were performed by the analytical chemistry laboratory and homogeneity studies of the 625 and 10,000 ppm dose formulations were performed by the study laboratory. Homogeneity and stability studies of the 100 ppm dose formulation were performed by the study laboratory using HPLC with mass spectrometry (MS) detection. Homogeneity was confirmed and the stability of the dose formulations was confirmed for at least 42 days when stored in sealed containers, protected from light.

Periodic analyses of the dose formulations of senna (quantitating sennoside A as the analytical marker) were conducted by the study laboratory using HPLC/UV (5-week study) or HPLC/MS/MS (40-week study). During the 5-week study, the dose formulations were analyzed once, and all five dose formulations were within 10% of the target concentrations (Table D5). Animal room samples of these dose formulations were also analyzed, and four of five were within 10% of the target concentrations. During the 40-week study, the dose formulations were analyzed five times; animal room samples were also analyzed (Table D6). Of the dose formulations analyzed, 33 of 50 were within 10% of the target concentrations; four of 10 animal room samples were within 10% of the target concentrations.

5-Week Study in C57BL/6NTac Mice

Male and female C57BL/6NTac mice were obtained from Taconic Farms, Inc. (Germantown, NY). On receipt, the mice were 4 to 5 weeks old. Animals were quarantined for 13 days and were 6 to 7 weeks old on the first day of the study. Before the study began, three male and two female mice were randomly selected for parasite evaluation and gross observation for evidence of disease. At the end of the study, blood samples were collected from five male and five female sentinel mice. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al*., 1986; Rao *et al*., 1989a,b). All results were negative.

The oral route of administration was selected to mimic oral exposures in humans taking senna in laxative preparations. Because senna is taken several times a day in laxative preparations, feed was selected as the route of administration to mimic human exposures. A dose of 10,000 ppm senna was selected as the maximum dose in the 5-week study, a dose expected to deliver daily doses that overlapped those used in the 13-week and 2-year senna rat studies (Mengs *et al*., 2004; Mitchell *et al.,* 2006). The doses of 625, 1,250, 2,500, 5,000, and 10,000 ppm included a low dose estimated to deliver a dose similar to human exposure levels to a high dose estimated to be near a maximum tolerated dose for repeated exposure.

Groups of five male and five female mice were fed diets containing 0, 625, 1,250, 2,500, 5,000, or 10,000 ppm senna for 29 days. Feed and water were available *ad libitum*. Mice were housed individually. Clinical findings were recorded weekly. Feed consumption was recorded weekly by animal. The animals were weighed initially, weekly, and at the end of the study. Details of the study design and animal maintenance are summarized in Table 1.

Necropsies were performed on all study animals. The heart, right kidney, liver, lung, right testis, and thymus were weighed. Histopathologic examinations were performed on all mice. Table 1 lists the tissues and organs examined.

40-Week Study in Heterozygous F1 p53$^{+/-}$ Mice

Male and female heterozygous F1 p53$^{+/-}$ mice were obtained from Taconic Farms, Inc. (Germantown, NY). On receipt, the mice were 5 to 7 weeks old. Animals were quarantined for 6 or 7 days and were 6 to 8 weeks old on the first day of the study. At the end of the quarantine period, serum samples for viral titer testing were collected from five male and five female mice randomly selected for parasite evaluation and gross observation for evidence of disease. Blood samples were collected from five male and five female sentinel mice 4 weeks after the start of the study and from five additional sentinel males and females at the end of the study and analyzed for antibody titers to rodent viruses. All results were negative.

Groups of 25 male and 25 female mice were fed diets containing 0, 100, 300, 1,000, 3,000, or 10,000 ppm senna for 40 weeks. Feed and water were available

ad libitum. Mice were housed individually. Clinical findings were recorded weekly and at study termination. Feed consumption was recorded weekly by animal. The animals were weighed initially, weekly, and at the end of the study. Details of the study design and animal maintenance are summarized in Table 1.

Necropsies were performed on all animals. The heart, right kidney, liver, lung, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (except eyes were first fixed in Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed on all mice. Table 1 lists the tissues and organs routinely examined.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The report, slides, paraffin blocks, residual wet tissues, and pathology data were sent to the NTP Archives for inventory, slide/block match, wet tissue audit, and storage. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 40-week studies, a quality assessment pathologist evaluated slides from all tumors and all potential target organs, which included the cecum, colon, and rectum.

The quality assessment report and the reviewed slides were submitted to the NTP PWG coordinator, who reviewed the selected tissues and addressed any inconsistencies in the diagnoses made by the laboratory and quality assessment pathologists. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologists, or lesions of general interest were presented by the coordinator to the PWG for review. The PWG consisted of the quality assessment pathologist and other pathologists experienced in rodent toxicologic pathology. This group examined the tissues without any knowledge of dose groups or previously rendered diagnoses. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, reviewing pathologist(s), and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). For subsequent analyses of the pathology data, the decision of whether to evaluate the diagnosed lesions for each tissue type separately or combined was generally based on the guidelines of McConnell *et al.* (1986).

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Senna

5-Week Study	40-Week Study
Study Laboratory Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)
Strain and Species C57BL/6NTac mice	Heterozygous F1 $p53^{+/-}$ mice
Animal Source Taconic Farms, Inc. (Germantown, NY)	Taconic Farms, Inc. (Germantown, NY)
Time Held Before Studies 13 days	6 (females) or 7 (males) days
Average Age When Studies Began 6 to 7 weeks	6 to 8 weeks
Date of First Exposure October 12, 2005	June 25 (females) or 26 (males), 2007
Duration of Exposure 5 weeks	40 weeks
Date of Last Exposure November 9, 2005	March 25-26 (females) or 27-28 (males), 2008
Necropsy Dates November 9, 2005	March 25-26 (females) or 27-28 (males), 2008
Average Age at Necropsy 10 to 11 weeks	45 to 48 weeks
Size of Study Groups 5 males and 5 females	25 males and 25 females
Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 5-week study
Animals per Cage 1	1
Method of Animal Identification Tail tattoo	Tail tattoo
Diet Irradiated NTP-2000 open formula meal diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed weekly	Same as 5-week study
Water Tap water (Columbus municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*	Same as 5-week study
Cages Polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly	Same as 5-week study
Bedding Irradiated Sani-Chips® (P.J. Murphy Forest Products Corp., Montville, NJ), changed weekly	Same as 5-week study

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Senna

5-Week Study	40-Week Study
Rack Filters Spun-bonded polyester (Snow Filtration Co., Cincinnati, OH), changed every 2 weeks	Same as 5-week study
Racks Stainless steel (Lab Products, Inc., Seaford, DE), changed every 2 weeks	Same as 5-week study
Animal Room Environment Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour
Exposure Concentrations 0, 625, 1,250, 2,500, 5,000, or 10,000 ppm in feed, available *ad libitum*	0, 100, 300, 1,000, 3,000, or 10,000 ppm in feed, available *ad libitum*
Type and Frequency of Observation Observed twice daily; animals were weighed initially, weekly, and at the end of the study; clinical findings were recorded weekly. Feed consumption was recorded weekly by animal.	Observed twice daily; animals were weighed initially, weekly, and at the end of the study; clinical findings were recorded weekly and at the end of the study. Feed consumption was recorded weekly by animal.
Method of Sacrifice Carbon dioxide asphyxiation	Same as 5-week study
Necropsy Necropsies were performed on all animals. Organs weighed were heart, right kidney, liver, lung, right testis, and thymus	Necropsies were performed on all animals. Organs weighed were heart, right kidney, liver, lung, right testis, and thymus
Histopathology Histopathology was performed on all mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular, mediastinal, and mesenteric), ovary, pituitary gland, spleen, stomach (forestomach and glandular), testis with epididymis, thymus, thyroid gland, urinary bladder, and uterus.	Complete histopathology was performed on all mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, gallbladder, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, and uterus.

STATISTICAL METHODS

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes were censored; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation and Analysis of Lesion Incidence

The incidences of neoplasms or nonneoplastic lesions are presented in Tables A1, A2, A3, and A4 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. The Fisher exact test (Gart *et al*., 1979) and the Cochran-Armitage trend test (Armitage, 1971), procedures based on the overall proportion of affected animals, were used to determine significance.

Analysis of Continuous Variables

Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972).

QUALITY ASSURANCE METHODS

The 40-week study was conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 40-week study were submitted to the NTP Archives, this study was audited retrospectively by an independent quality assurance contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Report.

GENETIC TOXICOLOGY

Bacterial Mutagenicity Test Protocol

Testing procedures used for senna at SRI International and BioReliance Corporation and for rhein and chrysophanic acid followed protocols reported by Zeiger *et al*. (1992); the remaining tests for senna and for sennosides A and B used a slightly modified procedure as described below. Senna, rhein, and chrysophanic acid were tested as coded samples. Test samples were incubated with two or more *Salmonella typhimurium* tester strains (TA97, TA98, TA100, and/or TA1535) either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37º C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following 2 days incubation at 37º C.

The protocol used for senna at SITEK Research Laboratories and ILS, Inc., and for sennosides A and B used only 10% rat liver S9 for exogenous metabolic activation and employed *Escherichia coli* strain WP2 *uvrA*/pKM101 as a bacterial tester strain in addition to *S. typhimurium* strains TA98 and TA100. Incubation of bacterial strains with coded senna and sennosides A and B samples and subsequent plating were carried out as described above.

For all tests, each trial consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of test compound. In senna tests where no toxicity was observed, a high dose of 10,000 to 16,666 µg/plate was used. The lot of senna used in the 40-week study was tested to a maximum concentration of 10,000 µg/plate in one assay (sample 2), 250 µg/plate a second assay (sample three), and 6,000 µg/plate in the third assay (sample four). Rhein and chrysophanic acid were limited by toxicity to 666 µg/plate. Sennosides A and B were tested up to 6,000 µg/plate.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is

defined as an increase in revertants that is not dose-related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

Mouse Peripheral Blood Micronucleus Protocol

The procedures used for the mouse peripheral blood micronucleus assay are described in detail by Dertinger *et al*. (2004) and Witt *et al*. (2008). Briefly, at the termination of the 40-week study of senna, one to two drops of blood from male and female mice were collected in microtubes with EDTA and shipped on ice to the genetic toxicity testing laboratory for processing and fixation in ultracold methanol, as per procedures described in the MicroFlow BASIC kit for mouse blood samples (Litron Laboratories, Rochester, NY). Upon arrival at the laboratory, it was discovered that the blood samples from the female mice had frozen during transport and were not amenable to analysis. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used to carry out the analyses of the samples from male mice. Reticulocytes (polychromatic erythrocytes, PCEs) were identified by the presence of an active transferrin receptor (CD71+) on the cell surface and mature erythrocytes (normochromatic erythrocytes, NCEs) were CD71-negative (CD71–). Micronuclei were detected using propidium iodide (a DNA stain) in conjunction with RNase treatment. Approximately 1×10^6 erythrocytes and 20,000 reticulocytes were evaluated per male mouse for the presence of micronuclei (propidium iodide-associated fluorescence). In addition, for each blood sample, the percentage of reticulocytes in 1×10^6 erythrocytes was determined as a measure of bone marrow toxicity.

Based on prior experience with the large number of cells scored using flow cytometric scoring techniques (Kissling *et al*., 2007), it is reasonable to assume that the proportion of micronucleated reticulocytes is approximately normally distributed. The statistical tests selected for trend and for pairwise comparisons with the control group depend on whether the variances among the groups are equal. Levene's test at α=0.05 is used to test for equal variances. In the case of equal variances, linear regression is used to test for a linear trend with dose and Williams' test is used to test for pairwise differences between each treatment group and the control group. In the case of unequal variances, Jonckheere's test is used to test for linear trend and Dunn's test is used for pairwise comparisons of each treatment group with the control group. To correct for multiple pairwise comparisons, the P value for each comparison with the control group is multiplied by the number of comparisons made. In the event that this product is greater than 1.00, it is replaced with 1.00. Trend tests and pairwise comparisons with the controls are considered statistically significant at P=0.025.

Ultimately, the scientific staff determines the final call after considering the results of statistical analyses, reproducibility of any effects observed, and the magnitudes of those effects.

Evaluation Protocol

These are the basic guidelines for arriving at an overall assay result for assays performed by the NTP. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple aliquots of a chemical were tested in the same assay and different results were obtained among aliquots and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The summary table in the Abstract of this Report presents a result that represents a scientific judgment of the overall evidence for activity of the chemical in an assay.

RESULTS

5-WEEK STUDY IN C57BL/6NTAC MICE

All mice survived to the end of the study (Table 2). Final mean body weights and body weight gains of exposed groups were similar to those of the controls. Feed consumption by exposed groups was similar to that by the controls. Concentrations of 625, 1,250, 2,500, 5,000, and 10,000 ppm senna in feed resulted in average daily doses of 115, 245, 490, 975, and 2,075 mg senna/kg body weight to males and 160, 310, 625, 1,190, and 2,570 mg/kg to females. There were no treatment-related clinical findings. Significant decreases in absolute heart weights occurred in all exposed groups of males (Table C1). Relative heart weights were significantly decreased in 625, 5,000, and 10,000 ppm males.

TABLE 2
Survival, Body Weights, and Feed Consumption of C57BL/6NTac Mice in the 5-Week Feed Study of Senna[a]

Concentration (ppm)	Survival[b]	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)	Feed Consumption Week 1	Feed Consumption Week 5
Male							
0	5/5	19.4 ± 0.3	23.5 ± 0.3	4.1 ± 0.4		4.0	4.3
625	5/5	19.3 ± 0.3	23.2 ± 0.3	3.8 ± 0.3	99	3.9	3.9
1,250	5/5	19.3 ± 0.6	22.6 ± 0.5	3.3 ± 0.3	96	4.2	4.0
2,500	5/5	19.4 ± 0.5	23.2 ± 0.8	3.8 ± 0.4	99	4.2	4.3
5,000	5/5	19.4 ± 0.7	23.3 ± 0.3	4.0 ± 0.5	99	4.3	4.0
10,000	5/5	19.3 ± 0.5	22.5 ± 0.2	3.2 ± 0.4	96	4.3	4.3
Female							
0	5/5	16.0 ± 0.5	20.3 ± 0.4	4.2 ± 0.2		3.7	5.1
625	5/5	15.8 ± 0.5	19.2 ± 0.4	3.4 ± 0.2	95	4.5	4.9
1,250	5/5	16.0 ± 0.2	20.1 ± 0.3	4.1 ± 0.2	99	4.2	5.3
2,500	5/5	15.9 ± 0.6	20.2 ± 0.5	4.3 ± 0.3	100	4.4	4.8
5,000	5/5	15.7 ± 0.3	19.1 ± 0.6	3.4 ± 0.6	94	4.0	4.6
10,000	5/5	15.8 ± 0.6	19.5 ± 0.5	3.7 ± 0.3	96	4.7	4.6

[a] Weights and weight changes are given as mean ± standard error. Feed consumption is expressed as grams per animal per day. Differences in weights and weight changes from the control group are not significant by Dunnett's test.
[b] Number of animals surviving at 5 weeks/number initially in group

Exposure to senna resulted in epithelial hyperplasia of the cecum and colon in males and of the cecum, colon, and rectum in females (Table 3). A no-observed-effect level (NOEL) could not be determined for the male mice because the 625 ppm group had two mice with minimal hyperplasia of the cecum, and one animal in the 1,250 ppm group had hyperplasia of the cecum and colon. The NOEL for female mice was 1,250 ppm, based upon the lack of lesions in that group and the presence of two animals in the 2,500 ppm group with cecal hyperplasia and one with hyperplasia of the colon. The hyperplasia was graded for severity semi-quantitatively. In the cecum and colon, grade 1 (minimal) lesions were recorded when the epithelial thickness was approximately 150 to 200 µm, grade 2 (mild) when it was around 200 to 250 µm, and grade 3 (moderate) when sections of the epithelium were greater than 250 µm. None of the lesions had changes severe enough to warrant a severity score of 4 (marked). The severity grade also took into account the amount of the tissue in the section involved– minimal lesions might only involve a focal area, whereas lesions with higher severity scores involved more of the tissue on the section. The hyperplasia was characterized by a thickening of the mucosa by a lengthening of the crypts (Plates 1 and 2). This was due to increased numbers of epithelial cells, which were small, with basophilic cytoplasm and round to oval nuclei, which became more vesicular toward the luminal surface of the glands. Goblet cells were interspersed with these cells, but did not constitute an increased proportion of the epithelial cells in the colon or cecum. Mitotic figures were numerous. Due to the naturally thicker mucosa found in the rectum, the same criteria for diagnosis used for the cecum and colon could not be applied to the rectum; severity grading for the rectum was done more subjectively.

Exposure Concentration Selection Rationale: There were no treatment-related clinical signs of toxicity in the 5-week study. Final mean body weights of exposed mice were within 10% of those of the control mice. Epithelial hyperplasia was seen in the cecum of all exposed groups of males, in the colon of 1,250 ppm or greater males, in the cecum and colon of 2,500 ppm or greater females, and in the rectum of 10,000 ppm females. Because intestinal lesions were not considered to be of sufficient severity to cause mortality in a 40-week p53$^{(+/-)}$ mouse study, the high dose of 10,000 ppm was selected. In order to expose mice to senna in a broader range, 0, 100, 300, 1,000, 3,000, and 10,000 ppm were selected as the exposure concentrations for the 40-week study.

TABLE 3
Incidences of Hyperplasia in the Large Intestine in C57BL/6NTac Mice in the 5-Week Feed Study of Senna

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Male						
Cecum[a]	5	5	5	5	5	5
Hyperplasia, epithelial[b]	0	2 (1.0)[c]	1 (1.0)	1 (1.0)	3 (1.0)	5** (1.8)
Colon	5	5	5	5	5	5
Hyperplasia, epithelial	0	0	1 (1.0)	1 (1.0)	4* (1.3)	5** (2.0)
Female						
Cecum	5	5	5	5	5	5
Hyperplasia, epithelial	0	0	0	2 (1.0)	4* (1.0)	5** (1.8)
Colon	5	5	5	5	5	5
Hyperplasia, epithelial	0	0	0	1 (1.0)	5** (1.4)	5** (3.0)
Rectum	5	5	5	5	5	5
Hyperplasia, epithelial	0	0	0	0	0	3 (1.0)

* Significantly different (P≤0.05) from the control group by the Fisher exact test
** P≤0.01
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

40-WEEK STUDY IN HETEROZYGOUS F1 P53$^{+/-}$ MICE

Survival

Estimates of 40-week survival probabilities for male and female mice are shown in Table 4 and in the Kaplan-Meier survival curves (Figure 3). Survival of all exposed groups was similar to that of the control groups.

Body Weights, Feed and Compound Consumption, Organ Weights, and Clinical Findings

Mean body weights of exposed male and female mice were within 10% of those of the controls throughout the study (Figure 4; Tables 5 and 6). Feed consumption by exposed mice was generally similar to that by the controls throughout the study (Tables E1 and E2).

Concentrations of 100, 300, 1,000, 3,000, and 10,000 ppm in feed resulted in average daily doses of 12, 36, 120, 365, and 1,260 mg/kg to males and 14, 42, 140, 435, and 1,520 mg/kg to females. The absolute and relative liver weights of 10,000 ppm males were significantly less than those of the controls (Table C2). There were no exposure-related clinical findings.

TABLE 4
Survival of Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male						
Animals initially in study	25	25	25	25	25	25
Natural deaths	0	1	0	0	2	1
Animals surviving to study termination	25	24	25	25	23	24
Percent probability of survival at end of study[a]	100	96	100	100	92	96
Mean survival (days)[b]	276	274	276	276	275	266
Survival analysis[c]	P=0.657	P=1.000	—[d]	—[d]	P=0.471	P=1.000
Female						
Animals initially in study	25	25	25	25	25	25
Moribund	2	2	2	2	2	1
Natural deaths	0	0	0	1	1	0
Animals surviving to study termination	23	23	23	22	22	24
Percent probability of survival at end of study	92	92	92	88	88	96
Mean survival (days)	273	272	272	267	261	275
Survival analysis	P=0.679N	P=1.000	P=1.000	P=0.940	P=0.970	P=0.991N

[a] Kaplan-Meier determinations
[b] Mean of all deaths (uncensored, censored, and terminal sacrifice).
[c] The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed group columns. A negative trend or lower mortality in an exposure group is indicated by **N**.
[d] Value of the statistic cannot be computed.

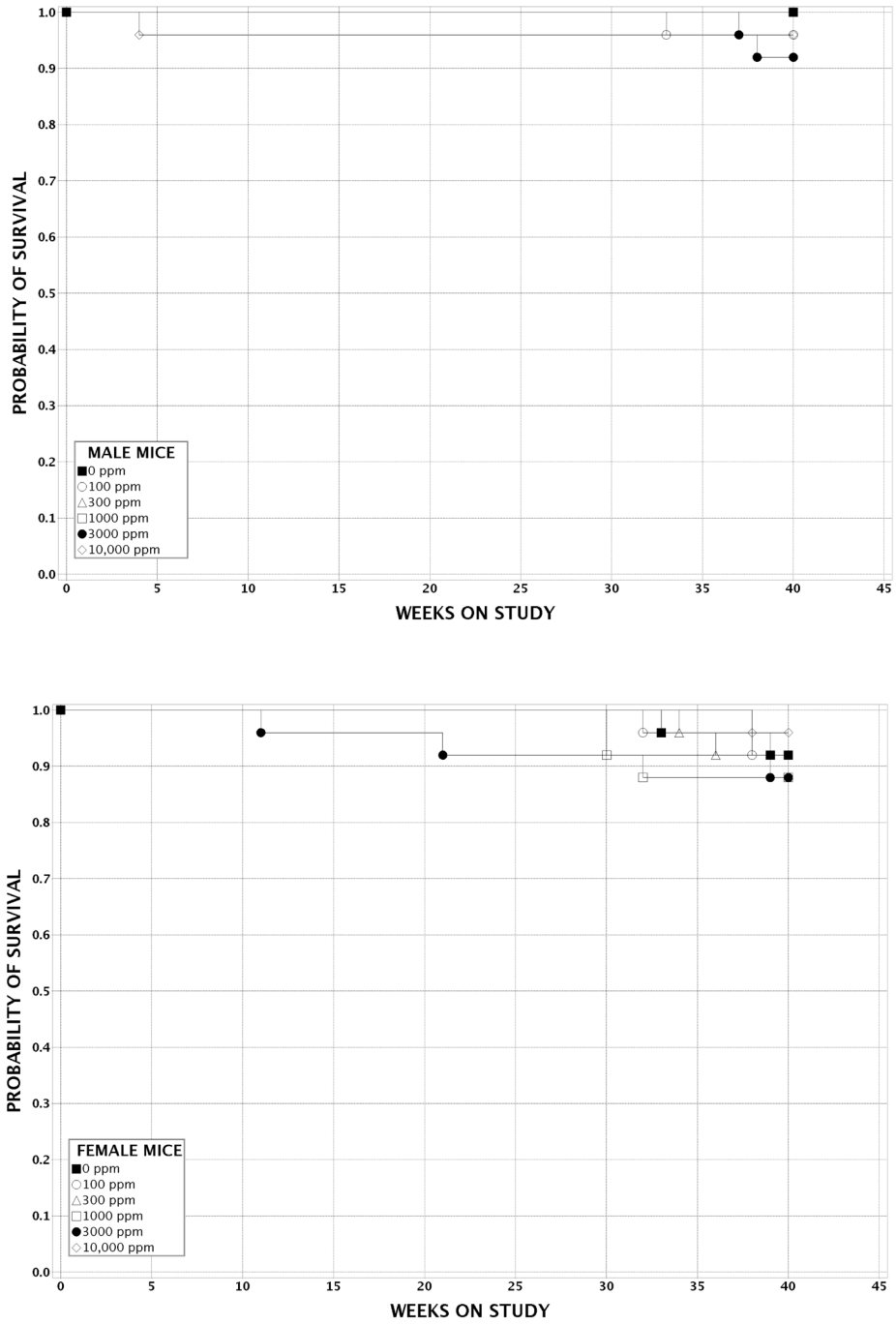


FIGURE 3
Kaplan-Meier Survival Curves for Heterozygous F1 p53$^{+/-}$ Mice Exposed to Senna in Feed for 40 Weeks

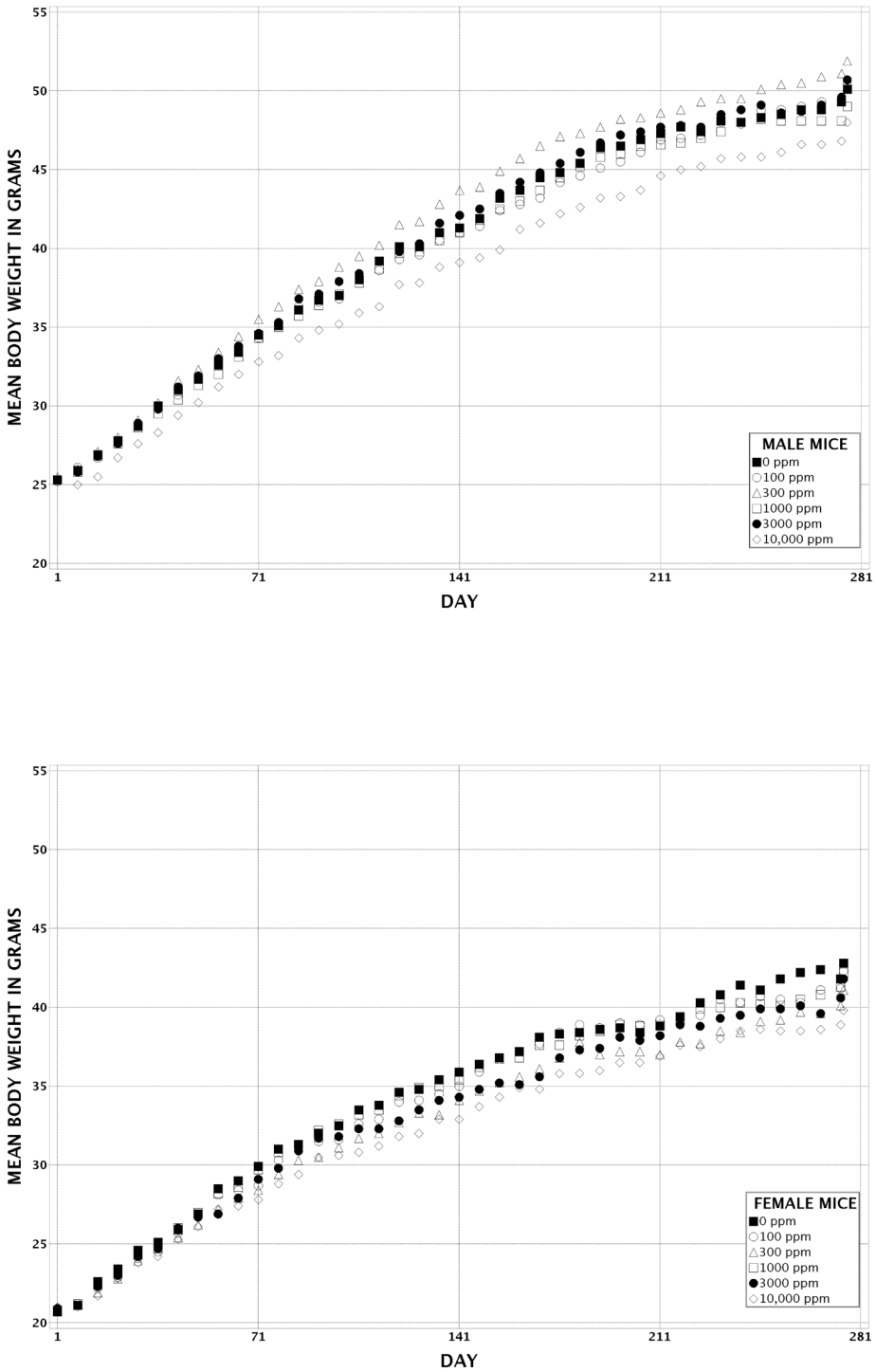


FIGURE 4
Growth Curves for Heterozygous F1 p53$^{+/-}$ Mice Exposed to Senna in Feed for 40 Weeks

TABLE 5
Mean Body Weights and Survival of Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm		100 ppm			300 ppm			1,000 ppm		
Days	Av. Wt (g)	No. of Survivors	Av. Wt (g)	Wt (% of) controls)	No. of Survivors	Av. Wt (g)	Wt (% of) controls)	No. of Survivors	Av. Wt (g)	Wt (% of) controls)	No. of Survivors
1	25.3	25	25.2	100	25	25.5	101	25	25.3	100	25
8	25.9	25	26.1	101	25	26.0	100	25	25.8	99	25
15	26.9	25	26.7	99	25	27.1	101	25	26.8	100	25
22	27.8	25	27.6	99	25	28.0	100	25	27.6	99	25
29	28.7	25	28.9	101	25	29.1	101	25	28.6	100	25
36	30.0	25	29.9	100	25	30.2	101	25	29.5	98	25
43	31.0	25	30.7	99	25	31.6	102	25	30.4	98	25
50	31.7	25	31.8	100	25	32.3	102	25	31.3	99	25
57	32.6	25	32.9	101	25	33.4	103	25	32.0	98	25
64	33.4	25	33.7	101	25	34.4	103	25	33.1	99	25
71	34.5	25	34.3	100	25	35.5	103	25	34.3	99	25
78	35.1	25	35.2	100	25	36.3	104	25	35.0	100	25
85	36.1	25	36.3	101	25	37.4	104	25	35.7	99	25
92	36.7	25	36.6	100	25	37.9	103	25	36.4	99	25
99	37.0	25	36.8	100	25	38.8	105	25	37.1	100	25
106	38.0	25	38.0	100	25	39.5	104	25	37.8	100	25
113	39.2	25	38.6	99	25	40.2	103	25	38.7	99	25
120	40.1	25	39.3	98	25	41.5	104	25	39.7	99	25
127	40.1	25	39.6	99	25	41.7	104	25	39.8	99	25
134	41.0	25	40.5	99	25	42.8	105	25	40.5	99	25
141	41.3	25	41.0	99	25	43.7	106	25	41.0	99	25
148	41.9	25	41.4	99	25	43.9	105	25	41.8	100	25
155	43.2	25	42.4	98	25	44.9	104	25	42.5	98	25
162	43.7	25	42.8	98	25	45.7	105	25	43.0	98	25
169	44.5	25	43.2	97	25	46.5	105	25	43.7	98	25
176	44.8	25	44.2	99	25	47.1	105	25	44.5	99	25
183	45.4	25	44.6	98	25	47.3	104	25	45.2	100	25
190	46.4	25	45.1	97	25	47.7	103	25	45.8	99	25
197	46.5	25	45.5	98	25	48.2	104	25	46.0	99	25
204	46.9	25	46.1	98	25	48.3	103	25	46.5	99	25
211	47.3	25	46.9	99	25	48.6	103	25	46.6	99	25
218	47.7	25	47.0	99	25	48.8	102	25	46.7	98	25
225	47.4	25	47.2	100	25	49.3	104	25	47.0	99	25
232	48.1	25	48.0	100	24	49.5	103	25	47.4	99	25
239	48.0	25	47.9	100	24	49.5	103	25	48.0	100	25
246	48.3	25	48.8	101	24	50.1	104	25	48.2	100	25
253	48.5	25	48.8	101	24	50.4	104	25	48.1	99	25
260	48.8	25	49.0	101	24	50.5	104	25	48.1	99	25
267	48.8	25	49.3	101	24	50.9	104	25	48.1	99	25
274	49.3	25	49.6	101	24	51.1	104	25	48.1	98	25
Mean for Weeks											
1-13	30.7		30.7	100		31.3	102		30.4	99	
14-40	44.4		44.0	99		46.1	104		43.9	99	

TABLE 5
Mean Body Weights and Survival of Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	3,000 ppm			10,000 ppm		
Days	Av. Wt (g)	Wt (% of) controls)	No. of Survivors	Av. Wt (g)	Wt (% of) controls)	No. of Survivors
1	25.3	100	25	25.2	100	25
8	25.8	100	25	25.0	96	25
15	26.8	100	25	25.5	95	25
22	27.6	99	25	26.7	96	24
29	28.9	101	25	27.6	96	24
36	29.8	99	25	28.3	94	24
43	31.2	101	25	29.4	95	24
50	31.9	101	25	30.2	95	24
57	33.0	101	25	31.2	96	24
64	33.8	101	25	32.0	96	24
71	34.6	101	25	32.8	95	24
78	35.3	101	25	33.2	95	24
85	36.8	102	25	34.3	95	24
92	37.1	101	25	34.8	95	24
99	37.9	103	25	35.2	95	24
106	38.4	101	25	35.9	95	24
113	39.2	100	25	36.3	93	24
120	39.8	99	25	37.7	94	24
127	40.3	101	25	37.8	94	24
134	41.6	101	25	38.8	95	24
141	42.1	102	25	39.1	95	24
148	42.5	101	25	39.4	94	24
155	43.5	101	25	39.9	92	24
162	44.2	101	25	41.2	94	24
169	44.8	101	25	41.6	94	24
176	45.4	101	25	42.2	94	24
183	46.1	102	25	42.6	94	24
190	46.7	101	25	43.2	93	24
197	47.2	102	25	43.3	93	24
204	47.4	101	25	43.7	93	24
211	47.7	101	25	44.6	94	24
218	47.8	100	25	45.0	94	24
225	47.7	101	25	45.2	95	24
232	48.5	101	25	45.7	95	24
239	48.8	102	25	45.8	95	24
246	49.1	102	25	45.8	95	24
253	48.6	100	25	46.1	95	24
260	48.7	100	24	46.6	96	24
267	49.1	101	23	46.6	96	24
274	49.6	101	23	46.8	95	24
Mean for Weeks						
1-13	30.8	101		29.3	96	
14-40	44.8	101		41.9	94	

TABLE 6
Mean Body Weights and Survival of Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm		100 ppm			300 ppm			1,000 ppm		
Days	Av. Wt (g)	No. of Survivors	Av. Wt (g)	Wt (% of) controls)	No. of Survivors	Av. Wt (g)	Wt (% of) controls)	No. of Survivors	Av. Wt (g)	Wt (% of) controls)	No. of Survivors
1	20.7	25	20.9	101	25	20.9	101	25	20.8	100	25
8	21.1	25	21.2	100	25	21.1	100	25	21.2	101	25
15	22.6	25	22.2	98	25	21.9	97	25	22.4	99	25
22	23.4	25	22.9	98	25	22.8	97	25	23.2	99	25
29	24.6	25	24.1	98	25	23.9	97	25	24.3	99	25
36	25.1	25	24.5	98	25	24.7	99	25	24.9	99	25
43	25.9	25	25.7	99	25	25.4	98	25	26.0	100	25
50	26.9	25	26.9	100	25	26.2	97	25	27.0	100	25
57	28.5	25	28.1	99	25	27.2	95	25	28.2	99	25
64	29.0	25	28.4	98	25	27.9	96	25	28.6	99	25
71	29.9	25	28.7	96	25	28.4	95	25	29.7	99	25
78	31.0	25	30.3	98	25	29.4	95	25	30.8	100	25
85	31.3	25	31.2	100	25	30.3	97	25	31.3	100	25
92	32.0	25	31.5	98	25	30.5	95	25	32.2	101	25
99	32.5	25	31.6	97	25	31.1	96	25	32.6	100	25
106	33.5	25	32.6	97	25	31.7	95	25	33.2	99	25
113	33.8	25	32.9	98	25	32.0	95	25	33.5	99	25
120	34.6	25	34.0	98	25	32.7	95	25	34.4	100	25
127	34.8	25	34.1	98	25	33.3	96	25	34.9	100	25
134	35.4	25	34.5	98	25	33.2	94	25	35.0	99	25
141	35.9	25	35.0	98	25	34.1	95	25	35.4	99	25
148	36.4	25	35.9	99	25	34.7	95	25	36.2	100	25
155	36.8	25	36.7	100	25	35.2	96	25	36.7	100	25
162	37.2	25	37.2	100	25	35.6	96	25	36.8	99	25
169	38.1	25	37.7	99	25	36.1	95	25	37.6	99	25
176	38.3	25	38.4	100	25	36.8	96	25	37.6	98	25
183	38.4	25	38.9	101	25	37.8	98	25	38.2	99	25
190	38.6	25	38.7	100	25	37.0	96	25	38.5	100	25
197	38.7	25	39.0	101	25	37.2	96	25	38.9	101	25
204	38.4	25	38.9	101	25	37.2	97	25	38.8	101	24
211	38.8	25	39.2	101	25	37.0	96	25	38.9	100	23
218	39.4	25	39.2	99	25	37.8	96	25	39.2	100	23
225	40.3	24	39.5	98	24	37.7	94	25	39.9	99	22
232	40.8	24	40.5	99	24	38.5	94	24	40.0	98	22
239	41.4	24	40.3	97	24	38.4	93	24	40.3	97	22
246	41.1	24	40.7	99	24	39.1	95	23	40.2	98	22
253	41.8	24	40.5	97	24	39.2	94	23	40.1	96	22
260	42.2	24	40.3	96	24	39.7	94	23	40.5	96	22
267	42.4	23	41.1	97	23	39.6	94	23	40.8	96	22
274	41.8	23	41.2	99	23	40.1	96	23	41.3	99	22
Mean for Weeks											
1-13	26.2		25.8	99		25.4	97		26.0	100	
14-40	37.9		37.4	99		36.0	95		37.5	99	

TABLE 6
Mean Body Weights and Survival of Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	3,000 ppm			10,000 ppm		
Days	Av. Wt (g)	Wt (% of) controls)	No. of Survivors	Av. Wt (g)	Wt (% of) controls)	No. of Survivors
1	20.9	101	25	21.0	102	25
8	21.1	100	25	21.0	100	25
15	22.3	99	25	21.7	96	25
22	23.0	98	25	22.8	97	25
29	24.2	98	25	23.8	96	25
36	24.7	99	25	24.2	97	25
43	26.0	100	25	25.3	98	25
50	26.7	99	25	26.1	97	25
57	26.9	94	25	27.2	95	25
64	27.9	96	25	27.4	95	25
71	29.1	97	24	27.8	93	25
78	29.8	96	24	28.8	93	25
85	30.9	99	24	29.4	94	25
92	31.7	99	24	30.5	95	25
99	31.8	98	24	30.6	94	25
106	32.3	96	24	30.8	92	25
113	32.3	96	24	31.2	92	25
120	32.8	95	24	31.8	92	25
127	33.5	96	24	32.0	92	25
134	34.1	96	24	32.9	93	25
141	34.3	96	23	32.9	92	25
148	34.8	96	23	33.7	93	25
155	35.2	96	23	34.3	93	25
162	35.1	94	23	34.9	94	25
169	35.6	93	23	34.8	91	25
176	36.8	96	23	35.8	93	25
183	37.3	97	23	35.8	93	25
190	37.4	97	23	36.0	93	25
197	38.1	99	23	36.5	94	25
204	37.9	99	23	36.5	95	25
211	38.2	99	23	36.9	95	25
218	38.9	99	23	37.6	95	25
225	38.8	97	23	37.5	93	25
232	39.3	96	23	38.0	93	25
239	39.5	95	23	38.5	93	25
246	39.9	97	23	38.6	94	25
253	39.9	96	23	38.5	92	25
260	40.1	95	23	38.5	91	25
267	39.6	94	23	38.6	91	24
274	40.6	97	22	38.9	93	24
Mean for Weeks						
1-13	25.7	98		25.1	96	
14-40	36.5	96		35.3	93	

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of bone and liver neoplasms and nonneoplastic lesions of the large intestine. Summaries of the incidences of neoplasms and nonneoplastic lesions are presented in Appendix A for male and female mice. Historical control incidences for neoplasms in heterozygous F1 $p53^{+/-}$ mice are shown in Appendix F.

Large Intestine: Significant increases in the incidences of epithelial hyperplasia of the colon and cecum occurred in 10,000 ppm males and females, and the incidence of hyperplasia of the colon was significantly increased in 3,000 ppm females (Tables 7, A2, and A4). Three 3,000 ppm males had epithelial hyperplasia of the colon and single occurrences of epithelial hyperplasia of the rectum were recorded in the 10,000 ppm males and females; although lacking statistical significance, these lesions are considered biologically noteworthy.

Epithelial hyperplasia of the colon, cecum, and rectum (Plates 3 through 8) was similar in character to that observed in C57BL/6NTac mice exposed to senna for 5 weeks. The changes were characterized by a thickening of the mucosa by a lengthening of the crypts. This was due to increased numbers of epithelial cells that appeared crowded together. These cells were small and had basophilic cytoplasm and round to oval nuclei, which became more vesicular toward the luminal surface of the glands. Goblet cells were interspersed with these cells, but did not constitute an increased proportion of the epithelial cells in the colon or cecum. Mitotic figures were numerous. The cecum was usually less severely affected than the colon.

Normal crypt depth in controls was up to 175 µm. Hyperplasia of the colon was graded based upon crypt depth, as follows: in minimal (grade 1) hyperplasia, crypt depth was approximately 200 µm (from basement membrane to surface of crypt epithelium, measured where an entire crypt was in longitudinal section), but only focally or multifocally; in mild (grade 2) hyperplasia, crypt depth was greater than or equal to 200 µm over much of the circumference, and was focally approximately 300 to 350 µm; in moderate (grade 3) hyperplasia almost the entire circumference of the section examined was involved with crypts 300 to 350 µm deep. Lesions severe enough to be recorded as marked (grade 4) hyperplasia were not observed. Due

TABLE 7
Incidences of Hyperplasia in the Large Intestine in Heterozygous F1 $p53^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male						
Cecum[a]	25	25	25	25	23	25
Epithelium, hyperplasia[b]	0	0	0	0	0	22** (1.4)[c]
Colon	25	25	25	25	24	25
Epithelium, hyperplasia	0	0	0	0	3 (1.3)	25** (2.8)
Rectum	24	25	25	25	24	25
Epithelium, hyperplasia	0	0	0	0	0	1 (2.0)
Female						
Cecum	25	25	25	25	25	25
Epithelium, hyperplasia	0	0	0	0	0	19** (1.3)
Colon	25	25	25	25	25	25
Epithelium, hyperplasia	0	0	0	0	7** (1.0)	25** (2.7)
Rectum	25	25	25	25	25	25
Epithelium, hyperplasia	0	0	0	0	0	1 (1.0)

** Significantly different ($P \leq 0.01$) from the control group by the Fisher exact test.
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

to the naturally thinner mucosa of the cecum and the naturally thicker mucosa found in the rectum, the same criteria for diagnosis used for the colon could not be applied; severity grading for the cecum and rectum was done more subjectively.

Bone: Osteosarcomas occurred in 1,000 and 3,000 ppm males but not in the control group; the incidences in these groups were within the historical control range (Tables 8, A1, and F1). In 1,000 ppm females, the incidence of osteosarcoma was greater than the historical control range but not significantly greater than the incidence in the concurrent control group (Tables 8, A3, and F2). Given that there was not a dose response and the incidences were generally within the historical and/or concurrent control ranges, the osteosarcomas were not considered to be related to senna exposure.

Liver: The incidence of hepatocellular adenoma in 300 ppm males was increased, but not significantly, and exceeded the historical control range (Tables 9, A1, and F1). The biological relevance of this finding is unknown.

GENETIC TOXICOLOGY

Four different samples of senna, including three samples of the same lot that was used in the 40-week mouse study, were tested for mutagenicity in bacterial test systems (Tables B1 and B2). The first sample produced a weak response in *Salmonella typhimurium* strain TA100 at high concentrations in the presence of induced rat or hamster liver S9. In strain TA98, significant increases in mutant colonies were seen with high concentrations of this senna sample in the presence of hamster or rat liver S9 mix. The second sample, from the same lot of senna used in the 40-week mouse study, was not mutagenic at concentrations up to 10,000 µg/plate, with or without S9, in *S. typhimurium* strains TA97, TA98, TA100, or TA1535; two trials, one in TA100 without S9 and one in TA98 with 10% hamster liver S9 gave equivocal responses that were not reproduced in the replicate trial. The third and fourth samples were also from the same lot of senna that was used in the 40-week mouse study. The third sample was tested only to a maximum concentration of 250 µg/plate; no evidence of mutagenicity was observed over this dose range, with or without S9, in any bacterial

TABLE 8
Incidences of Bone Neoplasms in Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male						
Number examined microscopically	25	25	25	25	25	25
Femur, osteoma	0	0	1 (4%)	0	0	0
Maxilla, osteosarcoma	0	0	0	3 (12%)	1 (4%)	0
Vertebra, osteosarcoma	0	0	0	0	1 (4%)	0
Osteoma or osteosarcoma[a]	0	0	1 (4%)	3 (12%)	2 (8%)	0
Female						
Number examined microscopically	25	25	25	25	25	25
Osteosarcoma (site not specified)	0	0	0	0	0	1 (4%)
Femur, osteosarcoma	0	0	0	0	0	1 (4%)
Maxilla, osteosarcoma	0	1 (4%)	0	1 (4%)	0	0
Rib, osteosarcoma	1 (4%)	0	0	0	0	0
Tibia, osteosarcoma	0	1 (4%)	0	0	0	0
Vertebra, osteosarcoma	1 (4%)	0	1 (4%)	2 (8%)	0	0
Osteosarcoma[b]	2 (8%)	2 (8%)	1 (4%)	3 (12%)	0	2 (8%)

[a] Historical incidence for control groups in 9-month heterozygous F1 p53$^{+/-}$ mouse studies: 5/77, range 0%-12%
[b] Historical incidence for control groups in 9-month heterozygous F1 p53$^{+/-}$ mouse studies: 3/76, range 0%-8%

TABLE 9
Incidences of Hepatocellular Adenoma in Heterozygous F1 p53$^{+/-}$ Male Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Hepatocellular Adenoma[a]						
Overall rate[b]	1/25 (4%)	0/25 (0%)	5/25 (20%)	0/25 (0%)	2/25 (8%)	1/25 (4%)
Terminal rate[c]	1/25 (4%)	0/24 (0%)	5/25 (20%)	0/25 (0%)	2/23 (9%)	1/24 (4%)
First incidence (days)	276 (T)	—[e]	276 (T)	—	276 (T)	276 (T)
Statistical analysis[d]	P=0.477N	P=0.500N	P=0.095	P=0.500N	P=0.500	P=0.755N

(T) Terminal sacrifice
[a] Historical incidence for control groups in 9-month heterozygous F1 p53$^{+/-}$ mouse studies: 5/76, range 4%-12%
[b] Number of animals with lesion per number of animals with liver examined microscopically
[c] Observed incidence at terminal kill
[d] The result of the Cochran-Armitage trend test (Armitage, 1971) is in the control column, and the results of the Fisher exact pairwise comparisons (Cox, 1972) with the controls are in the exposed group columns. A negative trend or a lower incidence in an exposure group is indicated by **N**.
[e] Not applicable; no lesions in animal group

strain. The fourth sample was tested up to a concentration of 6,000 µg/plate; significant increases in mutant colonies were observed in TA100 with rat liver S9 and in TA98 with and without rat liver S9. Significant increases were seen at lower concentrations in the tests conducted with the fourth sample of senna in the presence of S9. Overall, there is a general lack of reproducibility in the mutagenic activity observed among different lots of senna.

In addition to senna, the NTP also tested rhein (a component of senna) for mutagenicity in *S. typhimurium* strains TA98 and TA100; dose-related increases in mutant colonies were seen with both strains in the presence of rat or hamster liver S9 and at lower concentrations (approximately 100 to 166 µg/plate) than were required for the positive responses seen with senna samples (Table B3). Another component of senna, chrysophanic acid, was tested for mutagenicity in TA98, TA100, and TA1535; weak and inconsistent responses were seen in TA100 with rat and hamster liver S9 (Table B4). Sennosides A and B (up to 6,000 µg/plate) were also tested for mutagenicity in bacterial test systems; neither compound was mutagenic, with or without S9 metabolic activation, in *S. typhimurium* strains TA98 or TA100, or *Escherichia coli* strain WP2 *uvrA*/pKM101 (Tables B5 and B6).

In vivo, no significant increases in the frequencies of micronucleated reticulocytes (polychromatic erythrocytes; PCEs) or erythrocytes (normochromatic erythrocytes, NCEs) were seen in male mice exposed to senna (100 to 10,000 ppm) in dosed feed for 40 weeks (Table B7). Although the trend test was significant (P=0.016), the increase in micronucleated NCEs seen at the highest dose was small (less than 0.2 micronucleated cells per 1,000), no treatment groups were significantly elevated over the control group, and the frequency of micronucleated PCEs in the high dose group was not increased over the control. Furthermore, there was no dose-related increase in micronucleated PCEs (trend test P=0.825). Therefore, senna was judged to be negative in the erythrocyte micronucleus test. No significant changes in the percentage of PCEs among erythrocytes were observed in male mice suggesting that exposure to senna did not induce bone marrow toxicity.

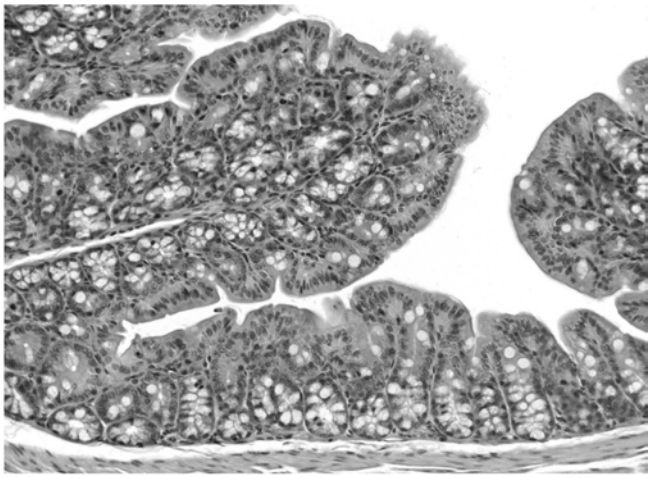


PLATE 1
Colon from a control female C57BL/6NTac mouse in the 5-week feed study of senna. Plates 1 and 2 were taken at the same magnification. H&E

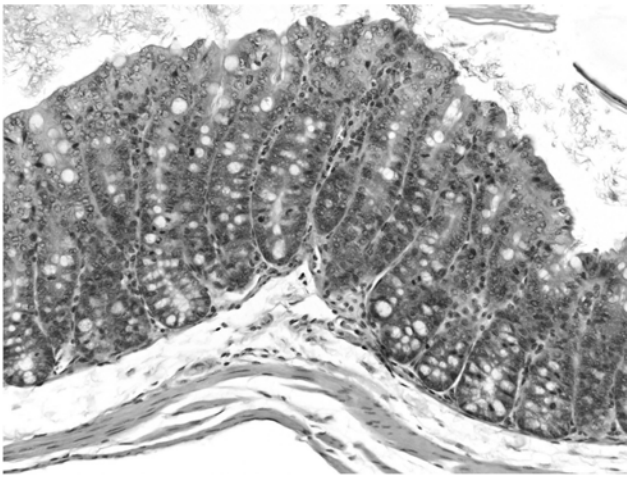


PLATE 2
Epithelial hyperplasia (grade 3) in the colon from a female C57BL/6NTac mouse exposed to 10,000 ppm senna in feed for 5 weeks. The lengths of the crypts and numbers of epithelial cells are increased, as evidenced by crowding, increased basophilia, and increased mitoses. H&E

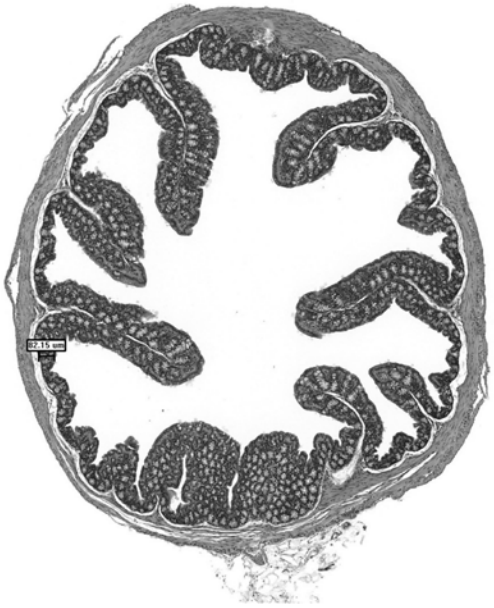


PLATE 3
Colon from a control male heterozygous F1 p53$^{+/-}$ mouse in the 40-week feed study of senna. Plates 3 and 4 were taken at the same magnification. H&E

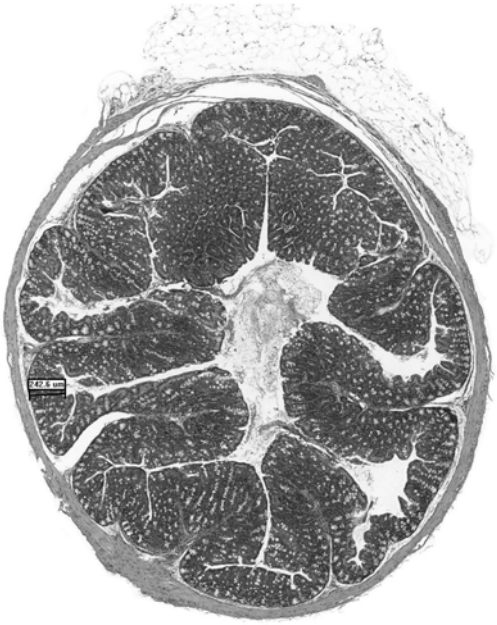


PLATE 4
Moderate epithelial hyperplasia (grade 3) in the colon from a male heterozygous F1 p53$^{+/-}$ mouse exposed to 10,000 ppm senna in feed for 40 weeks. H&E

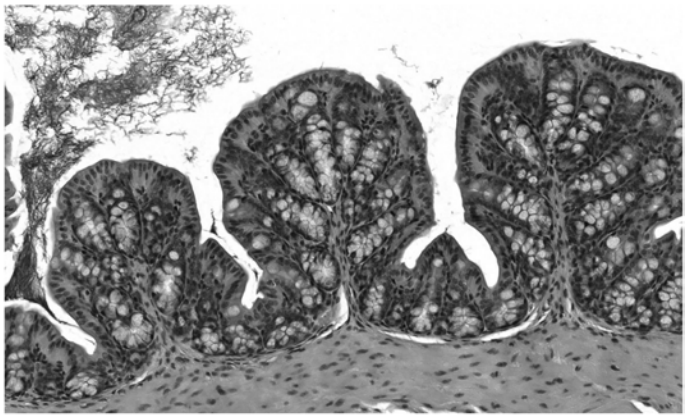


PLATE 5
Colon of a control male heterozygous F1 p53$^{+/-}$ mouse in the 40-week feed study of senna. H&E

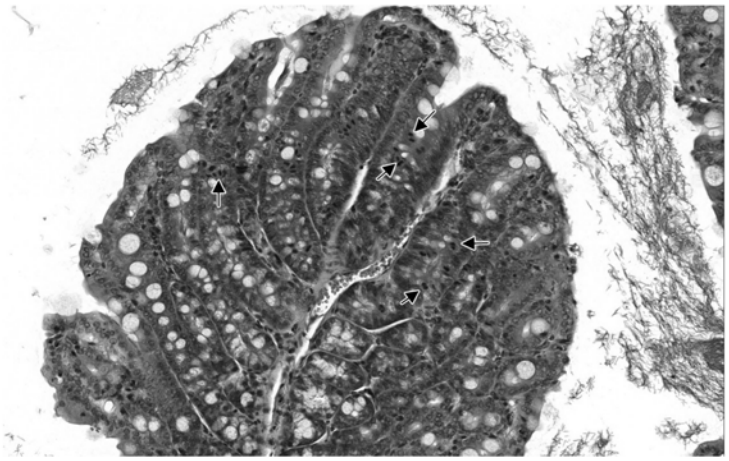


PLATE 6
Moderate (grade 3) epithelial hyperplasia in the colon of a male heterozygous F1 p53$^{+/-}$ mouse exposed to 10,000 ppm senna in feed for 40 weeks. The epithelium is thickened as evidenced by increased distance from the luminal surface to the base of the crypts. Epithelial cells are increased in number and are basophilic and crowded; the cells lack the orderly arrangement seen in the control mouse colon shown in Plate 5. There are numerous mitotic figures (arrows). H&E

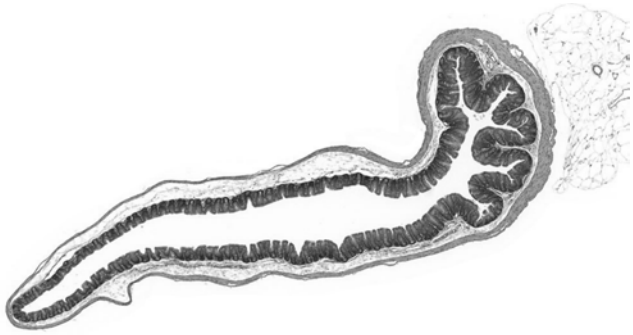


PLATE 7
Cecum of a control male heterozygous F1 p53$^{+/-}$ mouse in the 40-week feed study of senna. Plates 7 and 8 were taken at the same magnification. H&E

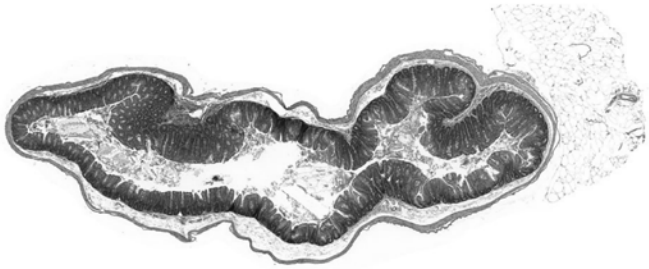


PLATE 8
Moderate hyperplasia (grade 3) in the cecum of a male heterozygous F1 p53$^{+/-}$ mouse exposed to 10,000 ppm senna in feed for 40 weeks. H&E

DISCUSSION AND CONCLUSIONS

These studies provide data on the carcinogenic potential of senna in a genetically-modified mouse model.

Treatment-related epithelial hyperplasia in the large intestine occurred in both the 5-week senna study in C57BL/6NTac mice and in the 40-week senna study in C3B6.129F1/Tac-*Trp53*^{tm1Brd} N12 haploinsufficient mice (also referred to in this study as heterozygous F1 p53$^{+/-}$ mice); in both studies, senna was administered in feed. The daily senna exposure concentrations in these studies overlapped daily exposures in humans taking senna in laxative preparations.

In the 40-week study, the average senna consumption of males exposed to 100, 300, 1,000, 3,000, or 10,000 ppm was 12, 36, 120, 365, or 1,260 mg/kg per day. Based on the percent total weight values for sennosides A and B (approximately 2% of senna by weight), the sennosides A and B consumption was approximately 0.24, 0.72, 2.4, 7.3, or 25.4 mg/kg or 0.72, 2.2, 7.2, 21.9, or 76.1 mg/m^3 (on a body surface area basis) per day (Freireich *et al*., 1966). Senna consumption by females was similar to that by males. For a typical over-the-counter senna laxative preparation taken twice daily, the amount of sennosides consumed is approximately 1.4 mg/kg per day or 51.8 mg/m^3 (body surface area basis) per day (Novartis, 2010). In some cases, laxatives may be taken in greater than recommended amounts (Cance *et al*., 2005; Bryant-Waugh *et al*., 2006; Mond *et al*., 2006; Tozzi *et al*., 2006).

Colon and cecum epithelial hyperplasias were seen in 5,000 and 10,000 ppm males and females in the 5-week C57BL/6NTac mouse study. A few female mice exposed to 10,000 ppm also had epithelial hyperplasia of the rectum. Colon/cecum epithelium hyperplastic lesions also occurred in male and female mice in the 40-week heterozygous F1 p53$^{+/-}$ mouse study. In humans, senna may be associated with increased cell proliferation in the intestine (Kleibuker *et al*., 1995; van Gorkom *et al*., 2000).

The C3B6.129F1/Tac-*Trp53*^{tm1Brd} N12 haploinsufficient mouse used in these senna studies was a different strain than that used in earlier NTP GMM studies. Those studies used the B6.129F1-*Trp53*^{tm1Brd} (N5) haploinsufficient mouse, a strain resulting from back-crossing the p53$^{+/-}$ mouse five times to the C57/B6 mouse (Pritchard *et al*., 2003; NTP, 2005a,b; 2007a,b,c,d,e; 2008). The source of the knockout gene is the same in both p53$^{+/-}$ mouse strains (Donehower *et al*., 1992, 1995). There have been no comparisons for chemical carcinogenic effects in these two p53$^{+/-}$ mouse trains.

Senna had a laxative effect in rats receiving daily doses of 300 mg/kg or greater by oral gavage for 13 consecutive weeks (Mengs *et al*., 2004); however, in the current studies no laxative effect was observed at comparable or higher doses in mice, this possibly indicates species-dependent differences in sensitivity or kinetics in formation of rhein anthrone, primarily responsible for the purgative activity of senna (Leng-Peschlow, 1992; Yamauchi *et al*., 1993), or difference in kinetics between gavage and feed. Rhein anthrone is formed by intestinal bacteria at the site of action and is purportedly metabolized through a free radical (Lemli and Lemmens, 1980; Dreessen *et al*., 1981; Lemli, 1988), a possible mechanism in the formation of the intestinal hyperplastic response observed at the high dose in the current studies.

Two different lots of senna were tested in four independent bacterial mutagenicity assays (Appendix B). Two of the samples, one of which was the same lot of senna used in the 40-week study, gave positive results. Most of the positive responses observed with these two senna samples occurred in tests conducted with rat or hamster liver S9 preparations. Results from the testing of the two other samples that came from the same lot of senna used in the 40-week mouse study were negative. The variability in responses among different lots may be related to sample constituents. Information from the literature indicates that some metabolites and/or constituents of senna are potentially genotoxic (Dreessen *et al*., 1981; Dreessen and Lemli, 1982; Grimminger and Leng-Peschlow, 1988; Krumbiegel and Schulz, 1993; Lang, 1993; Brusick and Mengs, 1997; Morales *et al*., 2009). Rhein is mutagenic in *Salmonella typhimurium* strains TA98 and TA100 when tested in the presence of induced rat or hamster liver S9 mix (Appendix B); no *in vivo* mutagenicity data are available for rhein. Aloe-emodin is a well documented *in vitro* mutagen, with or without S9, in both bacterial and mammalian systems (Heidemann *et al*., 1993; Müller *et al*., 1996; Nesslany *et al*., 2009); *in vivo* DNA damage studies with aloe-emodin in mice using the comet assay gave positive

results in both colon and kidney cells (Nesslany *et al*., 2009). Emodin is also a well documented *in vitro* mutagen but data from *in vivo* studies suggest that emodin does not induce chromosomal damage in erythrocytes (micronucleus test) (Mengs *et al*., 1997; NTP, 2001). Overall, *in vitro* studies provide evidence of mutagenicity of some senna constituents and metabolites. Additional *in vivo* studies may help to define any potential genotoxic hazard associated with senna exposure.

Other carcinogenicity studies have been conducted with senna. Mitchell *et al.* (2006) conducted a 2-year oral gavage study of senna in Sprague-Dawley rats (0, 25, 100, or 300 mg/kg per day). There were no treatment-related carcinogenic effects in any target organ and there was only a slight intestinal hyperplasia in the treated groups. In another 2-year study in Sprague-Dawley rats administered a senna extract (reported to contain up to 35% to 42% sennosides) in drinking water (to deliver 0, 5, 15, or 25 mg/kg per day of the test article), no intestinal lesions were observed in treated or control groups (Lydén-Sokolowski *et al*., 1993). Also, administration of 30 and 60 mg/kg senna extract by oral gavage (six times per week) for 110 weeks in male Wistar rats did not induce aberrant crypt foci or tumors (Borrelli *et al*., 2005).

Chronic exposure of rodents to specific anthraquinones has resulted in increased incidences of tumors in some tissues (Mori *et al*., 1985, 1986, 1990; Doi *et al*., 2005). In studies of a series of anthraquinones conducted by the NTP, the occurrence and site of carcinogenicity appeared to be directly related to the number, position, or type of side chain substitutions (Doi *et al*., 2005). Further, in rodent studies conducted by Mori *et al*. (1985, 1986, 1990), exposure to hydroxyanthraquinones (1-hydroxyanthraquinone and danthron), structurally similar to rhein, caused tumors in the large intestine of rats.

In the current study, senna administered in the feed caused intestinal hyperplasia in heterozygous F1 $p53^{+/-}$ mice. Although senna was evaluated in this current study, specific senna constituents or metabolites have not been evaluated in this model.

CONCLUSIONS

Under the conditions of this 40-week feed study, there was *no evidence of carcinogenic activity** of senna in male or female C3B6.129F1/Tac-*Trp53*^{tm1Brd} N12 haplo-insufficient mice exposed to 100, 300, 1,000, 3,000, or 10,000 ppm.

Senna induced epithelial hyperplasia of the large intestine (colon and cecum) in male and female mice.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 8. A summary of the Technical Reports Peer Review Panel comments and the public discussion on this Report appears on page 10.

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APPENDIX A
SUMMARY OF LESIONS
IN HETEROZYGOUS F1 p53$^{+/-}$ MICE
IN THE 40-WEEK FEED STUDY OF SENNA

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TABLE A1
Summary of the Incidence of Neoplasms in Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna[a]

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Disposition Summary						
Animals initially in study	25	25	25	25	25	25
Early deaths						
Natural deaths		1			2	1
Survivors						
Terminal sacrifice	25	24	25	25	23	24
Animals examined microscopically	25	25	25	25	25	25
Alimentary System						
Intestine large, cecum	(25)	(25)	(25)	(25)	(23)	(25)
Intestine large, colon	(25)	(25)	(25)	(25)	(24)	(25)
Intestine large, rectum	(24)	(25)	(25)	(25)	(24)	(25)
Intestine small, duodenum	(25)	(25)	(25)	(25)	(23)	(25)
Intestine small, jejunum	(25)	(25)	(25)	(25)	(23)	(24)
Liver	(25)	(25)	(25)	(25)	(25)	(25)
Hemangiosarcoma, metastatic, heart		1 (4%)				
Hepatocellular adenoma	1 (4%)		5 (20%)		1 (4%)	1 (4%)
Hepatocellular adenoma, multiple					1 (4%)	
Pancreas	(25)	(25)	(25)	(25)	(25)	(25)
Hemangiosarcoma, metastatic, heart		1 (4%)				
Stomach, forestomach	(25)	(25)	(25)	(25)	(25)	(25)
Stomach, glandular	(25)	(25)	(25)	(25)	(25)	(25)
Tooth	(1)	(0)	(0)	(0)	(0)	(0)
Cardiovascular System						
Blood vessel	(25)	(25)	(25)	(25)	(25)	(25)
Heart	(25)	(25)	(25)	(25)	(25)	(25)
Hemangiosarcoma		1 (4%)				
Endocrine System						
Adrenal cortex	(24)	(25)	(25)	(25)	(25)	(25)
Hemangiosarcoma, metastatic, heart		1 (4%)				
Adrenal medulla	(24)	(25)	(25)	(25)	(25)	(25)
Hemangiosarcoma, metastatic, heart		1 (4%)				
General Body System						
None						
Genital System						
Coagulating gland	(2)	(0)	(1)	(0)	(0)	(3)
Epididymis	(25)	(25)	(25)	(25)	(25)	(25)
Preputial gland	(25)	(25)	(25)	(25)	(25)	(23)
Prostate	(25)	(25)	(25)	(25)	(25)	(25)
Seminal vesicle	(25)	(25)	(25)	(25)	(25)	(25)
Hemangiosarcoma, metastatic, heart		1 (4%)				
Testes	(25)	(25)	(25)	(25)	(25)	(25)
Hemangiosarcoma, metastatic, heart		1 (4%)				

TABLE A1
Summary of the Incidence of Neoplasms in Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Hematopoietic System						
Bone marrow	(25)	(25)	(25)	(25)	(25)	(25)
Lymph node, mandibular	(25)	(25)	(25)	(25)	(24)	(25)
Lymph node, mesenteric	(24)	(25)	(25)	(25)	(25)	(25)
Spleen	(25)	(25)	(25)	(25)	(23)	(25)
Thymus	(25)	(24)	(25)	(25)	(23)	(25)
Integumentary System						
Skin	(25)	(25)	(25)	(25)	(25)	(25)
Hemangioma					1 (4%)	
Subcutaneous tissue, sarcoma			1 (4%)			
Musculoskeletal System						
Bone	(25)	(25)	(25)	(25)	(25)	(25)
Femur, osteoma			1 (4%)			
Maxilla, osteosarcoma				3 (12%)	1 (4%)	
Vertebra, osteosarcoma					1 (4%)	
Nervous System						
Brain	(25)	(25)	(25)	(25)	(25)	(25)
Hemangiosarcoma, metastatic, heart		1 (4%)				
Respiratory System						
Lung	(25)	(25)	(25)	(25)	(25)	(25)
Alveolar/bronchiolar adenoma	1 (4%)		1 (4%)			
Hemangiosarcoma, metastatic, heart		1 (4%)				
Nose	(25)	(25)	(25)	(25)	(25)	(25)
Special Senses System						
Harderian gland	(0)	(0)	(0)	(0)	(1)	(0)
Carcinoma					1 (100%)	
Urinary System						
Kidney	(25)	(25)	(25)	(25)	(25)	(25)
Hemangiosarcoma, metastatic, heart		1 (4%)				
Urinary bladder	(25)	(25)	(25)	(25)	(25)	(25)

TABLE A1
Summary of the Incidence of Neoplasms in Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Systemic Lesions						
Multiple organs[b]	(25)	(25)	(25)	(25)	(25)	(25)
Lymphoma, malignant	1 (4%)					
Neoplasm Summary						
Total animals with primary neoplasms[c]	3	1	8	3	5	1
Total primary neoplasms	3	1	8	3	6	1
Total animals with benign neoplasms	2		7		3	1
Total benign neoplasms	2		7		3	1
Total animals with malignant neoplasms	1	1	1	3	3	
Total malignant neoplasms	1	1	1	3	3	
Total animals with metastatic neoplasms		1				
Total metastatic neoplasms		9				

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna[a]

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Disposition Summary						
Animals initially in study	25	25	25	25	25	25
Early deaths						
Natural deaths		1			2	1
Survivors						
Terminal sacrifice	25	24	25	25	23	24
Animals examined microscopically	25	25	25	25	25	25
Alimentary System						
Intestine large, cecum	(25)	(25)	(25)	(25)	(23)	(25)
Epithelium, hyperplasia						22 (88%)
Intestine large, colon	(25)	(25)	(25)	(25)	(24)	(25)
Epithelium, hyperplasia					3 (13%)	25 (100%)
Intestine large, rectum	(24)	(25)	(25)	(25)	(24)	(25)
Epithelium, hyperplasia						1 (4%)
Intestine small, duodenum	(25)	(25)	(25)	(25)	(23)	(25)
Ectopic tissue					1 (4%)	
Intestine small, jejunum	(25)	(25)	(25)	(25)	(23)	(24)
Epithelium, hyperplasia			1 (4%)			
Liver	(25)	(25)	(25)	(25)	(25)	(25)
Basophilic focus	2 (8%)	3 (12%)	2 (8%)			4 (16%)
Clear cell focus				1 (4%)		
Degeneration, cystic				1 (4%)		
Eosinophilic focus		1 (4%)			1 (4%)	
Fibrosis				1 (4%)		
Hematopoietic cell proliferation		2 (8%)				
Inflammation, chronic active	15 (60%)	17 (68%)	19 (76%)	22 (88%)	14 (56%)	10 (40%)
Mixed cell focus			1 (4%)	1 (4%)	1 (4%)	
Necrosis	1 (4%)	1 (4%)		1 (4%)	1 (4%)	1 (4%)
Tension lipidosis	1 (4%)					
Kupffer cell, hyperplasia, multifocal					1 (4%)	
Pancreas	(25)	(25)	(25)	(25)	(25)	(25)
Stomach, forestomach	(25)	(25)	(25)	(25)	(25)	(25)
Inflammation				1 (4%)		
Epithelium, hyperplasia				1 (4%)		
Stomach, glandular	(25)	(25)	(25)	(25)	(25)	(25)
Mineralization			1 (4%)	1 (4%)		
Glands, cyst	2 (8%)	2 (8%)	2 (8%)		1 (4%)	
Tooth	(1)	(0)	(0)	(0)	(0)	(0)
Malformation	1 (100%)					
Cardiovascular System						
Blood vessel	(25)	(25)	(25)	(25)	(25)	(25)
Inflammation		1 (4%)				
Heart	(25)	(25)	(25)	(25)	(25)	(25)
Mineralization					1 (4%)	
Valve, pigmentation, melanin	1 (4%)					

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Endocrine System						
Adrenal cortex	(24)	(25)	(25)	(25)	(25)	(25)
Atrophy				1 (4%)		
Embolus bacterial					1 (4%)	
Hypertrophy	3 (13%)	1 (4%)	2 (8%)	1 (4%)	1 (4%)	4 (16%)
Subcapsular, hyperplasia	4 (17%)					8 (32%)
Adrenal medulla	(24)	(25)	(25)	(25)	(25)	(25)
General Body System						
None						
Genital System						
Coagulating gland	(2)	(0)	(1)	(0)	(0)	(3)
Epithelium, hyperplasia	2 (100%)		1 (100%)			3 (100%)
Epididymis	(25)	(25)	(25)	(25)	(25)	(25)
Inflammation						1 (4%)
Epithelium, karyomegaly	14 (56%)				1 (4%)	14 (56%)
Preputial gland	(25)	(25)	(25)	(25)	(25)	(23)
Ectasia						1 (4%)
Hyperplasia					1 (4%)	
Duct, ectasia	4 (16%)					2 (9%)
Prostate	(25)	(25)	(25)	(25)	(25)	(25)
Infiltration cellular, mononuclear cell	1 (4%)		2 (8%)	1 (4%)		
Inflammation					1 (4%)	1 (4%)
Seminal vesicle	(25)	(25)	(25)	(25)	(25)	(25)
Testes	(25)	(25)	(25)	(25)	(25)	(25)
Germinal epithelium, degeneration			1 (4%)			
Rete testes, cyst	1 (4%)					
Hematopoietic System						
Bone marrow	(25)	(25)	(25)	(25)	(25)	(25)
Hyperplasia		1 (4%)				1 (4%)
Lymph node, mandibular	(25)	(25)	(25)	(25)	(24)	(25)
Atrophy				3 (12%)	2 (8%)	
Hyperplasia, lymphoid			1 (4%)	2 (8%)		
Lymph node, mesenteric	(24)	(25)	(25)	(25)	(25)	(25)
Atrophy		2 (8%)				
Hyperplasia, lymphoid				1 (4%)		
Spleen	(25)	(25)	(25)	(25)	(23)	(25)
Hematopoietic cell proliferation		1 (4%)	1 (4%)			
Infiltration cellular, plasma cell						1 (4%)
Lymphoid follicle, atrophy		2 (8%)				1 (4%)
Lymphoid follicle, hyperplasia	1 (4%)	1 (4%)				1 (4%)
Thymus	(25)	(24)	(25)	(25)	(23)	(25)
Atrophy		1 (4%)		2 (8%)		1 (4%)
Cyst		1 (4%)	1 (4%)			
Cortex, hyperplasia, lymphoid					1 (4%)	
Epithelial cell, hyperplasia				1 (4%)		
Medulla, hyperplasia, lymphoid		2 (8%)	3 (12%)	2 (8%)	4 (17%)	
Integumentary System						
Skin	(25)	(25)	(25)	(25)	(25)	(25)

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Musculoskeletal System						
Bone	(25)	(25)	(25)	(25)	(25)	(25)
Hyperostosis						1 (4%)
Nervous System						
Brain	(25)	(25)	(25)	(25)	(25)	(25)
Respiratory System						
Lung	(25)	(25)	(25)	(25)	(25)	(25)
Thrombosis					1 (4%)	
Alveolar epithelium, hyperplasia						1 (4%)
Nose	(25)	(25)	(25)	(25)	(25)	(25)
Inflammation	2 (8%)	1 (4%)	2 (8%)		1 (4%)	
Glands, olfactory epithelium, hyperplasia			1 (4%)			
Glands, respiratory epithelium, hyperplasia	1 (4%)	6 (24%)	2 (8%)	2 (8%)	3 (12%)	
Respiratory epithelium, cyst					1 (4%)	
Respiratory epithelium, hyperplasia	2 (8%)		1 (4%)	2 (8%)		1 (4%)
Septum, hyalinization	15 (60%)	1 (4%)			2 (8%)	16 (64%)
Special Senses System						
Harderian gland	(0)	(0)	(0)	(0)	(1)	(0)
Urinary System						
Kidney	(25)	(25)	(25)	(25)	(25)	(25)
Infiltration cellular, mononuclear cell	1 (4%)					
Inflammation					1 (4%)	1 (4%)
Mineralization		4 (16%)	1 (4%)		1 (4%)	
Necrosis					1 (4%)	
Nephropathy	7 (28%)	7 (28%)	7 (28%)	7 (28%)	9 (36%)	8 (32%)
Cortex, cyst					1 (4%)	
Papilla, necrosis						1 (4%)
Urinary bladder	(25)	(25)	(25)	(25)	(25)	(25)
Inflammation					1 (4%)	1 (4%)

TABLE A3
Summary of the Incidence of Neoplasms in Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna[a]

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Disposition Summary						
Animals initially in study	25	25	25	25	25	25
Early deaths						
Moribund sacrifice	2	2	2	2	2	1
Natural deaths				1	1	
Survivors						
Terminal sacrifice	23	23	23	22	22	24
Animals examined microscopically	25	25	25	25	25	25
Alimentary System						
Gallbladder	(25)	(25)	(25)	(25)	(25)	(25)
Intestine large, cecum	(25)	(25)	(25)	(25)	(25)	(25)
Intestine large, colon	(25)	(25)	(25)	(25)	(25)	(25)
Intestine large, rectum	(25)	(25)	(25)	(25)	(25)	(25)
Intestine small, duodenum	(25)	(25)	(25)	(24)	(25)	(25)
Intestine small, jejunum	(25)	(25)	(25)	(25)	(25)	(25)
Liver	(25)	(25)	(25)	(25)	(25)	(25)
Pancreas	(25)	(25)	(25)	(25)	(25)	(25)
Salivary glands	(25)	(25)	(25)	(25)	(25)	(25)
Stomach, forestomach	(25)	(25)	(25)	(25)	(25)	(25)
Stomach, glandular	(25)	(25)	(25)	(25)	(25)	(25)
Granulosa cell tumor malignant, metastatic, ovary	1 (4%)					
Tooth	(0)	(1)	(0)	(0)	(0)	(0)
Cardiovascular System						
Heart	(25)	(25)	(25)	(25)	(25)	(25)
Endocrine System						
Adrenal cortex	(25)	(25)	(25)	(25)	(25)	(25)
Adrenal medulla	(25)	(25)	(25)	(25)	(25)	(25)
Pheochromocytoma benign	1 (4%)					
Pituitary gland	(25)	(25)	(25)	(25)	(25)	(25)
Thyroid gland	(25)	(25)	(25)	(25)	(25)	(25)
General Body System						
Peritoneum	(1)	(0)	(0)	(0)	(0)	(0)
Granulosa cell tumor malignant, metastatic, ovary	1 (100%)					
Genital System						
Ovary	(25)	(25)	(25)	(25)	(25)	(25)
Cystadenoma	1 (4%)					
Granulosa cell tumor malignant	1 (4%)					
Uterus	(25)	(25)	(25)	(25)	(25)	(25)
Polyp, stromal			1 (4%)		1 (4%)	

TABLE A3
Summary of the Incidence of Neoplasms in Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Hematopoietic System						
Bone marrow	(25)	(25)	(25)	(25)	(25)	(25)
Lymph node	(2)	(1)	(0)	(0)	(0)	(0)
Renal, granulosa cell tumor malignant, metastatic, ovary	1 (50%)					
Lymph node, mandibular	(25)	(25)	(25)	(25)	(25)	(25)
Lymph node, mesenteric	(23)	(25)	(25)	(25)	(25)	(25)
Spleen	(25)	(25)	(25)	(25)	(25)	(25)
Thymus	(24)	(22)	(22)	(22)	(22)	(24)
Integumentary System						
Mammary gland	(25)	(25)	(25)	(25)	(25)	(25)
Carcinoma				1 (4%)		
Musculoskeletal System						
Bone	(25)	(25)	(25)	(25)	(25)	(25)
Osteosarcoma						1 (4%)
Femur, osteosarcoma						1 (4%)
Maxilla, osteosarcoma		1 (4%)		1 (4%)		
Rib, osteosarcoma	1 (4%)					
Tibia, osteosarcoma		1 (4%)				
Vertebra, osteosarcoma	1 (4%)		1 (4%)	2 (8%)		
Nervous System						
Brain	(25)	(25)	(25)	(25)	(25)	(25)
Olfactory neuroblastoma, metastatic, nose						1 (4%)
Peripheral nerve	(1)	(0)	(1)	(1)	(1)	(0)
Spinal cord	(1)	(0)	(2)	(2)	(1)	(1)
Respiratory System						
Lung	(25)	(25)	(25)	(25)	(25)	(25)
Osteosarcoma, metastatic, bone		1 (4%)				1 (4%)
Nose	(25)	(25)	(25)	(25)	(25)	(25)
Olfactory epithelium, meuroblastoma malignant						1 (4%)
Special Senses System						
None						
Urinary System						
Kidney	(25)	(25)	(25)	(25)	(25)	(25)
Granulosa cell tumor malignant, metastatic, ovary	1 (4%)					
Urinary bladder	(25)	(25)	(25)	(25)	(25)	(25)

TABLE A3
Summary of the Incidence of Neoplasms in Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Systemic Lesions						
Multiple organs[b]	(25)	(25)	(25)	(25)	(25)	(25)
Histiocytic sarcoma		1 (4%)				
Lymphoma malignant	1 (4%)		1 (4%)		1 (4%)	
Neoplasm Summary						
Total animals with primary neoplasms[c]	6	3	3	4	2	3
Total primary neoplasms	6	3	3	4	2	3
Total animals with benign neoplasms	2		1		1	
Total benign neoplasms	2		1		1	
Total animals with malignant neoplasms	4	3	2	4	1	3
Total malignant neoplasms	4	3	2	4	1	3
Total animals with metastatic neoplasms	1	1				2
Total metastatic neoplasms	4	1				2

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Number of animals with any tissue examined microscopically
[c] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna[a]

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Disposition Summary						
Animals initially in study	25	25	25	25	25	25
Early deaths						
Moribund sacrifice	2	2	2	2	2	1
Natural deaths				1	1	
Survivors						
Terminal sacrifice	23	23	23	22	22	24
Animals examined microscopically	25	25	25	25	25	25
Alimentary System						
Gallbladder	(25)	(25)	(25)	(25)	(25)	(25)
Inflammation						1 (4%)
Intestine large, cecum	(25)	(25)	(25)	(25)	(25)	(25)
Epithelium, hyperplasia						19 (76%)
Intestine large, colon	(25)	(25)	(25)	(25)	(25)	(25)
Epithelium, hyperplasia					7 (28%)	25 (100%)
Intestine large, rectum	(25)	(25)	(25)	(25)	(25)	(25)
Epithelium, hyperplasia						1 (4%)
Intestine small, duodenum	(25)	(25)	(25)	(24)	(25)	(25)
Intestine small, jejunum	(25)	(25)	(25)	(25)	(25)	(25)
Diverticulum			1 (4%)	1 (4%)		
Liver	(25)	(25)	(25)	(25)	(25)	(25)
Basophilic focus						1 (4%)
Clear cell focus						1 (4%)
Hematopoietic cell proliferation	1 (4%)				1 (4%)	1 (4%)
Infiltration cellular, mixed cell				2 (8%)		
Inflammation, chronic active	13 (52%)	2 (8%)	1 (4%)	5 (20%)	6 (24%)	9 (36%)
Mixed cell focus	1 (4%)					
Necrosis					1 (4%)	
Necrosis, focal	1 (4%)					
Tension lipidosis				1 (4%)		
Pancreas	(25)	(25)	(25)	(25)	(25)	(25)
Atrophy					1 (4%)	
Salivary glands	(25)	(25)	(25)	(25)	(25)	(25)
Hyperplasia, lymphoid	1 (4%)			1 (4%)		1 (4%)
Stomach, forestomach	(25)	(25)	(25)	(25)	(25)	(25)
Inflammation			1 (4%)			
Epithelium, hyperplasia			1 (4%)			
Stomach, glandular	(25)	(25)	(25)	(25)	(25)	(25)
Epithelium, metaplasia, squamous				1 (4%)	1 (4%)	
Glands, cyst	1 (4%)	4 (16%)	3 (12%)	2 (8%)	1 (4%)	
Tooth	(0)	(1)	(0)	(0)	(0)	(0)
Cardiovascular System						
Heart	(25)	(25)	(25)	(25)	(25)	(25)
Cardiomyopathy					2 (8%)	1 (4%)
Embolus tumor		1 (4%)				
Infiltration cellular, lymphocyte					1 (4%)	
Epicardium, inflammation					1 (4%)	
Valve, thrombosis				1 (4%)		

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Endocrine System						
Adrenal cortex	(25)	(25)	(25)	(25)	(25)	(25)
Hypertrophy			1 (4%)			
Subcapsular, hyperplasia	16 (64%)	2 (8%)	1 (4%)	2 (8%)	1 (4%)	16 (64%)
Adrenal medulla	(25)	(25)	(25)	(25)	(25)	(25)
Pituitary gland	(25)	(25)	(25)	(25)	(25)	(25)
Pars distalis, hyperplasia			1 (4%)			
Thyroid gland	(25)	(25)	(25)	(25)	(25)	(25)
Cyst		1 (4%)				
General Body System						
Peritoneum	(1)	(0)	(0)	(0)	(0)	(0)
Genital System						
Ovary	(25)	(25)	(25)	(25)	(25)	(25)
Angiectasis		1 (4%)				
Atrophy	3 (12%)	3 (12%)	2 (8%)	3 (12%)	2 (8%)	4 (16%)
Cyst	1 (4%)	2 (8%)		3 (12%)	1 (4%)	1 (4%)
Mineralization			1 (4%)			
Pigmentation, hemosiderin			1 (4%)			
Uterus	(25)	(25)	(25)	(25)	(25)	(25)
Endometrium, hyperplasia, cystic	15 (60%)	7 (28%)	3 (12%)	5 (20%)	6 (24%)	14 (56%)
Hematopoietic System						
Bone marrow	(25)	(25)	(25)	(25)	(25)	(25)
Hyperplasia	1 (4%)	1 (4%)		1 (4%)		1 (4%)
Inflammation		1 (4%)				
Necrosis					1 (4%)	
Lymph node	(2)	(1)	(0)	(0)	(0)	(0)
Lymph node, mandibular	(25)	(25)	(25)	(25)	(25)	(25)
Atrophy			1 (4%)	2 (8%)	1 (4%)	1 (4%)
Hyperplasia, lymphoid	1 (4%)					
Infiltration cellular, mast cell		1 (4%)				
Lymph node, mesenteric	(23)	(25)	(25)	(25)	(25)	(25)
Atrophy	1 (4%)	1 (4%)	2 (8%)	2 (8%)	2 (8%)	
Hyperplasia, lymphoid						1 (4%)
Inflammation, suppurative				1 (4%)		
Spleen	(25)	(25)	(25)	(25)	(25)	(25)
Hematopoietic cell proliferation	1 (4%)	1 (4%)		1 (4%)	1 (4%)	
Lymphoid follicle, atrophy	1 (4%)	1 (4%)				
Lymphoid follicle, hyperplasia		1 (4%)		2 (8%)		
Thymus	(24)	(22)	(22)	(22)	(22)	(24)
Atrophy	3 (13%)	1 (5%)	1 (5%)	1 (5%)	1 (5%)	2 (8%)
Hyperplasia, lymphoid	1 (4%)					
Medulla, hyperplasia, lymphoid	4 (17%)	3 (14%)	3 (14%)	6 (27%)	5 (23%)	4 (17%)
Integumentary System						
Mammary gland	(25)	(25)	(25)	(25)	(25)	(25)
Duct, ectasia	1 (14%)				1 (4%)	1 (4%)

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Musculoskeletal System						
Bone	(25)	(25)	(25)	(25)	(25)	(25)
Callus	1 (4%)					
Fibro-osseous lesion		2 (8%)		2 (8%)	1 (4%)	1 (4%)
Nervous System						
Brain	(25)	(25)	(25)	(25)	(25)	(25)
Peripheral nerve	(1)	(0)	(1)	(1)	(1)	(0)
Degeneration	1 (100%)					
Spinal cord	(1)	(0)	(2)	(2)	(1)	(1)
Degeneration			2 (100%)	2 (100%)		1 (100%)
Nerve, degeneration	1 (100%)					
Respiratory System						
Lung	(25)	(25)	(25)	(25)	(25)	(25)
Alveolar epithelium, hyperplasia	1 (4%)					
Artery, infiltration cellular, mixed cell		1 (4%)				
Serosa, inflammation					1 (4%)	
Nose	(25)	(25)	(25)	(25)	(25)	(25)
Inflammation	1 (4%)	1 (4%)	1 (4%)	2 (8%)		
Inflammation, suppurative				1 (4%)		
Glands, olfactory epithelium, dilatation			1 (4%)			
Glands, respiratory epithelium, hyperplasia	1 (4%)	6 (24%)	4 (16%)	4 (16%)	4 (16%)	
Olfactory epithelium, degeneration						1 (4%)
Septum, hyalinization	17 (68%)	2 (8%)	3 (12%)	2 (8%)	3 (12%)	15 (60%)
Special Senses System						
None						
Urinary System						
Kidney	(25)	(25)	(25)	(25)	(25)	(25)
Hyperplasia, lymphoid						1 (4%)
Mineralization	1 (4%)		1 (4%)			
Nephropathy	3 (12%)	6 (24%)	5 (20%)	4 (16%)	9 (36%)	2 (8%)
Urinary bladder	(25)	(25)	(25)	(25)	(25)	(25)
Infiltration, cellular, lymphocyte		1 (4%)	3 (12%)	2 (8%)		
Inflammation			1 (4%)			
Transitional epithelium, vacuolization cytoplasmic					1 (4%)	

APPENDIX B
GENETIC TOXICOLOGY

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TABLE B1
Mutagenicity of Senna in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Without S9	With 30% hamster S9	With 30% hamster S9	With 30% hamster S9	With 30% hamster S9	
Assay performed at SRI International (sample one)							
TA100	0	90 ± 1	113 ± 14	95 ± 10	122 ± 4	126 ± 7	
	100	94 ± 3	95 ± 4				
	333	105 ± 5	130 ± 4				
	1,000	90 ± 4	119 ± 10	103 ± 7	131 ± 4	111 ± 3	
	3,333	89 ± 9[b]	123 ± 12[b]	91 ± 3	144 ± 6	146 ± 8	
	6,666			103 ± 5	158 ± 5	174 ± 12	
	10,000	74 ± 1[b]	165 ± 3[b]	138 ± 6	174 ± 9	186 ± 8	
	16,666			121 ± 9	199 ± 12	195 ± 4	
Trial summary		Negative	Equivocal	Equivocal	Weakly Positive	Weakly Positive	
Positive control[c]		1,569 ± 22	676 ± 63	610 ± 26	775 ± 25	561 ± 39	
		With 30% rat S9	**With 30% rat S9**	**With 30% rat S9**	**With 30% rat S9**		
TA100 (continued)	0	134 ± 10	120 ± 7	145 ± 2	135 ± 10		
	100	140 ± 9					
	333	135 ± 2					
	1,000	134 ± 3	121 ± 12	151 ± 5	145 ± 10		
	3,333	154 ± 3[b]	133 ± 1	151 ± 8	174 ± 1		
	6,666		129 ± 5	172 ± 9	209 ± 12		
	10,000	166 ± 9	144 ± 7	204 ± 8	238 ± 12		
	16,666		142 ± 4	248 ± 18	231 ± 18[d]		
Trial summary		Negative	Negative	Weakly Positive	Weakly Positive		
Positive control		488 ± 9	474 ± 17	470 ± 12	396 ± 13		
		Without S9	**With 30% hamster S9**	**With 30% hamster S9**	**With 30% hamster S9**	**With 30% hamster S9**	**With 30% hamster S9**
TA98	0	24 ± 4	25 ± 6	26 ± 3	25 ± 4	24 ± 3	18 ±3
	100	28 ± 2	21 ± 4	12 ± 3	20 ± 2	21 ± 3	
	333	25 ± 3	24 ± 4	18 ± 3	20 ± 3	16 ± 1	
	1,000	27 ± 2	28 ± 5	24 ± 4	28 ± 1	35 ± 4	30 ±1
	1,666						35 ±2
	3,333	24 ± 1[b]	51 ± 4[b]	39 ± 7	47 ± 8	71 ± 3	59 ±4
	6,666						75 ±1
	10,000	26 ± 2[b]	70 ± 2[b]	50 ± 1	101 ± 9	137 ± 5	117 ±8
Trial summary		Negative	Positive	Positive	Positive	Positive	Positive
Positive control		557 ± 17	308 ± 22	529 ± 43	417 ± 13	354 ± 17	272 ±8

TABLE B1
Mutagenicity of Senna in *Salmonella typhimurium*

Strain	Dose (µg/plate)	With 30% rat S9	With 30% rat S9	With 30% rat S9	With 30% rat S9	With 30% rat S9	
TA98 (continued)	0	41 ± 2	21 ± 3	31 ± 2	36 ± 2	38 ± 3	
	100	32 ± 5	24 ± 4	30 ± 4	33 ± 4		
	333	21 ± 1	23 ± 5	29 ± 5	30 ± 4		
	1,000	24 ± 3	31 ± 1	26 ± 1	34 ± 3	54 ± 2	
	1,666					64 ± 12	
	3,333	82 ± 6[b]	34 ± 4	69 ± 4	107 ± 11	121 ± 11	
	6,666					150 ± 7	
	10,000	77 ± 39[e]	59 ± 9	167 ± 9	237 ± 16	143 ± 14	
Trial summary		Positive	Positive	Positive	Positive	Positive	
Positive control		122 ± 13	119 ± 6	133 ± 9	104 ± 10	81 ± 3	
Assay performed at BioReliance Corporation (sample two)							
		Without S9	**Without S9**	**With 10% hamster S9**	**With 30% hamster S9**	**With 10% rat S9**	**With 30% rat S9**
TA100	0	108 ± 3	168 ± 4	190 ± 17	105 ± 2	181 ± 4	120 ± 1
	100	110 ± 7[b]	198 ± 9[b]	173 ± 3[b]	109 ± 3[b]	216 ± 6[b]	109 ± 3[b]
	333	122 ± 8[b]	241 ± 27[b]	201 ± 4[b]	116 ± 3[b]	216 ± 8[b]	114 ± 6[b]
	1,000	109 ± 1[b]	201 ± 10[b]	211 ± 36[b]	114 ± 3[b]	230 ± 13[b]	111 ± 3[b]
	3,333	107 ± 2[b]	221 ± 9[b]	190 ± 4[b]	115 ± 6[b]	197 ± 11[b]	129 ± 10[b]
	10,000	106 ± 1[b]	192 ± 8[b]	193 ± 3[b]	119 ± 6[b]	195 ± 10[b]	106 ± 2[b]
Trial summary		Negative	Equivocal	Negative	Negative	Negative	Negative
Positive control		606 ± 3	511 ± 19	1,291 ± 70	842 ± 41	915 ± 28	632 ± 14
TA1535	0	10 ± 5[e]	12 ± 2	9 ± 2	13 ± 1	15 ± 3	16 ± 0
	100	17 ± 1[b]	11 ± 3[b]	11 ± 2[b]	15 ± 1[b]	8 ± 1[b]	17 ± 1[b]
	333	16 ± 1[b]	10 ± 2[b]	12 ± 0[b]	14 ± 1[b]	10 ± 1[b]	17 ± 1[b]
	1,000	15 ± 0[b]	12 ± 2[b]	12 ± 3[b]	16 ± 2[b]	12 ± 3[b]	16 ± 1[b]
	3,333	18 ± 1[b]	12 ± 1[b]	9 ± 0[b]	16 ± 0[b]	8 ± 0[b]	16 ± 0[b]
	10,000	16 ± 0[b]	14 ± 2[b]	11 ± 2[b]	17 ± 1[b]	13 ± 1[b]	16 ± 1[b]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		655 ± 27	244 ± 11	145 ± 10	494 ± 62	70 ± 2	79 ± 4
TA97	0	91 ± 6	140 ± 22	177 ± 3	101 ± 4	144 ± 16	108 ± 4
	100	108 ± 5[b]	200 ± 2[b]	150 ± 5[b]	97 ± 4[b]	164 ± 6[b]	115 ± 7[b]
	333	111 ± 2[b]	198 ± 3[b]	163 ± 5[b]	101 ± 1[b]	180 ± 10[b]	116 ± 7[b]
	1,000	106 ± 1[b]	196 ± 9[b]	170 ± 4[b]	119 ± 5[b]	189 ± 4[b]	105 ± 1[b]
	3,333	111 ± 4[b]	189 ± 5[b]	166 ± 2[b]	121 ± 4[b]	191 ± 2[b]	119 ± 7[b]
	10,000	122 ± 8[b]	193 ± 4[b]	174 ± 4[b]	120 ± 9[b]	193 ± 6[b]	114 ± 3[b]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		982 ± 172	714 ± 80	1,456 ± 33	1,700 ± 34	1,741 ± 109	534 ± 74

TABLE B1
Mutagenicity of Senna in *Salmonella typhimurium*

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% hamster S9	With 10% hamster S9	With 30% hamster S9
TA98	0	13 ± 0	13 ± 1	22 ± 2	19 ± 2	11 ± 1
	100	13 ± 1[b]	13 ± 2[b]	18 ± 1[b]	24 ± 5[b]	11 ± 1[b]
	333	14 ± 0[b]	12 ± 1[b]	17 ± 1[b]	20 ± 7[b]	16 ± 1[b]
	1,000	14 ± 1[b]	17 ± 1[b]	31 ± 4[b]	21 ± 2[b]	13 ± 1[b]
	3,333	16 ± 1[b]	15 ± 1[b]	40 ± 2[b]	21 ± 3[b]	15 ± 1[b]
	10,000	16 ± 1[b]	16 ± 1[b]	38 ± 8[b]	28 ± 1[b]	17 ± 1
Trial summary		Negative	Negative	Equivocal	Negative	Negative
Positive control		118 ± 3	115 ± 7	1,307 ± 61	817 ± 87	906 ± 56
		With 10% rat S9	**With 30% rat S9**			
TA98	0	16 ± 4	13 ± 1			
(continued)	100	15 ± 2[b]	14 ± 2[b]			
	333	25 ± 3[b]	13 ± 1[b]			
	1,000	22 ± 5[b]	14 ± 0[b]			
	3,333	22 ± 1[b]	18 ± 1[b]			
	10,000	25 ± 2[b]	19 ± 1[b]			
Trial summary		Negative	Negative			
Positive control		379 ± 12	191 ± 13			

[a] Data are presented as revertants/plate (mean ± standard error) from three plates. The detailed protocol is presented by Zeiger *et al*. (1992). 0 µg/plate was the solvent control.
[b] Precipitate on plate
[c] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA97), and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.
[d] Contamination
[e] Slight toxicity and precipitate on plate

TABLE B2
Mutagenicity of Senna in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% rat S9	With 10% rat S9	With 30% rat S9	With 30% rat S9
Assay performed at SITEK Research Laboratories (sample three)							
TA100	0	99 ± 6	83 ± 3	63 ± 4	60 ± 0		
	10	80 ± 6	61 ± 2	61 ± 3	58 ± 1		
	25	82 ± 2	65 ± 8	73 ± 5	56 ± 3		
	50	71 ± 3	61 ± 7	77 ± 4	62 ± 4		
	100	80 ± 4	46 ± 2	64 ± 4	48 ± 3		
	250	104 ± 5	56 ± 5	65 ± 1	36 ± 5		
Trial summary		Negative	Negative	Negative	Negative		
Positive control[b]		274 ± 28	556 ± 21	498 ± 27	936 ± 167		
TA98	0	26 ± 4	25 ± 2	32 ± 2	42 ± 6	34 ± 4	51 ± 4
	10	28 ± 4	22 ± 2	29 ± 1	28 ± 4	37 ± 5	46 ± 10
	25	24 ± 3	25 ± 2	23 ± 4	22 ± 2	26 ± 8	62 ± 4
	50	40 ± 5	26 ± 1	24 ± 1	34 ± 1	42 ± 1	56 ± 8
	100	23 ± 5	20 ± 1	18 ± 1	28 ± 4	35 ± 7	42 ± 8
	250	28 ± 4	18 ± 1	17 ± 2	22 ± 2	29 ± 1	39 ± 8
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		432 ± 27	431 ± 19	1,102 ± 31	544 ± 35	285 ± 7	317 ± 23
***Escherichia coli* WP2 *uvrA*/pKM101**							
	0	160 ± 15	166 ± 5	219 ± 10	206 ± 5		
	10	202 ± 7	202 ± 7	235 ± 12	330 ± 12		
	25	205 ± 9	208 ± 49	287 ± 13	244 ± 16		
	50	163 ± 5	116 ± 12	206 ± 5	186 ± 12		
	100	176 ± 9	107 ± 4	219 ± 5	153 ± 3		
	250	219 ± 23	53 ± 1	270 ± 21	126 ± 10		
Trial summary		Negative	Negative	Negative	Equivocal		
Positive control		1,568 ± 66	1,734 ± 46	824 ± 47	906 ± 29		
Assay performed at ILS, Inc. (sample four)							
TA100	0	110 ± 9	76 ± 6	77 ± 9	111 ± 7		
	125	108 ± 8	87 ± 4	89 ± 2	97 ± 1		
	250	104 ± 3[c]	84 ± 3	80 ± 3	103 ± 10[c]		
	500	141 ± 6[c]	91 ± 11	93 ± 4[c]	139 ± 3[c]		
	1,500	105 ± 11[c]	91 ± 6[c]	119 ± 6[c]	125 ± 6[c]		
	3,000	130 ± 3[c]	95 ± 3[c]	132 ± 3[c]	161 ± 6[c]		
	6,000	120 ± 11[c]	97 ± 3[c]	142 ± 6[c]	165 ± 2[c]		
Trial summary		Negative	Negative	Weakly Positive	Weakly Positive		
Positive control		858 ± 48	665 ± 54	2,065 ± 90	1,889 ± 34		

TABLE B2
Mutagenicity of Senna in Bacterial Tester Strains

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% rat S9	With 10% rat S9
TA98	0	21 ± 3	24 ± 2	31 ± 4	30 ± 3
	125	22 ± 2	23 ± 2	34 ± 1	23 ± 3
	250	22 ± 4[c]	25 ± 2	35 ± 2	44 ± 6[c]
	500	19 ± 2[c]	30 ± 1[c]	49 ± 6[c]	50 ± 3[c]
	1,500	22 ± 3[c]	36 ± 2[c]	64 ± 9[c]	48 ± 5[c]
	3,000	36 ± 3[c]	31 ± 3[c]	80 ± 9[c]	68 ± 14[c]
	6,000	59 ± 6[c]	63 ± 14[c]	90 ± 5[c]	85 ± 7[c]
Trial summary		Positive	Weakly Positive	Positive	Positive
Positive control		735 ± 11	611 ± 21	1,990 ± 168	1,456 ± 20
***Escherichia coli* WP2 *uvrA*/pKM101**					
	0	182 ± 6	192 ± 18	231 ± 3	243 ± 16
	125	175 ± 9	188 ± 12	232 ± 14	206 ± 10
	250	161 ± 6	172 ± 5	204 ± 8	222 ± 7
	500	161 ± 10	140 ± 9[c]	225 ± 19	223 ± 4[c]
	1,500	158 ± 11[c]	133 ± 14[c]	185 ± 44[c]	165 ± 7[c]
	3,000	155 ± 9[c]	136 ± 4[c]	158 ± 17[c]	177 ± 8[c]
	6,000	156 ± 10[c]	172 ± 20[c]	204 ± 6[c]	170 ± 9[c]
Trial summary		Negative	Negative	Negative	Negative
Positive control		1,302 ± 38	1,066 ± 47	1,740 ± 17	1,552 ± 31

[a] Assays using two different samples from the same lot of senna that was used in the 40-week study. Data are presented as revertants/plate (mean ± standard error) from three plates. 0 µg/plate was the solvent control.

[b] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

[c] Precipitate on plate

TABLE B3
Mutagenicity of Rhein in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Without S9	With 30% hamster S9	With 30% hamster S9	With 30% rat S9	With 30% rat S9
TA100	0	85 ± 3	101 ± 6	119 ± 5	129 ± 5	104 ± 10
	3	85 ± 3				
	10	94 ± 5	134 ± 8		131 ± 6	
	33	94 ± 5	122 ± 5	100 ± 3	120 ± 5	116 ± 18
	66			140 ± 3		134 ± 2
	100	91 ± 4	140 ± 6	172 ± 14	138 ± 7	156 ± 4
	166			198 ± 9		191 ± 4
	333	66 ± 5	200 ± 47	208 ± 8	169 ± 14	225 ± 19
	666		246 ± 26	180 ± 3	219 ± 35	74 ± 12
Trial summary		Negative	Positive	Positive	Positive	Positive
Positive control[b]		1,569 ± 22	676 ± 63	696 ± 31	488 ± 9	430 ± 20
TA98	0	19 ± 2	33 ± 2	21 ± 2	45 ± 3	26 ± 5
	3	21 ± 2				
	10	24 ± 3	22 ± 2		41 ± 7	
	33	26 ± 3	28 ± 0	21 ± 3	42 ± 6	37 ± 2
	66			29 ± 4		48 ± 4
	100	23 ± 1	30 ± 2	44 ± 2	45 ± 3	61 ± 4
	166			57 ± 14		78 ± 9
	333	27 ± 2	94 ± 8	68 ± 12	72 ± 5	111 ± 7
	666		124 ± 11	98 ± 8	143 ± 12	73 ± 5
Trial summary		Negative	Positive	Positive	Positive	Positive
Positive control		557 ± 17	308 ± 22	549 ± 19	122 ± 13	144 ± 6

[a] Study was performed at SRI International. Data are presented as revertants/plate (mean ± standard error) from three plates. The detailed protocol is presented by Zeiger *et al*. (1992). 0 µg/plate was the solvent control.

[b] The positive controls in the absence of metabolic activation were sodium azide (TA100) and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with both strains was 2-aminoanthracene.

TABLE B4
Mutagenicity of Chrysophanic Acid in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Without S9	With 10% hamster S9	With 10% hamster S9	With 30% hamster S9	With 5% hamster S9	With 5% hamster S9
TA100	0	111 ± 6	86 ± 3	118 ± 14	107 ± 10	98 ± 5	121 ± 3
	1	115 ± 5					
	3	91 ± 6		108 ± 6			110 ± 11
	10	94 ± 3	105 ± 15	126 ± 8	134 ± 10	104 ± 13	144 ± 16
	16			138 ± 1			133 ± 10
	33	86 ± 6	126 ± 7	143 ± 6	122 ± 10	131 ± 8	155 ± 3
	66			148 ± 8			142 ± 5
	100	83 ± 3	132 ± 5	127 ± 4	152 ± 1	124 ± 5	99 ± 13
	166			134 ± 2			124 ± 7
	333		123 ± 8	114 ± 8	160 ± 11	112 ± 7	132 ± 9
	666		118 ± 6[b]		165 ± 10[b]	81 ± 3[b]	
Trial summary		Negative	Equivocal	Negative	Weakly Positive	Negative	Negative
Positive control[c]		1,384 ± 50	856 ± 73	987 ± 39	590 ± 26	1,458 ± 109	1,058 ± 14
		With 30% hamster S9	**With 30% hamster S9**	**With 10% rat S9**	**With 10% rat S9**	**With 5% rat S9**	**With 5% rat S9**
TA100 (continued)	0	125 ± 8	132 ± 10	99 ± 12	114 ± 13	95 ± 11	105 ± 17
	3		129 ± 10		130 ± 8		131 ± 11
	10	102 ± 7	147 ± 3	126 ± 6	134 ± 4	94 ± 4	158 ± 6
	16		146 ± 4		163 ± 2		157 ± 3
	33	125 ± 9	151 ± 12	145 ± 10	157 ± 8	135 ± 6	147 ± 8
	66		162 ± 9		166 ± 4		142 ± 7
	100	145 ± 11	156 ± 10	169 ± 5	163 ± 8	148 ± 3	124 ± 12
	166		157 ± 2		151 ± 13		134 ± 10
	333	154 ± 3	123 ± 7	148 ± 4	137 ± 6	107 ± 4	109 ± 7
	666	142 ± 12[b]		121 ± 4[b]		98 ± 2[b]	
Trial summary		Negative	Negative	Weakly Positive	Equivocal	Equivocal	Equivocal
Positive control		696 ± 31	731 ± 40	369 ± 18	477 ± 35	706 ± 33	908 ± 79
		With 30% rat S9	**With 30% rat S9**	**With 30% rat S9**			
TA100 (continued)	0	135 ± 6	134 ± 5	119 ± 4			
	3			126 ± 7			
	10	154 ± 6	110 ± 11	147 ± 8			
	16			143 ± 4			
	33	139 ± 15	136 ± 5	145 ± 11			
	66			182 ± 4			
	100	189 ± 13	154 ± 10	178 ± 10			
	166			177 ± 12			
	333	193 ± 7	156 ± 6	178 ± 11			
	666	185 ± 18[b]	146 ± 4[b]				
Trial summary		Weakly Positive	Negative	Equivocal			
Positive control		393 ± 23	430 ± 20	434 ± 25			

TABLE B4
Mutagenicity of Chrysophanic Acid in *Salmonella typhimurium*

Strain	Dose (µg/plate)	Without S9	With 30% hamster S9	With 30% rat S9			
TA98	0	20 ± 1	21 ± 3	40 ± 5			
	1	22 ± 0					
	3	14 ± 3					
	10	10 ± 1	20 ± 4	32 ± 2			
	33	10 ± 1	27 ± 3	31 ± 4			
	100	13 ± 0	29 ± 1	35 ± 2			
	333		28 ± 3	31 ± 2			
	666		28 ± 2[b]	37 ± 6[b]			
Trial summary		Negative	Negative	Negative			
Positive control		515 ± 29	482 ± 30	138 ± 13			
		With 10% hamster S9	**With 30% hamster S9**	**With 5% hamster S9**	**With 10% rat S9**	**With 30% rat S9**	**With 5% rat S9**
TA1535	0	7 ± 1	7 ± 2	6 ± 1	5 ± 1	6 ± 2	5 ± 2
	3	5 ± 1	7 ± 1	7 ± 1	4 ± 2	4 ± 2	6 ± 1
	10	4 ± 1	7 ± 3	7 ± 1	3 ± 1	6 ± 1	5 ± 2
	16	6 ± 1	7 ± 2	4 ± 0	5 ± 1	6 ± 1	4 ± 1
	33	4 ± 2	9 ± 1	5 ± 1	7 ± 1	5 ± 1	4 ± 1
	66	6 ± 3	4 ± 1	6 ± 0	5 ± 0	7 ± 1	6 ± 1
	100	8 ± 2	7 ± 1	7 ± 1	7 ± 1	8 ± 1	9 ± 2
	166	6 ± 1	10 ± 1	8 ± 3	12 ± 2	10 ± 2	7 ± 1
	333	6 ± 1	9 ± 1	5 ± 0	13 ± 1	7 ± 2	8 ± 1
Trial summary		Negative	Negative	Negative	Equivocal	Negative	Negative
Positive control		68 ± 6	109 ± 16	89 ± 9	36 ± 2	48 ± 4	51 ± 3

[a] Study was performed at ILS, Inc. Data are presented as revertants/plate (mean ± standard error) from three plates. This detailed protocol is presented by Zeiger *et al*. (1992). 0 µg/plate was the solvent control.

[b] Precipitate on plate

[c] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535) and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE B5
Mutagenicity of Sennoside A in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% rat S9	With 10% rat S9
TA100	0	94 ± 5	121 ± 5	112 ± 4	113 ± 4
	12.5	112 ± 7	110 ± 3	116 ± 6	107 ± 7
	50	87 ± 8	104 ± 4	119 ± 9	119 ± 9
	125	91 ± 3	110 ± 6	105 ± 5	108 ± 2
	500	105 ± 8	100 ± 9	121 ± 6	100 ± 4
	1,500	102 ± 6	122 ± 5	113 ± 2	100 ± 4
	6,000	85 ± 9	91 ± 4	109 ± 4	107 ± 7
Trial summary		Negative	Negative	Negative	Negative
Positive control[b]		823 ± 17	955 ± 35	2,374 ± 146	1,608 ± 112
TA98	0	36 ± 1	28 ± 5	45 ± 3	36 ± 1
	12.5	28 ± 0	26 ± 2	43 ± 5	43 ± 5
	50	34 ± 3	28 ± 1	40 ± 2	47 ± 6
	125	35 ± 8	24 ± 5	51 ± 3	47 ± 6
	500	29 ± 6	37 ± 4	41 ± 5	38 ± 4
	1,500	28 ± 4	35 ± 12	36 ± 2	39 ± 5
	6,000	27 ± 7	24 ± 1	23 ± 3	27 ± 6
Trial summary		Negative	Negative	Negative	Negative
Positive control		598 ± 43	788 ± 36	1,690 ± 127	1,768 ± 71
***Escherichia coli* WP2 *uvrA*/pKM101**					
	0	185 ± 9	170 ± 12	209 ± 10	211 ± 4
	12.5	192 ± 6	184 ± 5	222 ± 3	212 ± 3
	50	189 ± 6	172 ± 8	238 ± 15	212 ± 6
	125	181 ± 9	190 ± 2	229 ± 6	234 ± 7
	500	182 ± 9	175 ± 5	222 ± 13	224 ± 17
	1,500	197 ± 11	191 ± 4	242 ± 3	219 ± 9
	6,000	194 ± 5	176 ± 14	223 ± 9	219 ± 6
Trial summary		Negative	Negative	Negative	Negative
Positive control		1,336 ± 63	1,166 ± 10	1,561 ± 26	1,654 ± 17

[a] Study was performed at ILS, Inc. Data are presented as revertants/plate (mean ± standard error) from three plates. 0 µg/plate was the solvent control.

[b] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE B6
Mutagenicity of Sennoside B in Bacterial Tester Strains[a]

Strain	Dose (µg/plate)	Without S9	Without S9	With 10% rat S9	With 10% rat S9
TA100	0	104 ± 4	104 ± 3	105 ± 3	110 ± 10
	12.5	113 ± 6	97 ± 9	102 ± 3	120 ± 1
	50	105 ± 4	91 ± 3	101 ± 4	104 ± 2
	125	104 ± 9	101 ± 7	103 ± 10	115 ± 5
	500	103 ± 6	95 ± 5	96 ± 8	119 ± 15
	1,500	101 ± 4	107 ± 4	87 ± 3	105 ± 3
	6,000	96 ± 4	103 ± 3	85 ± 1	114 ± 4
Trial summary		Negative	Negative	Negative	Negative
Positive control[b]		824 ± 21	796 ± 11	1,662 ± 139	3,046 ± 65
TA98	0	29 ± 5	28 ± 5	38 ± 3	37 ± 3
	12.5	21 ± 6	24 ± 4	48 ± 5	27 ± 2
	50	21 ± 3	20 ± 4	42 ± 9	29 ± 1
	125	22 ± 4	28 ± 3	47 ± 4	36 ± 3
	500	22 ± 2	24 ± 4	37 ± 3	36 ± 5
	1,500	26 ± 3	25 ± 1	37 ± 3	41 ± 5
	6,000	23 ± 4	30 ± 4	29 ± 0	31 ± 3
Trial summary		Negative	Negative	Negative	Negative
Positive control		688 ± 100	610 ± 17	1,956 ± 62	1,550 ± 72
***Escherichia coli* WP2 *uvrA*/pKM101**					
	0	161 ± 7	179 ± 5	232 ± 14	196 ± 8
	12.5	151 ± 10	140 ± 4	216 ± 9	212 ± 4
	50	162 ± 2	165 ± 13	213 ± 15	213 ± 2
	125	157 ± 5	192 ± 19	216 ± 7	194 ± 6
	500	167 ± 4	183 ± 13	219 ± 5	198 ± 4
	1,500	165 ± 3	191 ± 0	217 ± 9	202 ± 8
	6,000	151 ± 9	198 ± 11	210 ± 14	191 ± 10
Trial summary		Negative	Negative	Negative	Negative
Positive control		1,066 ± 36	1,225 ± 102	1,973 ± 111	1,629 ± 54

[a] Study was performed at ILS, Inc. Data are presented as revertants/plate (mean ± standard error) from three plates. 0 µg/plate was the solvent control.

[b] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4 nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE B7
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Heterozygous F1 p53$^{+/-}$ Male Mice Following Administration of Senna in Feed for 40 Weeks[a]

	Concentration (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/ 1,000 NCEs[b]	P Value[c]	PCEs[b] (%)	P Value[c]
Feed[d]	0	4	3.24 ± 0.17		1.67 ± 0.04		1.961 ± 0.20	
Senna	100	4	2.98 ± 0.25	0.5766	1.70 ± 0.09	0.5196	2.582 ± 0.81	1.000
	300	4	4.13 ± 0.61	0.3897	1.64 ± 0.02	0.6045	2.098 ± 0.35	1.000
	1,000	3	3.42 ± 0.45	0.4383	1.65 ± 0.06	0.6544	1.955 ± 0.07	1.000
	3,000	5	3.79 ± 0.72	0.4127	1.72 ± 0.11	0.4288	1.643 ± 0.22	1.000
	10,000	5	2.93 ± 0.17	0.4202	1.84 ± 0.04	0.0847	2.778 ± 0.76	0.442
			P=0.825[e]		P=0.016[e]		P=0.360[e]	

[a] Study was performed at ILS, Inc. The detailed protocol is presented by Dertinger *et al.* (2004) and Witt *et al.* (2008). NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the control group; exposed group values are significant at P≤0.025 by Williams' test.
[d] Control
[e] Significance tested by linear regression; significant at P≤0.025

APPENDIX C
ORGAN WEIGHTS
AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE C1 **Organ Weights and Organ-Weight-to-Body-Weight Ratios for C57BL/6NTac Mice in the 5-Week Feed Study of Senna** **82**
TABLE C2 **Organ Weights and Organ-Weight-to-Body-Weight Ratios for Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna** **83**

TABLE C1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for C57BL/6NTac Mice in the 5-Week Feed Study of Senna[a]

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
n	5	5	5	5	5	5
Male						
Necropsy body wt	23.5 ± 0.3	23.2 ± 0.3	22.6 ± 0.5	23.2 ± 0.8	23.3 ± 0.3	22.5 ± 0.2
Heart						
Absolute	0.15 ± 0.01	0.13 ± 0.00**	0.13 ± 0.01**	0.13 ± 0.01**	0.13 ± 0.01**	0.12 ± 0.00**
Relative	6.559 ± 0.384	5.403 ± 0.140*	5.721 ± 0.251	5.662 ± 0.205	5.466 ± 0.229*	5.265 ± 0.199**
R. Kidney						
Absolute	0.19 ± 0.01	0.19 ± 0.00	0.19 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.19 ± 0.00
Relative	8.012 ± 0.253	7.980 ± 0.115	8.208 ± 0.308	8.205 ± 0.217	8.442 ± 0.391	8.326 ± 0.237
Liver						
Absolute	1.39 ± 0.05	1.28 ± 0.03	1.31 ± 0.03	1.35 ± 0.05	1.39 ± 0.02	1.29 ± 0.02
Relative	59.293 ± 1.345	55.090 ± 1.226*	58.075 ± 0.925	57.978 ± 0.943	59.744 ± 0.834	57.344 ± 1.061
Lung						
Absolute	0.20 ± 0.01	0.20 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.18 ± 0.01
Relative	8.487 ± 0.323	8.489 ± 0.309	8.123 ± 0.234	7.962 ± 0.150	7.942 ± 0.283	8.022 ± 0.319
R. Testis						
Absolute	0.081 ± 0.004	0.080 ± 0.004	0.084 ± 0.004	0.081 ± 0.003	0.082 ± 0.003	0.078 ± 0.004
Relative	3.459 ± 0.141	3.458 ± 0.115	3.714 ± 0.134	3.491 ± 0.121	3.501 ± 0.108	3.461 ± 0.170
Thymus						
Absolute	0.074 ± 0.005	0.073 ± 0.004	0.064 ± 0.008	0.062 ± 0.004	0.078 ± 0.007	0.063 ± 0.004
Relative	3.132 ± 0.196	3.166 ± 0.174	2.826 ± 0.392	2.668 ± 0.109	3.358 ± 0.284	2.807 ± 0.196
Female						
Necropsy body wt	20.3 ± 0.4	19.2 ± 0.4	20.1 ± 0.3	20.2 ± 0.5	19.1 ± 0.6	19.5 ± 0.5
Heart						
Absolute	0.12 ± 0.00	0.12 ± 0.01	0.12 ± 0.00	0.13 ± 0.01	0.13 ± 0.01	0.11 ± 0.00
Relative	5.990 ± 0.114	6.037 ± 0.326	5.788 ± 0.163	6.498 ± 0.440	6.885 ± 0.589	5.669 ± 0.163
R. Kidney						
Absolute	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.00	0.16 ± 0.00	0.15 ± 0.00	0.16 ± 0.01
Relative	7.876 ± 0.340	8.041 ± 0.297	7.740 ± 0.191	7.709 ± 0.256	7.574 ± 0.193	8.092 ± 0.168
Liver						
Absolute	1.18 ± 0.03	1.09 ± 0.04	1.11 ± 0.02	1.16 ± 0.04	1.15 ± 0.04	1.14 ± 0.05
Relative	58.099 ± 1.034	56.813 ± 1.411	55.333 ± 0.719	57.069 ± 0.912	60.165 ± 3.511	58.299 ± 1.831
Lung						
Absolute	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.22 ± 0.03	0.21 ± 0.02	0.17 ± 0.01
Relative	9.070 ± 0.353	8.840 ± 0.342	9.052 ± 0.301	10.821 ± 1.240	10.902 ± 1.596	8.702 ± 0.278
Thymus						
Absolute	0.090 ± 0.005	0.084 ± 0.003	0.086 ± 0.003	0.086 ± 0.003	0.074 ± 0.011	0.087 ± 0.003
Relative	4.435 ± 0.182	4.348 ± 0.109	4.293 ± 0.142	4.274 ± 0.212	3.815 ± 0.514	4.453 ± 0.123

* Significantly different (P≤0.05) from the control group by Williams' or Dunnett's test
** P≤0.01
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE C2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna[a]

	0 ppm	100 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male						
n	25	24	25	25	23	24
Necropsy body wt	50.1 ± 0.7	50.4 ± 0.5	51.9 ± 0.6	49.0 ± 0.9	50.7 ± 0.8	48.0 ± 0.6
Heart						
Absolute	0.21 ± 0.01	0.24 ± 0.01	0.24 ± 0.01*	0.21 ± 0.01	0.21 ± 0.01	0.20 ± 0.01
Relative	4.236 ± 0.111	4.673 ± 0.142	4.589 ± 0.136	4.215 ± 0.147	4.175 ± 0.115	4.237 ± 0.124
R. Kidney						
Absolute	0.41 ± 0.01	0.42 ± 0.01	0.44 ± 0.01	0.39 ± 0.01	0.41 ± 0.01	0.39 ± 0.01
Relative	8.151 ± 0.147	8.232 ± 0.226	8.460 ± 0.151	8.021 ± 0.171	8.058 ± 0.156	8.216 ± 0.133
Liver						
Absolute	2.42 ± 0.08	2.36 ± 0.08	2.66 ± 0.10	2.31 ± 0.10	2.40 ± 0.11	2.07 ± 0.04**
Relative	48.167 ± 1.188	46.553 ± 1.208	50.897 ± 1.553	46.753 ± 1.485	46.940 ± 1.620	43.060 ± 0.499**
Lung						
Absolute	0.37 ± 0.01	0.37 ± 0.01	0.40 ± 0.01[b]	0.34 ± 0.01	0.37 ± 0.02	0.36 ± 0.02
Relative	7.401 ± 0.262	7.286 ± 0.278	7.669 ± 0.251[b]	6.984 ± 0.309	7.325 ± 0.255	7.521 ± 0.298
R. Testis						
Absolute	0.112 ± 0.001	0.111 ± 0.002	0.113 ± 0.002	0.108 ± 0.001	0.111 ± 0.002	0.113 ± 0.001
Relative	2.242 ± 0.044	2.201 ± 0.029	2.173 ± 0.029	2.231 ± 0.048	2.206 ± 0.040	2.353 ± 0.036
Thymus						
Absolute	0.069 ± 0.005	0.064 ± 0.006	0.061 ± 0.004	0.064 ± 0.006	0.062 ± 0.005	0.058 ± 0.003
Relative	1.359 ± 0.098	1.271 ± 0.109	1.185 ± 0.075	1.297 ± 0.116	1.207 ± 0.098	1.200 ± 0.055
Female						
n	23	23	23	22	22	24
Necropsy body wt	42.8 ± 0.9	42.3 ± 0.8	41.1 ± 0.7	42.2 ± 0.9	41.8 ± 0.6	39.8 ± 0.7*
Heart						
Absolute	0.17 ± 0.01	0.18 ± 0.01	0.17 ± 0.00	0.17 ± 0.01	0.16 ± 0.00	0.17 ± 0.00
Relative	4.107 ± 0.204	4.307 ± 0.207	4.249 ± 0.097	4.073 ± 0.157	3.948 ± 0.100	4.326 ± 0.139
R. Kidney						
Absolute	0.24 ± 0.00	0.24 ± 0.00	0.24 ± 0.00	0.24 ± 0.01	0.24 ± 0.00	0.24 ± 0.01
Relative	5.670 ± 0.124	5.717 ± 0.126	5.790 ± 0.120	5.726 ± 0.153	5.810 ± 0.107	6.092 ± 0.135
Liver						
Absolute	1.64 ± 0.03	1.70 ± 0.04	1.63 ± 0.03	1.69 ± 0.03	1.70 ± 0.04	1.72 ± 0.03
Relative	38.561 ± 0.870	40.364 ± 1.031	39.909 ± 0.803	40.276 ± 0.901	40.711 ± 0.685	43.492 ± 0.770**
Lung						
Absolute	0.32 ± 0.01	0.35 ± 0.01	0.33 ± 0.01	0.32 ± 0.01	0.33 ± 0.01	0.32 ± 0.01
Relative	7.593 ± 0.374	8.396 ± 0.318	8.138 ± 0.298	7.616 ± 0.284	7.945 ± 0.276	8.175 ± 0.375
Thymus						
Absolute	0.060 ± 0.003[c]	0.049 ± 0.003	0.055 ± 0.003	0.058 ± 0.004	0.053 ± 0.003	0.052 ± 0.003
Relative	1.413 ± 0.081[c]	1.160 ± 0.070	1.348 ± 0.074	1.365 ± 0.079	1.263 ± 0.081	1.300 ± 0.060

* Significantly different (P≤0.05) from the control group by Williams' or Dunnett's test
** P≤0.01
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] n=24
[c] n=22

APPENDIX D
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

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CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF SENNA

Senna was obtained from Madaus AG (Köln, Germany) in one lot (1999000) that was used in the 5- and 40-week studies. Identity and purity analyses were conducted by the analytical chemistry laboratory at Research Triangle Institute (Research Triangle Park, NC) and by the study laboratory at Battelle Columbus Operations (Columbus, OH). Reports on analyses performed in support of the senna studies are on file at the National Institute of Environmental Health Sciences.

Lot 1999000 of the chemical, a brown, powdered raw plant material, was identified as senna by the analytical chemistry laboratory using high-performance liquid chromatography with ultraviolet detection (HPLC/UV) by system A (Table D1), and the identity was confirmed by the study laboratory using a similar HPLC/UV system.

The bulk test material was characterized by the analytical chemistry laboratory as a multi-component natural product with a complex mixture of constituents that were confirmed by identifying as many components as possible in methanol:water (50:50) extracts of the raw material. Chromatographic comparisons were made to reference standard solutions in the same solvent of sennosides A and B and sennidins A and B purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ); aloe-emodin, emodin, and rhein from Sigma-Aldrich, Inc. (St. Louis, MO); and aloe-emodin-8-glucoside, chrysophanic acid, and physcione from Madaus AG. In a parallel study conducted by the Natural Products Group of the analytical chemistry laboratory, nine additional reference standards that assisted in identifying constituents in lot 1999000 were isolated and identified in methanol:water (50:50) extracts of RTI lot 10001-86-01 of senna obtained from Frontier Natural Products Co-op (Norway, IA). Forty-seven components with a peak area of at least 0.1% of the total peak area were observed using system A, accounting for 99.6% of the total peak area for the test material extract (Figure D1). Of these 47, 18 were considered major components (≥ 1% each). By comparisons to the chromatographic profiles of the purchased and isolated reference standards, peaks were confirmed in lot 1999000 for sennosides A and B, sennidins A and B, rhein, aloe-emodin, quercetin 3-gentiobioside, kaempferol 3-gentiobioside, torachrysone-8-O-ß-D-glucopyranoside, quercetin, and torachrysone (Figures D2 and D3). Weight percentages of four postulated active components in the test article were estimated to be: 0.7% sennoside A, 1.3% sennoside B, 0.06% sennidin A, and 0.03% sennidin B. The identities of the marker compounds sennoside A and sennoside B in lot 1999000 were confirmed by the study laboratory using resolution standard solutions of these chemicals in methanol:water (50:50).

Lot 1999000 was sterilized by exposure to cobalt-60. The irradiation procedure was performed by Neutron Products, Inc. (Dickerson, MD). Comparison of the irradiated material with the nonirradiated material indicated that all 10 major peaks present in the nonirradiated material were present in the irradiated material at a relative concentration of ≥ 90.7%. Examination of the chromatograms before and after indicated that no new significant peaks appeared after irradiation (Figure D4, Tables D2 and D3).

The water content of lot 1999000 was determined by Covance Laboratories, Inc. (Madison, WI), using weight loss on drying and by the analytical laboratory using Karl Fischer titration. The bulk purity of the test material was determined by the analytical chemistry laboratory using broad gradient HPLC/UV by system B and a system similar to system B that monitored absorbance at 210 nm and utilized mobile phase modifiers that were compatible with low-ultraviolet wavelengths (i.e., phosphoric acid instead of ammonium formate). Broad screening assays for residual volatiles were conducted on dimethylsulfoxide extracts of the test material by the analytical chemistry laboratory using gas chromatography (GC) with flame ionization detection. These assays used a Varian 3800 instrument (Varian, Inc., Santa Clara, CA), a DB-624 (Agilent Technologies, Inc., Santa Clara, CA) or a Rtx®-200 (Restex, Bellefonte, PA) 30 m × 0.53 mm column with a 3.0 µm film, a temperature program of 40° C for 20 minutes followed by a 20° C/minute increase to 240° C and then a 20 minute hold, and helium carrier gas flowing at 4.3 to 4.6 mL/minute. Nutritional testing, trace metals screening, nitrosamines assays, and pesticide screening were performed on lot 1999000 by Covance Laboratories, Inc., using standard methods; trace metals were also assayed by the Trace Inorganics Department at the analytical chemistry laboratory.

For lot 1999000, moisture content was determined to be 6.0% (w/w) by weight loss on drying and 5.8% by weight by Karl Fischer titration. Broad gradient HPLC/UV analyses with monitoring at 210 or 270 nm each detected 13 major (≥ 1% of the total peak areas) components accounting for 91% of the total peak areas and 25 minor (< 1%) components. GC for residual volatiles detected methanol and isopropanol, both at levels below their estimated limits of quantitation (0.1% and 0.09% by weight, respectively), and a single unknown. Organochlorine and organophophorus pesticide levels were below the detection limits of 200 and 50 ppb, respectively. Mercury, thallium, and cadmium levels were less than the limits of detection, which were respectively 25, 20, and 500 ppb. Lead and selenium were present at 299 and 690 ppb, respectively. Nitrosamines were not present above the detection limit of 1.0 ppb. Results of the nutritional and contaminant tests were deemed acceptable for use in these studies.

To ensure stability, the bulk chemical was stored at approximately 5° C in a sealed plastic bag inside a 6-gallon metal pail (5-week study) or in amber glass bottles (40-week study). All transferring and handling of the study material was done under yellow lights. Periodic reanalyses of the bulk chemical were performed during the 5- and 40-week studies by the study laboratory using HPLC/UV by system A, and no degradation of the test material was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared by mixing senna with feed (Table D4). A premix was prepared by hand and then blended with additional feed in a Patterson-Kelly twin-shell blender for 15 minutes. Formulations were stored in sealed plastic buckets containing plastic liners at approximately 5° C and used within 42 days.

Homogeneity and stability studies of the 300 and 10,000 ppm dose formulations were performed by the analytical chemistry laboratory using HPLC/UV by system C or a similar system, and the study laboratory performed homogeneity studies of the 625 and 10,000 ppm dose formulations using HPLC/UV by system C. Homogeneity and stability studies of the 100 ppm dose formulation were performed by the study laboratory using HPLC with mass spectrometry (MS) detection by system D; the more sensitive HPLC/MS/MS enabled the quantification of sennoside A in the 100 ppm dose formulation. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in sealed containers, protected from light, at 5° C, and for at least 7 days under simulated animal room conditions.

Periodic analyses of the dose formulations of senna (quantitating sennoside A as the analytical marker) were conducted by the study laboratory using HPLC/UV by system C (5-week study) or HPLC/MS/MS by system D (40-week study). During the 5-week study, the dose formulations were analyzed once; all five dose formulations were within 10% of the target concentrations (Table D5). Animal room samples of these dose formulations were also analyzed, and four of five were within 10% of the target concentrations. During the 40-week study, the dose formulations were analyzed five times; animal room samples were also analyzed (Table D6). Of the dose formulations analyzed, 33 of 50 were within 10% of the target concentrations; four of 10 animal room samples were within 10% of the target concentrations. Variability of the slope of the standard curve for the analytical assay was determined to account for the discrepancies between observed and expected sample values rather than incorrect formulation preparation or changing formulation concentrations during use.

TABLE D1
High-Performance Liquid Chromatography Systems Used in the Feed Studies of Senna[a]

Detection System	Column	Solvent System
System A Ultraviolet (270 nm) light	Luna® C18, 150 mm × 4.6 mm, 5 µm particle size (Phenomenex, Torrance, CA)	A) acetonitrile:25 mM aqueous ammonium formate (10:90) and B) acetonitrile:25 mM aqueous ammonium formate (90:10); 100% A for 10 minutes, then linear gradient to 96% A:4% B in 5 minutes, held for 10 minutes, then linear gradient to 10% A:90% B in 25 minutes, held for 10 minutes, then linear gradient to 100% A in 10 minutes, held for 5 minutes; flow rate 1.0 mL/minute
System B Ultraviolet (270 nm) light	Luna® C18, 150 mm × 4.6 mm, 5 µm particle size (Phenomenex)	A) acetonitrile:25 mM aqueous ammonium formate (10:90) and B) acetonitrile:25 mM aqueous ammonium formate (90:10); 100% A to 100% B in 25 minutes, held at 100% B for 20 minutes, then to 100% A in 10 minutes, held for 5 minutes; flow rate 1.0 mL/minute
System C Ultraviolet (270 nm) light	Nucleosil® C8, 250 mm × 4.6 mm, 5 µm particle size (Phenomenex or MACHERERY-NAGEL, Inc., Bethlehem, PA)	A) methanol:25 mM aqueous ammonium formate: (10:90) and B) methanol:25 mM aqueous ammonium formate: (90:10); linear gradient from 100% A to 60% A:40% B in 20 minutes, held for 10 minutes, then linear gradient to 100% B in 15 minutes, held for 10 minutes, then linear gradient to 100% A in 5 minutes, held for 5 minutes; flow rate 1.0 mL/minute
System D Mass spectrometry	Gemini® C18, 100 mm x 4.6 mm, 3.0 µm particle size (Phenomenex)	A) 0.1% acetic acid in water and B) 0.1% acetic acid in methanol; (40% A:60% B), isocratic; flow rate 0.5 mL/minute

[a] The high-performance liquid chromatographs were manufactured by Waters Corporation (Milford, MA) (systems A, B, and C) or Agilent Technologies, Inc. (Palo Alto, CA) (systems A, C, and D), and the mass spectrometer was manufactured by Micromass, Inc. (Manchester, UK) (system D).

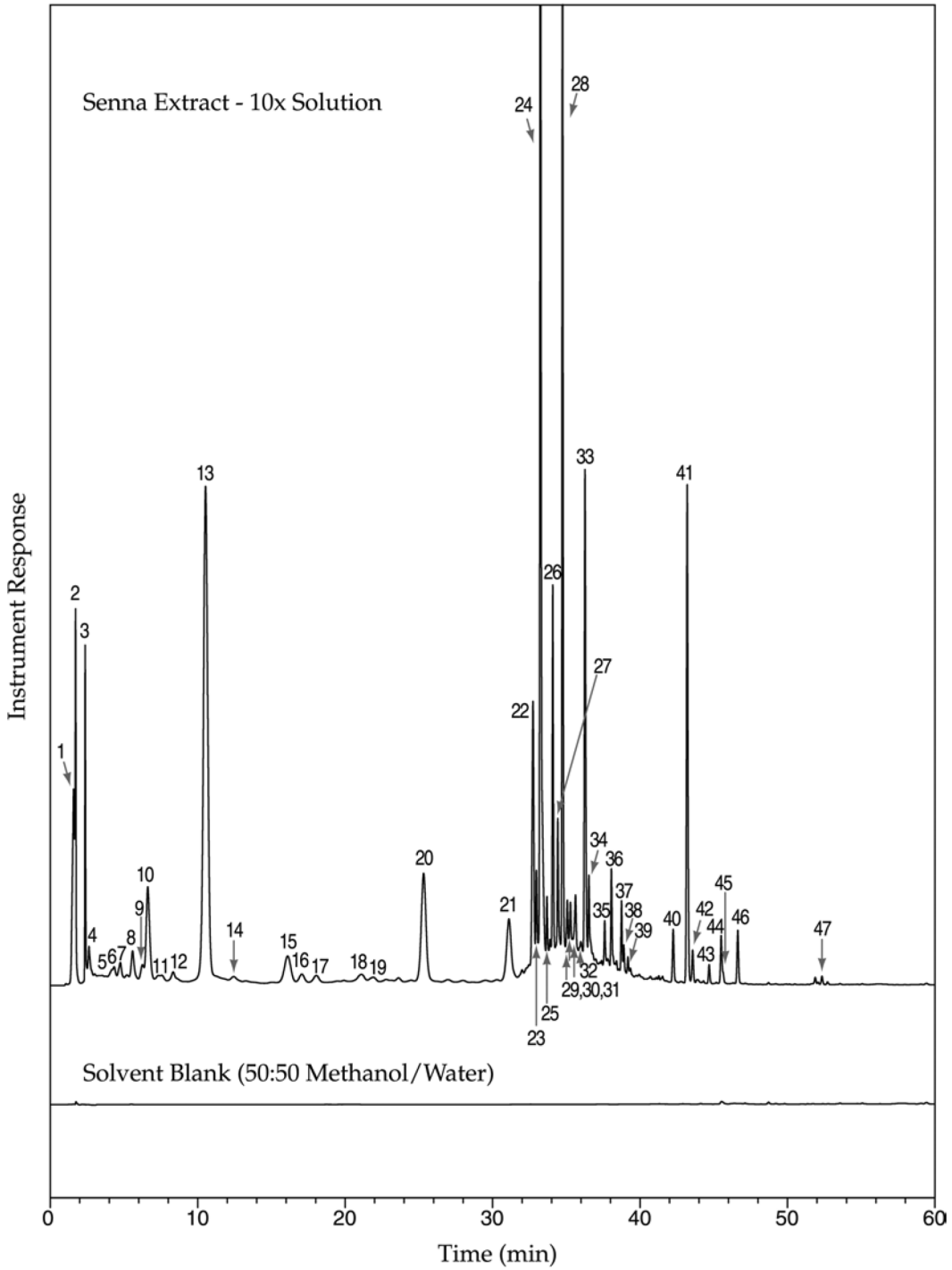

FIGURE D1
High-Performance Liquid Chromatography Profile of an Extracted Senna Sample and a Solvent Blank

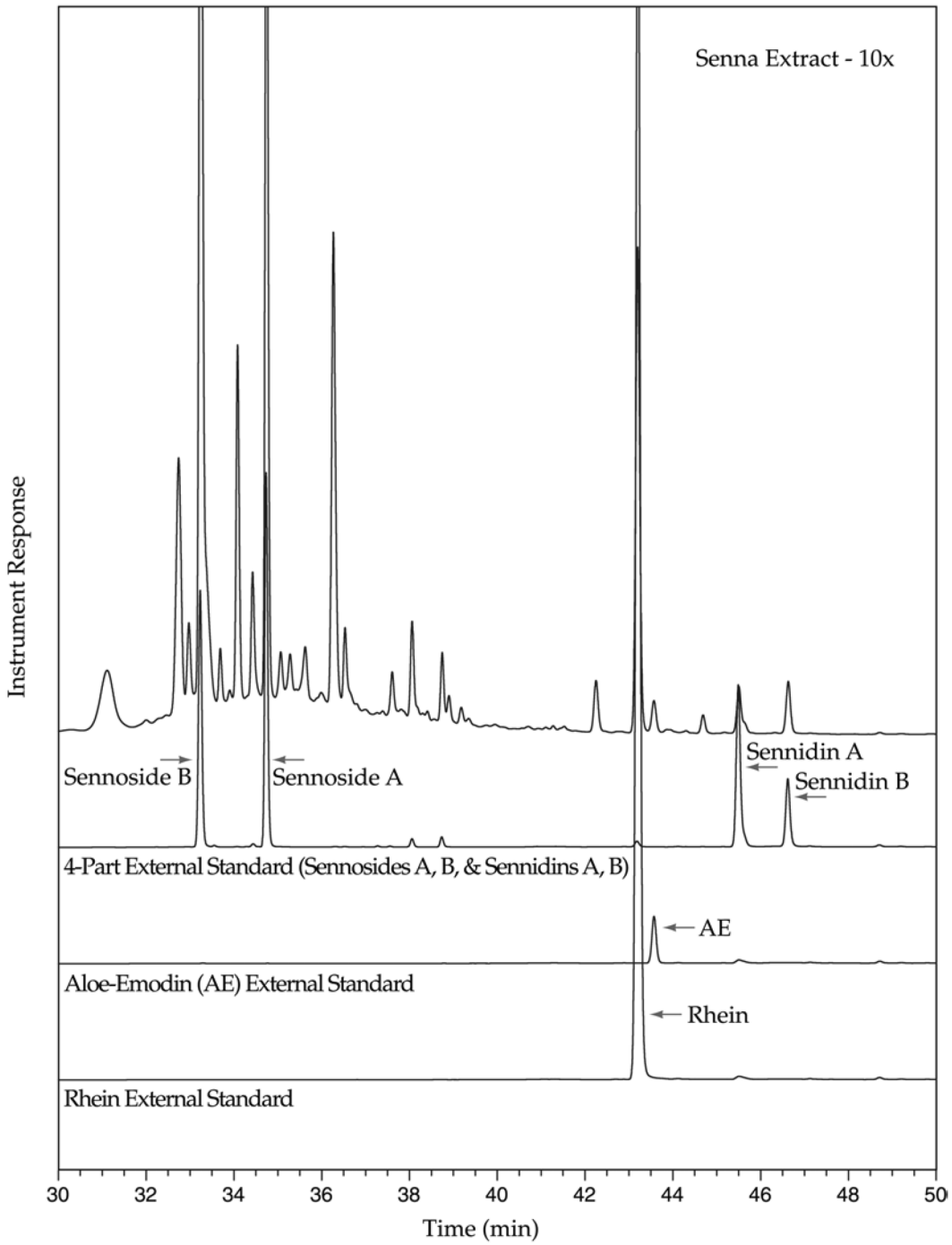

FIGURE D2
High-Performance Liquid Chromatography Profile of an Extracted Senna Sample; Component Confirmation for Commercially Acquired Components

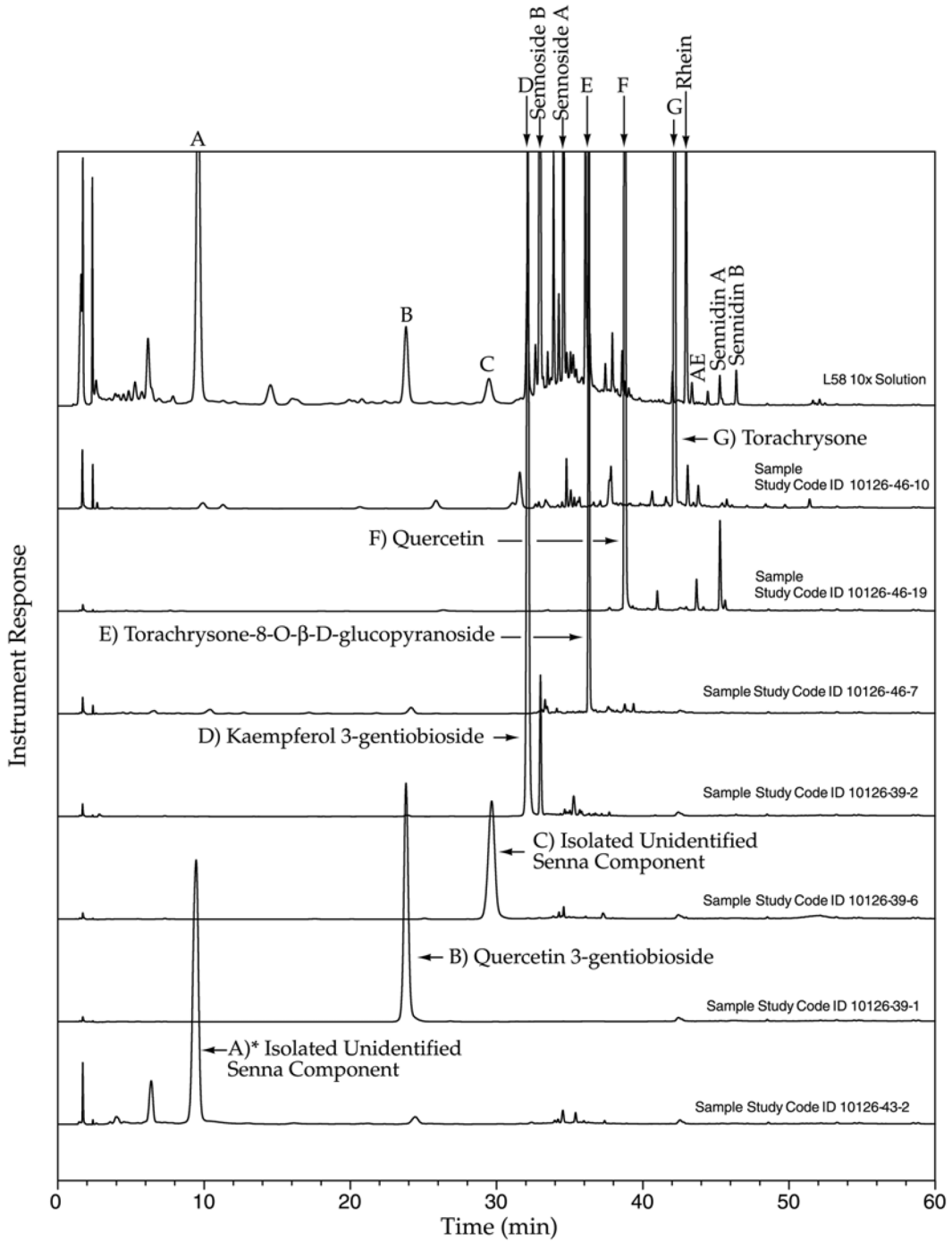

FIGURE D3
High-Performance Liquid Chromatography Profile of an Extracted Senna Sample; Component Confirmation for RTI-Isolated Components

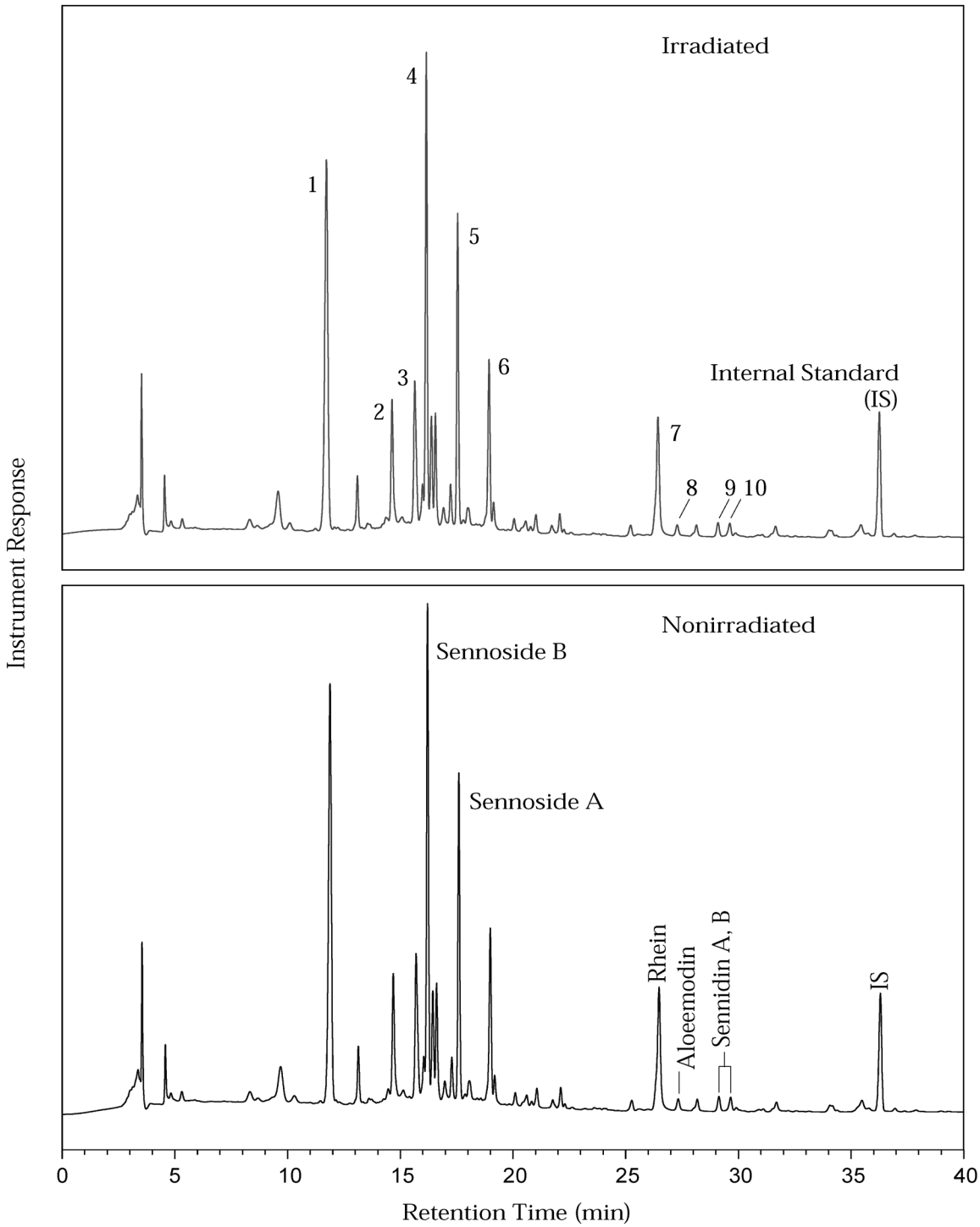

FIGURE D4
High Performance Liquid Chromatography Profiles of the Irradiated and Nonirradiated Senna Sample

TABLE D2
Results of Analyses of Irradiated Study Sample in the 5-Week Feed Study of Senna

Peak No. (Identity)	Replicate 1 RRF[a]	Replicate 2 RRF	Mean RRF	Relative Purity (%)
1	3.383	3.635	3.509	95.3
2	0.7461	0.8072	0.7767	91.4
3	1.014	1.064	1.039	91.8
4 (sennoside B)	2.519	2.690	2.605	91.5
5 (sennoside A)	1.581	1.696	1.639	91.2
6	1.031	1.117	1.074	92.5
7 (rhein)	1.093	1.177	1.135	92.4
8 (aloe-emodin)	0.07147	0.08064	0.07606	90.7
9 (sennidin A)	0.09410	0.10110	0.09760	92.6
10 (sennidin B)	0.08326	0.09143	0.08735	91.7

[a] RRF=relative response factor
[b] % Relative purity = (mean RRF, study material/mean RRF, comparison material) × 100

TABLE D3
Results of Analyses of Nonirradiated Study Sample in the 5-Week Feed Study of Senna

Peak No. (Identity)	Replicate 1 RRF[a]	Replicate 2 RRF	Mean RRF
1	3.710	3.656	3.683
2	0.8511	0.8482	0.8497
3	1.136	1.127	1.132
4 (sennoside B)	2.849	2.847	2.848
5 (sennoside A)	1.804	1.792	1.798
6	1.174	1.147	1.161
7 (rhein)	1.252	1.204	1.228
8 (aloe-emodin)	0.08655	0.08118	0.08387
9 (sennidin A)	0.1072	0.1036	0.1054
10 (sennidin B)	0.09814	0.09246	0.09530

[a] RRF=relative response factor

TABLE D4
Preparation and Storage of Dose Formulations in the Feed Studies of Senna

5-Week Study	40-Week Study
Preparation	
A premix of feed and senna was prepared, then layered into the remaining feed and blended in a Patterson-Kelly twin-shell V-blender for 15 minutes. The dose formulations were prepared once during the study.	Same as 5-week study. The dose formulations were prepared approximately monthly.
Chemical Lot Number	
1999000	1999000
Maximum Storage Time	
42 days	42 days
Storage Conditions	
Stored in sealed plastic buckets lined with plastic liners at approximately 5° C	Stored in sealed plastic buckets lined with plastic liners at approximately 5° C
Study Laboratory	
Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)

TABLE D5
Results of Analyses of Dose Formulations Administered to C57BL/6NTac Mice in the 5-Week Feed Study of Senna

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
September 28, 2005	September 28-30, 2005	625	642	+3
		1,250	1,310	+5
		2,500	2,500	0
		5,000	5,160	+3
		10,000	10,200	+2
	November 10-13, 2005[b]	625	605	–3
		1,250	1,260	+1
		2,500	2,550	+2
		5,000	5,770	+15
		10,000	9,340	–7

[a] Results of duplicate analyses
[b] Animal room samples

TABLE D6
Results of Analyses of Dose Formulations Administered to Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
June 12, 2007	June 14-18, 2007	100	112 ± 1	+12
		100	108 ± 3	+8
		300	335 ± 15	+12
		300	345 ± 16	+15
		1,000	1,140 ± 90	+14
		1,000	1,140 ± 50	+14
		3,000	3,460 ± 270	+15
		3,000	3,260 ± 270	+9
		10,000	9,550 ± 230	–5
		10,000	9,090 ± 70	–9
	August 5, 2007[b]	100	106 ± 2	+6
		300	349 ± 11	+16
		1,000	1,220 ± 10	+22
		3,000	3,730 ± 10	+24
		10,000	12,100 ± 0	+21
August 7, 2007	August 13, 2007	100	88.0 ± 1.5	–12
		100	86.9 ± 3.5	–13
		300	311 ± 4	+4
		300	312 ± 3	+4
		1,000	1,040 ± 20	+4
		1,000	1,030 ± 20	+3
		3,000	2,970 ± 20	–1
		3,000	3,020 ± 10	+1
		10,000	10,500 ± 100	+5
		10,000	10,400 ± 200	+4
November 9, 2007	November 13, 2007	100	95.7 ± 3.5	–4
		100	104 ± 5	+4
		300	282 ± 1	–6
		300	306 ± 13	+2
		1,000	923 ± 45	–8
		1,000	940 ± 27	–6
		3,000	2,830 ± 70	–6
		3,000	3,120 ± 50	+4
		10,000	9,970 ± 250	0
		10,000	10,400 ± 300	+4
January 11, 2008	January 16, 2008	100	72.7 ± 7.1[c]	–27
		100	73.3 ± 22.0[c]	–27
		300	233 ± 29[c]	–22
		300	253 ± 24	–16
		1,000	1,010 ± 130	+1
		1,000	1,120 ± 30	+12
		3,000	3,280 ± 20	+9
		3,000	3,320 ± 170	+11
		10,000	11,100 ± 800	+11
		10,000	10,700 ± 100	+7
	February 22, 2008[b]	100	99.4 ± 0.6	–1
		300	298 ± 6	–1
		1,000	869 ± 65	–13
		3,000	2,600 ± 160	–13
		10,000	9,350 ± 320	–7

TABLE D6
Results of Analyses of Dose Formulations Administered to Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
February 19, 2008	February 29, 2008	100	118 ± 2	+18
		100	101 ± 15	+1
		300	329 ± 28	+10
		300	295 ± 9	–2
		1,000	926 ± 41	–7
		1,000	950 ± 53	–5
		3,000	2,730 ± 60	–9
		3,000	2,590 ± 130	–14
		10,000	9,700 ± 590	–3
		10,000	10,300 ± 250	+3

[a] Results of triplicate analyses (mean ± standard deviation).
[b] Animal room samples
[c] Formulation was outside the acceptable range of ± 20% of target concentration, but used at NTP's direction.

APPENDIX E
FEED AND COMPOUND CONSUMPTION IN THE 40-WEEK FEED STUDY OF SENNA

TABLE E1 **Feed and Compound Consumption by Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna** **98**
TABLE E2 **Feed and Compound Consumption by Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna** **100**

TABLE E1
Feed and Compound Consumption by Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm		100 ppm			300 ppm			1,000 ppm		
Weeks	Feed[a] (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
2	4.6	25.9	4.7	26.1	18	4.7	26.0	54	4.7	25.8	183
3	4.8	26.9	4.6	26.7	17	4.9	27.1	54	4.7	26.8	175
4	4.6	27.8	4.7	27.6	17	4.8	28.0	52	4.5	27.6	163
5	4.7	28.7	4.7	28.9	16	4.7	29.1	49	4.5	28.6	158
6	4.6	30.0	4.7	29.9	16	4.6	30.2	48	4.5	29.5	153
7	4.4	31.0	4.7	30.7	15	5.0	31.6	48	4.5	30.4	148
8	4.5	31.7	4.8	31.8	15	4.8	32.3	45	4.6	31.3	147
9	4.9	32.6	4.8	32.9	15	4.8	33.4	43	4.6	32.0	144
10	4.6	33.4	4.7	33.7	14	4.7	34.4	41	4.8	33.1	145
11	4.5	34.5	4.5	34.3	13	4.7	35.5	40	4.6	34.3	134
12	4.7	35.1	4.7	35.2	13	4.8	36.3	40	4.7	35.0	134
13	4.7	36.1	4.7	36.3	13	4.8	37.4	39	4.5	35.7	126
14	4.6	36.7	4.4	36.6	12	4.8	37.9	38	4.6	36.4	126
15	4.5	37.0	4.4	36.8	12	4.7	38.8	36	4.6	37.1	124
16	4.5	38.0	4.7	38.0	12	4.7	39.5	36	4.6	37.8	122
17	4.6	39.2	4.5	38.6	12	4.7	40.2	35	4.8	38.7	124
18	4.7	40.1	4.6	39.3	12	4.9	41.5	35	4.8	39.7	121
19	4.4	40.1	4.4	39.6	11	4.5	41.7	32	4.4	39.8	111
20	4.6	41.0	4.6	40.5	11	4.8	42.8	34	4.4	40.5	109
21	4.4	41.3	4.5	41.0	11	4.8	43.7	33	4.6	41.0	112
22	4.6	41.9	4.5	41.4	11	4.6	43.9	31	4.6	41.8	110
23	4.8	43.2	4.9	42.4	12	4.9	44.9	33	4.7	42.5	111
24	4.7	43.7	4.7	42.8	11	4.9	45.7	32	4.6	43.0	107
25	4.7	44.5	4.6	43.2	11	4.7	46.5	30	4.6	43.7	105
26	4.6	44.8	4.8	44.2	11	4.8	47.1	31	4.7	44.5	106
27	4.8	45.4	4.6	44.6	10	4.7	47.3	30	4.6	45.2	102
28	4.8	46.4	4.6	45.1	10	4.7	47.7	30	4.7	45.8	103
29	4.5	46.5	4.6	45.5	10	4.8	48.2	30	4.7	46.0	102
30	4.5	46.9	4.7	46.1	10	4.7	48.3	29	4.6	46.5	99
31	4.6	47.3	4.8	46.9	10	4.9	48.6	30	4.7	46.6	101
32	5.0	47.7	4.9	47.0	10	5.1	48.8	31	4.7	46.7	101
33	4.6	47.4	4.7	47.2	10	5.2	49.3	32	4.9	47.0	104
34	5.1	48.1	5.1	48.0	11	5.2	49.5	32	5.1	47.4	108
35	4.8	48.0	4.8	47.9	10	5.0	49.5	30	5.1	48.0	106
36	4.9	48.3	5.1	48.8	11	5.2	50.1	31	5.0	48.2	104
37	4.9	48.5	4.9	48.8	10	5.2	50.4	31	4.9	48.1	102
38	4.9	48.8	4.9	49.0	10	5.1	50.5	30	5.0	48.1	104
39	4.9	48.8	5.0	49.3	10	5.2	50.9	31	4.8	48.1	100
40	5.0	49.3	5.0	49.6	10	5.1	51.1	30	4.9	48.1	102
Mean for Weeks											
1-13	4.6	31.1	4.7	31.2	15	4.8	31.8	46	4.6	30.8	151
14-40	4.7	44.4	4.7	44.0	11	4.9	46.1	32	4.7	43.9	108

TABLE E1
Feed and Compound Consumption by Male Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	3,000 ppm			10,000 ppm		
Weeks	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
2	4.7	25.8	546	4.6	25.0	1,844
3	4.8	26.8	537	4.8	25.5	1,884
4	4.7	27.6	510	4.5	26.7	1,689
5	4.7	28.9	487	4.5	27.6	1,632
6	4.7	29.8	473	4.6	28.3	1,627
7	4.8	31.2	462	4.7	29.4	1,600
8	4.8	31.9	451	4.6	30.2	1,523
9	4.7	33.0	427	4.7	31.2	1,509
10	4.7	33.8	417	4.7	32.0	1,468
11	4.8	34.6	416	4.6	32.8	1,402
12	4.7	35.3	399	4.4	33.2	1,326
13	4.9	36.8	400	4.6	34.3	1,341
14	4.6	37.1	372	4.7	34.8	1,351
15	4.7	37.9	372	4.6	35.2	1,307
16	4.6	38.4	359	4.6	35.9	1,280
17	4.7	39.2	360	4.4	36.3	1,211
18	4.7	39.8	355	4.7	37.7	1,248
19	4.5	40.3	335	4.3	37.8	1,139
20	4.8	41.6	346	4.6	38.8	1,186
21	4.6	42.1	328	4.5	39.1	1,151
22	4.6	42.5	325	4.4	39.4	1,118
23	4.8	43.5	331	4.5	39.9	1,127
24	4.8	44.2	326	4.8	41.2	1,166
25	4.7	44.8	315	4.5	41.6	1,082
26	4.7	45.4	311	4.6	42.2	1,091
27	4.8	46.1	312	4.6	42.6	1,079
28	4.8	46.7	309	4.6	43.2	1,064
29	4.8	47.2	305	4.5	43.3	1,039
30	4.7	47.4	297	4.5	43.7	1,030
31	4.8	47.7	302	4.8	44.6	1,076
32	4.8	47.8	301	4.8	45.0	1,066
33	4.8	47.7	302	4.8	45.2	1,062
34	5.2	48.5	322	5.0	45.7	1,095
35	5.0	48.8	308	4.9	45.8	1,071
36	5.0	49.1	306	4.7	45.8	1,026
37	4.8	48.6	296	4.8	46.1	1,041
38	4.6	48.7	284	4.9	46.6	1,052
39	5.1	49.1	311	4.8	46.6	1,029
40	5.1	49.6	308	4.9	46.8	1,046
Mean for Weeks						
1-13	4.8	31.3	460	4.6	29.7	1,570
14-40	4.8	44.8	322	4.7	41.9	1,120

[a] Grams of feed consumed per animal per day
[b] Milligrams of senna consumed per kilogram body weight per day

TABLE E2
Feed and Compound Consumption by Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	0 ppm		100 ppm			300 ppm			1,000 ppm		
Weeks	Feed[a] (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
2	4.4	21.1	4.5	21.2	21	4.7	21.1	67	4.5	21.2	212
3	4.6	22.6	4.5	22.2	20	4.6	21.9	63	4.8	22.4	214
4	4.5	23.4	4.5	22.9	20	4.2	22.8	55	4.5	23.2	194
5	4.7	24.6	4.5	24.1	19	4.4	23.9	55	4.6	24.3	189
6	4.1	25.1	4.5	24.5	18	4.5	24.7	55	4.5	24.9	181
7	4.3	25.9	4.3	25.7	17	4.2	25.4	50	4.2	26.0	162
8	4.4	26.9	5.0	26.9	19	4.6	26.2	53	4.7	27.0	174
9	4.8	28.5	4.9	28.1	17	4.7	27.2	52	4.8	28.2	170
10	4.6	29.0	4.7	28.4	17	4.6	27.9	49	4.7	28.6	164
11	4.6	29.9	4.4	28.7	15	4.4	28.4	47	4.8	29.7	161
12	4.8	31.0	5.0	30.3	17	4.7	29.4	48	4.8	30.8	156
13	4.6	31.3	4.5	31.2	14	4.4	30.3	44	4.7	31.3	150
14	4.6	32.0	4.9	31.5	16	4.5	30.5	44	4.6	32.2	143
15	4.6	32.5	4.3	31.6	14	4.5	31.1	43	4.5	32.6	138
16	4.6	33.5	4.4	32.6	14	4.5	31.7	43	4.5	33.2	135
17	4.3	33.8	4.5	32.9	14	4.1	32.0	38	4.3	33.5	128
18	4.8	34.6	4.6	34.0	14	4.4	32.7	40	4.6	34.4	134
19	4.2	34.8	4.3	34.1	13	4.2	33.3	38	4.2	34.9	121
20	4.4	35.4	4.3	34.5	13	4.0	33.2	36	4.0	35.0	114
21	4.4	35.9	4.5	35.0	13	4.4	34.1	39	4.2	35.4	119
22	4.6	36.4	4.7	35.9	13	4.4	34.7	38	4.6	36.2	127
23	4.8	36.8	5.1	36.7	14	4.6	35.2	39	4.6	36.7	125
24	4.6	37.2	4.7	37.2	13	4.4	35.6	37	4.5	36.8	122
25	4.9	38.1	4.7	37.7	13	4.5	36.1	37	4.7	37.6	125
26	4.5	38.3	4.8	38.4	13	4.7	36.8	38	4.5	37.6	120
27	4.4	38.4	4.6	38.9	12	4.9	37.8	39	4.6	38.2	120
28	4.3	38.6	4.3	38.7	11	4.1	37.0	33	4.4	38.5	114
29	4.3	38.7	4.6	39.0	12	4.3	37.2	35	4.5	38.9	116
30	4.4	38.4	4.4	38.9	11	4.3	37.2	35	4.2	38.8	108
31	4.5	38.8	4.7	39.2	12	4.3	37.0	35	4.4	38.9	113
32	4.8	39.4	4.6	39.2	12	4.7	37.8	37	4.6	39.2	117
33	4.8	39.8	4.8	39.5	12	4.5	37.7	36	4.8	39.9	120
34	4.8	40.8	5.2	40.5	13	4.5	38.0	36	4.9	40.0	123
35	4.9	41.4	4.8	40.3	12	4.4	38.4	34	4.6	40.3	114
36	4.5	41.1	5.0	40.7	12	4.6	38.5	36	4.7	40.2	117
37	4.9	41.8	4.7	40.5	12	4.8	39.2	37	4.7	40.1	117
38	4.9	42.2	4.8	40.3	12	4.8	39.7	36	4.9	40.5	121
39	4.6	42.3	4.6	41.1	11	4.5	39.6	34	4.9	40.8	120
40	4.5	41.8	4.9	41.2	12	4.8	40.1	36	4.8	41.3	116
Mean for Weeks											
1-13	4.5	26.6	4.6	26.2	18	4.5	25.8	53	4.6	26.5	177
14-40	4.6	37.9	4.7	37.4	13	4.5	36.0	37	4.5	37.5	122

TABLE E2
Feed and Compound Consumption by Female Heterozygous F1 p53$^{+/-}$ Mice in the 40-Week Feed Study of Senna

	3,000 ppm			10,000 ppm		
Weeks	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
2	4.5	21.1	639	4.7	21.0	2,239
3	4.7	22.3	631	4.9	21.7	2,258
4	4.6	23.0	601	4.7	22.8	2,065
5	4.5	24.2	558	4.9	23.8	2,062
6	4.5	24.7	546	4.8	24.2	1,984
7	4.3	26.0	497	4.7	25.3	1,858
8	4.8	26.7	540	4.9	26.1	1,876
9	4.8	26.9	536	5.0	27.2	1,840
10	4.6	27.9	495	4.8	27.4	1,749
11	4.7	29.1	484	4.7	27.8	1,694
12	4.6	29.8	463	5.1	28.8	1,773
13	4.7	30.9	456	4.9	29.4	1,669
14	4.9	31.7	463	5.2	30.5	1,705
15	4.7	31.8	444	4.6	30.6	1,504
16	4.6	32.3	427	4.5	30.8	1,461
17	4.3	32.2	400	4.3	31.2	1,379
18	4.6	32.8	421	4.6	31.8	1,445
19	4.3	33.5	385	4.2	32.0	1,312
20	4.4	34.1	387	4.5	32.9	1,370
21	4.3	33.9	380	4.5	32.9	1,367
22	4.5	34.8	388	4.6	33.7	1,364
23	5.0	35.2	426	5.0	34.3	1,459
24	4.5	35.1	385	4.9	34.9	1,403
25	4.6	35.6	388	4.7	34.8	1,352
26	4.9	36.8	400	5.0	35.8	1,398
27	4.9	37.3	394	4.6	35.8	1,285
28	4.5	37.4	361	4.6	36.0	1,279
29	4.7	38.1	370	4.7	36.5	1,287
30	4.5	37.9	356	4.4	36.5	1,206
31	4.7	38.2	367	4.5	36.9	1,221
32	4.8	38.9	371	4.9	37.6	1,305
33	4.8	38.8	371	4.8	37.5	1,279
34	5.0	39.3	382	5.2	38.0	1,369
35	4.9	39.5	372	5.0	38.5	1,299
36	4.9	39.9	368	4.7	38.6	1,218
37	4.9	39.9	368	4.8	38.5	1,248
38	4.9	40.1	367	4.8	38.5	1,248
39	4.5	39.6	341	4.7	38.6	1,217
40	4.9	40.6	362	4.8	38.9	1,233
Mean for Weeks						
1-13	4.6	26.1	537	4.8	25.5	1,922
14-40	4.7	36.5	387	4.7	35.3	1,341

[a] Grams of feed consumed per animal per day
[b] Milligrams of senna consumed per kilogram body weight per day

APPENDIX F
HISTORICAL CONTROL INCIDENCES

TABLE F1 **Historical Incidences of Neoplasms in Control Male Heterozygous F1 p53$^{+/-}$ Mice in the 9-Month NCTR Studies of AZT and AZT/3TC and the 40-Week NTP Study of Senna** **104**
TABLE F2 **Historical Incidences of Neoplasms in Control Female Heterozygous F1 p53$^{+/-}$ Mice in the 9-Month NCTR Studies of AZT and AZT/3TC and the 40-Week NTP Study of Senna** **105**

TABLE F1
Historical Incidences of Neoplasms in Control Male Heterozygous F1 p53$^{+/-}$ Mice in the 9-Month NCTR Studies of AZT and AZT/3TC and the 40-Week NTP Study of Senna[a]

Neoplasm	AZT	AZT/3TC	Senna	Overall
Bone				
Osteosarcoma	0/27	1/25	0/25	1/77
Humerus, osteosarcoma	1/27	1/25	0/25	2/77
Femur, osteosarcoma	1/27	0/25	0/25	1/77
Tibia, osteosarcoma	0/27	1/25	0/25	1/77
Any location, osteosarcoma	2/27	3/25	0/25	5/77
Harderian gland				
Adenoma	1/27	0/25	0/25	1/77
Liver				
Hepatocellular adenoma	3/26	1/25	1/25	5/76
Lung				
Alveolar/bronchiolar adenoma	0/27	0/25	1/25	1/77
Lymph node				
Mediastinal, sarcoma	0/27	1/25	0/25	1/77
Mesenteric, sarcoma	0/26	1/24	0/24	1/74
Mesentery				
Sarcoma	0/27	1/25	0/25	1/77
Pancreas				
Sarcoma	0/27	1/24	0/25	1/76
Skeletal muscle, diaphragm				
Sarcoma	0/27	1/25	0/25	1/77
Small intestine				
Duodenum, leiomyosarcoma	0/26	1/23	0/25	1/74
Jejunum, adenocarcinoma	0/26	1/23	0/25	1/74
Tissue NOS				
Sarcoma	1/26	0/25	0/25	1/76
Abdominal sarcoma	0/27	1/25	0/25	1/77
All organs				
Malignant lymphoma	1/27	1/25	1/25	3/77

[a] Data as of February 18, 2010. The AZT studies involved transplacental dosing and may not be comparable to the current NTP senna study.

TABLE F2
Historical Incidences of Neoplasms in Control Female Heterozygous F1 p53$^{+/-}$ Mice in the 9-Month NCTR Studies of AZT and AZT/3TC and the 40-Week NTP Study of Senna[a]

Neoplasm	AZT	AZT/3TC	Senna	Overall
Adrenal medulla				
Pheochromocytoma, benign	0/25	0/25	1/25	1/75
Bone				
Femur, osteosarcoma	1/26	0/25	0/25	1/76
Rib, osteosarcoma	0/26	0/25	1/25	1/76
Vertebra, osteosarcoma	0/26	0/25	1/25	1/76
Any location, osteosarcoma	1/26	0/25	2/25	3/76
Bone marrow				
Sarcoma	1/26	0/25	0/25	1/76
Brain, cerebrum				
Sarcoma	1/26	0/25	0/25	1/76
Large intestine, cecum				
Polyp	0/25	0/25	0/25	0/75
Liver				
Sarcoma	1/26	0/25	0/25	1/76
Lung				
Alveolar/bronchiolar adenoma	0/26	1/25	0/25	1/76
Mediastinum, sarcoma	1/26	0/25	0/25	1/76
Lymph node				
Mandibular, sarcoma	1/26	0/25	0/25	1/76
Mesenteric, sarcoma	1/26	0/25	0/23	1/74
Mammary gland				
Adenocarcinoma	0/25	1/25	0/25	1/75
Nose				
Sarcoma	1/26	0/25	0/25	1/76
Ovary				
Cystadenoma	0/26	0/25	1/25	1/76
Granuloma cell tumor, malignant	0/26	0/25	1/25	1/76
Luteoma	1/26	0/25	0/25	1/76
Spleen				
Sarcoma	1/26	0/25	0/25	1/76
All organs				
Malignant lymphoma	0/26	2/25	1/25	3/76
Malignant mesothelioma	1/26	0/25	0/25	1/76

[a] Data as of February 18, 2010. The AZT studies involved transplacental dosing and may not be comparable to the current NTP senna study.

APPENDIX G
INGREDIENTS, NUTRIENT COMPOSITION, AND CONTAMINANT LEVELS IN NTP-2000 RAT AND MOUSE RATION

TABLE G1 Ingredients of NTP-2000 Rat and Mouse Ration 108
TABLE G2 Vitamins and Minerals in NTP-2000 Rat and Mouse Ration 108
TABLE G3 Nutrient Composition of NTP-2000 Rat and Mouse Ration 109
TABLE G4 Contaminant Levels in NTP-2000 Rat and Mouse Ration 110

TABLE G1
Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground hard winter wheat	22.26
Ground #2 yellow shelled corn	22.18
Wheat middlings	15.0
Oat hulls	8.5
Alfalfa meal (dehydrated, 17% protein)	7.5
Purified cellulose	5.5
Soybean meal (49% protein)	5.0
Fish meal (60% protein)	4.0
Corn oil (without preservatives)	3.0
Soy oil (without preservatives)	3.0
Dried brewer's yeast	1.0
Calcium carbonate (USP)	0.9
Vitamin premix[a]	0.5
Mineral premix[b]	0.5
Calcium phosphate, dibasic (USP)	0.4
Sodium chloride	0.3
Choline chloride (70% choline)	0.26
Methionine	0.2

[a] Wheat middlings as carrier
[b] Calcium carbonate as carrier

TABLE G2
Vitamins and Minerals in NTP-2000 Rat and Mouse Ration[a]

	Amount	Source
Vitamins		
A	4,000 IU	Stabilized vitamin A palmitate or acetate
D	1,000 IU	D-activated animal sterol
K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl acetate	100 IU	
Niacin	23 mg	
Folic acid	1.1 mg	
d-Pantothenic acid	10 mg	*d*-Calcium pantothenate
Riboflavin	3.3 mg	
Thiamine	4 mg	Thiamine mononitrate
B_{12}	52 µg	
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	*d*-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a] Per kg of finished product

TABLE G3
Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	15.1 ± 0.46	14.4 – 15.7	7
Crude fat (% by weight)	8.2 ± 0.24	7.8 – 8.5	7
Crude fiber (% by weight)	8.9 ± 0.68	8.0 – 9.7	7
Ash (% by weight)	5.0 ± 0.24	4.6 – 5.4	7
Amino Acids (% of total diet)			
Arginine	0.775 ± 0.068	0.670 – 0.970	20
Cystine	0.222 ± 0.025	0.150 – 0.250	20
Glycine	0.701 ± 0.043	0.620 – 0.800	20
Histidine	0.356 ± 0.081	0.270 – 0.680	20
Isoleucine	0.543 ± 0.045	0.430 – 0.660	20
Leucine	1.094 ± 0.069	0.960 – 1.240	20
Lysine	0.706 ± 0.115	0.310 – 0.840	20
Methionine	0.408 ± 0.048	0.260 – 0.490	20
Phenylalanine	0.626 ± 0.041	0.540 – 0.720	20
Threonine	0.502 ± 0.044	0.430 – 0.610	20
Tryptophan	0.147 ± 0.027	0.110 – 0.200	20
Tyrosine	0.394 ± 0.058	0.280 – 0.540	20
Valine	0.666 ± 0.045	0.550 – 0.730	20
Essential Fatty Acids (% of total diet)			
Linoleic	3.92 ± 0.231	3.49 – 4.54	20
Linolenic	0.30 ± 0.031	0.21 – 0.35	20
Vitamins			
Vitamin A (IU/kg)	4,223 ± 1,888	2,890 – 8,400	7
Vitamin D (IU/kg)	1,000[a]		
α-Tocopherol (ppm)	82.8 ± 19.39	52.0 – 124.0	20
Thiamine (ppm)[b]	8.7 ± 3.86	6.2 – 17.3	7
Riboflavin (ppm)	7.1 ± 1.96	4.20 – 11.20	20
Niacin (ppm)	78.5 ± 9.39	66.4 – 98.2	20
Pantothenic acid (ppm)	26.8 ± 13.16	17.4 – 81.0	20
Pyridoxine (ppm)[b]	9.46 ± 2.06	6.4 – 13.7	20
Folic acid (ppm)	1.65 ± 0.50	1.15 – 3.27	20
Biotin (ppm)	0.319 ± 0.11	0.200 – 0.704	20
Vitamin B_{12} (ppb)	53.9 ± 41.6	18.3 – 174.0	20
Choline (ppm)[b]	2,939 ± 399	2,000 – 3,790	20
Minerals			
Calcium (%)	0.937 ± 0.060	0.872 – 1.040	7
Phosphorus (%)	0.560 ± 0.044	0.481 – 0.625	7
Potassium (%)	0.664 ± 0.028	0.626 – 0.732	20
Chloride (%)	0.386 ± 0.040	0.300 – 0.474	20
Sodium (%)	0.190 ± 0.016	0.160 – 0.222	20
Magnesium (%)	0.217 ± 0.065	0.185 – 0.490	20
Sulfur (%)	0.170 ± 0.029	0.116 – 0.209	14
Iron (ppm)	184 ± 40.7	135 – 311	20
Manganese (ppm)	51.8 ± 7.31	21.0 – 73.1	20
Zinc (ppm)	53.5 ± 8.85	43.3 – 78.5	20
Copper (ppm)	7.05 ± 2.677	3.21 – 16.30	20
Iodine (ppm)	0.496 ± 0.215	0.158 – 0.972	20
Chromium (ppm)	0.674 ± 0.283	0.330 – 1.380	19
Cobalt (ppm)	0.27 ± 0.164	0.133 – 0.864	18

[a] From formulation
[b] As hydrochloride (thiamine and pyridoxine) or chloride (choline)

TABLE G4
Contaminant Levels in NTP-2000 Rat and Mouse Ration[a]

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.26 ± 0.054	0.20 – 0.36	7
Cadmium (ppm)	0.07 ± 0.012	0.05 – 0.08	7
Lead (ppm)	0.10 ± 0.009	0.08 – 0.11	7
Mercury (ppm)	<0.02		7
Selenium (ppm)	0.27 ± 0.160	0.16 – 0.63	7
Aflatoxins (ppb)	<5.00		7
Nitrate nitrogen (ppm)[c]	21.9 ± 7.65	10.0 – 29.0	7
Nitrite nitrogen (ppm)[c]	<0.61		7
BHA (ppm)[d]	<1.0		7
BHT (ppm)[d]	<1.0		7
Aerobic plate count (CFU/g)	10 ± 0	10	7
Coliform (MPN/g)	3 ± 0	3	7
Escherichia coli (MPN/g)	<10		7
Salmonella (MPN/g)	Negative		7
Total nitrosoamines (ppb)[e]	11.6 ± 4.66	7.9 – 20.4	7
N-Nitrosodimethylamine (ppb)[e]	4.4 ± 4.37	1.0 – 13.5	7
N-Nitrosopyrrolidine (ppb)[e]	7.2 ± 5.3	1.2 – 16.2	7
Pesticides (ppm)			
α-BHC	<0.01		7
β-BHC	<0.02		7
γ-BHC	<0.01		7
δ-BHC	<0.01		7
Heptachlor	<0.01		7
Aldrin	<0.01		7
Heptachlor epoxide	<0.01		7
DDE	<0.01		7
DDD	<0.01		7
DDT	<0.01		7
HCB	<0.01		7
Mirex	<0.01		7
Methoxychlor	<0.05		7
Dieldrin	<0.01		7
Endrin	<0.01		7
Telodrin	<0.01		7
Chlordane	<0.05		7
Toxaphene	<0.10		7
Estimated PCBs	<0.20		7
Ronnel	<0.01		7
Ethion	<0.02		7
Trithion	<0.05		7
Diazinon	<0.10		7
Methyl chlorpyrifos	0.048 ± 0.039	0.020 – 0.113	7
Methyl parathion	<0.02		7
Ethyl parathion	<0.02		7
Malathion	0.082 ± 0.070	0.020 – 0.187	7
Endosulfan I	<0.01		7
Endosulfan II	<0.01		7
Endosulfan sulfate	<0.03		7

[a] All samples were irradiated. CFU=colony-forming units; MPN=most probable number; BHC=hexachlorocyclohexane or benzene hexachloride
[b] For values less than the limit of detection, the detection limit is given as the mean.
[c] Sources of contamination: alfalfa, grains, and fish meal
[d] Sources of contamination: soy oil and fish meal
[e] All values were corrected for percent recovery.

APPENDIX H
SENTINEL ANIMAL PROGRAM

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SENTINEL ANIMAL PROGRAM

METHODS

Rodents used in the Carcinogenesis Program of the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of chemical compounds. Under this program, the disease state of the rodents is monitored via serology on sera from extra (sentinel) animals in the study rooms. These animals and the study animals are subject to identical environmental conditions. The sentinel animals come from the same production source and weanling groups as the animals used for the studies of chemical compounds.

In the 5-week study, serum samples were collected from five male and five female sentinel mice at the end of the study. In the 40-week study, serum samples were collected from five male and five female sentinel mice at 1 month and from an additional five male and five female mice at the end of the study. Blood from each animal was collected and allowed to clot, and the serum was separated. The samples were processed appropriately and sent to BioReliance Corporation (Rockville, MD) for determination of antibody titers. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

Method and Test	Time of Collection
MICE	
5-Week Study	
ELISA	
Ectromelia virus	Study termination
EDIM (epizootic diarrhea of infant mice)	Study termination
GDVII (mouse encephalomyelitis virus)	Study termination
LCM (lymphocytic choriomeningitis virus)	Study termination
Mouse adenoma virus-1	Study termination
MHV (mouse hepatitis virus)	Study termination
MMV VP2 (mouse minute virus, viral protein 2)	Study termination
MPV VP2 (mouse parvovirus, viral protein 2)	Study termination
PVM (pneumonia virus of mice)	Study termination
Reovirus 3	Study termination
Sendai	Study termination
Immunofluorescence Assay	
Parvovirus	Study termination
40-Week Study	
ELISA	
Ectromelia virus	1 month
EDIM	1 month
GDVII	1 month
LCM	1 month
Mouse adenoma virus-1	1 month
MHV	1 month
MMV	1 month
MPV	1 month
PVM	1 month
Reovirus 3	1 month
Sendai	1 month

Method and Test	Time of Collection
40-Week Study (continued)	
Immunofluorescence Assay	
MMV	1 month
Multiplex Flourescent Immunoassay	
Ectromelia virus	Study termination
EDIM	Study termination
GDVII	Study termination
LCM	Study termination
MHV	Study termination
MMV	Study termination
MNV (mouse norovirus)	Study termination
MPV	Study termination
Mycoplasma pulmonis	Study termination
Parvovirus NS-1	Study termination
PVM	Study termination
Reovirus 3	Study termination
Sendai	Study termination

RESULTS

All test results were negative.