Mutagenesis and Antimutagenesis in Big Blue® *lacI* Transgenic Rats

by

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A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming
to the required standard

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Abstract

The initiation of the cancer process is associated with mutations. Analysis of environmental exposure to chemical or physical agents causing these genetic alterations is of great importance in order to develop strategies for avoiding or reducing cancer risk in humans. The causality between mutagenesis and carcinogenesis also prompts the concept that the modifying effect on mutagenesis by a compound would be predictive of the cancer preventive potential of that compound. The Big Blue® transgenic assay, using the *E. coli lacI* gene as the mutational target provides an opportunity to evaluate mutagenesis and its modulation *in vivo*. This model system was used to study the tissue-specific effect of the potential chemopreventive agent conjugated linoleic acid (CLA), on the mutagenicity of the suspected human carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). PhIP and CLA were selected for study since both compounds are consumed by humans on a daily basis, and are suspected to be related to the human risk of colon, breast, and prostate cancers.

The mutagenicity of PhIP in Big Blue® rats was shown to be tissue-, sex-, and dose-dependent. PhIP was found to be a potent mutagen in the colon, followed by the cecum, prostate, and kidney. Compared with the background mutational spectra, the PhIP-induced spectra were characterized by an elevated proportion of –1 frameshifts, consisting mainly of deletions of single G:C base pair. However, the induced spectra varied among tissues. A sex-dependent induction of mutation by PhIP was observed in the kidney such that the PhIP-induced mutation frequency was twice as high in male rats as in female rats; the biological significance of this difference is not clear. In contrast,

although PhIP has been shown to induce colon tumors preferentially in male rats, and only rarely in female rats, no difference in mutational response was detected between the colons of male and female rats treated with PhIP.

Experiments were performed to examine the *in vivo* effect of CLA on mutagenesis. Similar to what is seen for the mutagenicity of PhIP, the modification by CLA depends on tissue, sex, and dose of administration. CLA showed a modest protection against PhIP-induced mutagenesis in the distal part of the colon, in the prostate, and in the kidney of female rats. However, significant changes in the overall PhIP-induced mutation spectrum were seen only in the prostate. The antimutagenic effect of CLA may be directly responsible for its cancer prevention capability, since PhIP-induced aberrant crypt foci in the colon of male rats were completely inhibited by CLA. However, CLA was not totally innocuous. When supplemented at 0.5%, CLA acted as a comutagen of PhIP, increasing the PhIP-induced MF in the cecum, although this effect was not observed when CLA was supplemented at 1%. The differences in effect may be related to the antioxidant or pro-oxidant activities of CLA isomers under experimental conditions.

Due to the artificial nature of the lambda/LIZ *lacI* transgene and the possible absence of DNA repair in this transgene, the suitability of the Big Blue® transgenic assay as a mutational test system has been questioned. We examined the repair of UV- and benzo(*a*)pyrene diol epoxide-induced DNA damage in this non-transcribed lambda construct of the Big Blue® rat-2 transgenic cell line and demonstrated that DNA damage is indeed repaired in this transgenic construct. Lastly, since CLA altered the mutational spectra in the prostate in a way consistent with an effect of mismatch repair, the

possibility of an effect of CLA on mismatch repair was explored in bacteria. Although CLA was found to increase mutant frequency in a mismatch repair proficient *E. coli* strain, but not in deficient strains, the mechanism by which CLA operates remains unclear.

Altogether, the data demonstrate the mutagenicity of PhIP and its modulation by CLA as a function of tissue, sex, and dose of administration, and support the application of the Big Blue® transgenic assay as a screening tool for mutagens and chemopreventive agents.

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List of abbreviations

A	adenosine
AαC	2-amino-9H-pyrido[2,3-b]indole
ACF	Aberrant crypt foci
ACs	Aberrant crypts
ANOVA	analysis of variance
Apc	adenomatous polyposis coli (gene)
B(*a*)P	benzo(*a*)pyrene
BPDE	benzo(*a*)pyrene diol epoxide
C	cytosine
c9,t11-CLA	*cis*-9,*tran*-11 CLA
CAs	chromosomal aberrations
CHO	Chinese hamster ovary
CLA	conjugated linoleic acid
CPDs	cyclobutane pyrimidine dimers
CYP	cytochrome P450
DC	distal part of the colon
dhfr	dihydrofolate reductase (gene)
dNTP	deoxynucleotide triphosphate
dG-C8-PhIP	N2-(2'-deoxyguanosin-8-yl)-PhIP
DMBA	dimethylbenz[a]anthracene
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DTT	1,2-dithiole-3-thione
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ENU	N-ethyl-N-nitrosourea
ES	effective size
F344	Fischer 344 rat strain
FBS	fetal bovine serum
G	guanosine
GCA	Generalized Cochran-Armitage
Glu-P-1	2-amino-6-methyl-dipyrido[1,2-a:3',2'-d]imidazole
Glu-P-2	2-aminodipyrido[1,2-a:3',2'-d]imidazole
GSH	glutathione
GST	glutathione-*S*-transferases
HCA	heterocyclic amine
hprt	hypoxanthine-guanine phosphoribosyltransferase
i.p.	intraperitoneal
IQ	2-amino-3-methylimidazo[4,5-f]quinoline
LA	linoleic acid
lacI	lactose repressor (*E. coli* gene)
lacZ	β-galactosidase (*E. coli* gene)
λLIZ/*lacI*	Big Blue® λ shuttle vector encoding the *lacI* gene

MeAαC	2-amino-3-methyl-9H-pyrido[2,3-b]indole
MeIQ	2-amino-3,4-dimethylimidazo[4,5-f]quinoline
MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
MF	mutation frequency
MMR	mismatch repair
MNU	N-methyl-N-nitrosourea
MS	mutational spectrum
NAT	*N*-acetyltransferase
NER	nucleotide excision repair
NMR	nuclear magnetic resonance
nt	nucleotide
PAH	polycyclic aromatic hydrocarbon
PC	proximal part of the colon
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pfu	plaque forming units
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
QPCR	quantitative polymerase chain reaction
Rif^r	rifampicin resistant
ROD	relative optical density
SCEs	sister-chromatid exchanges
SDS	sodium dodecyl sulfate
SM	(bacteriophage) storage medium
SD	standard deviation
R2λLIZ	Big Blue® rat-2 λLIZ cell
T	thymidine
t10,c12-CLA	*trans*-10,*cis*-12 CLA
TCR	transcription-coupled excision repair
TE	tris-HCl EDTA (buffer)
TEBs	terminal end buds
tRNA	transfer ribonucleic acid
Trp-P-1	3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole
Trp-P-2	3-amino-1-methyl-5H-pyrido[4,3-b]indole
UDPGT	UDP-glucuronosyltransferases
UV	ultraviolet light
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Acknowledgements

I would like to express my gratitude to my supervisor, Dr. Barry Glickman, for his support, patience, guidance, and encouragement throughout my graduate studies. He has provided me with opportunities and freedom to explore different research directions, and has opened my eyes and mind to the importance, excitement and challenge of scientific research.

A special thanks goes to my co-supervisor, Dr. Johan de Boer, who is most responsible for helping me design and perform the research project, and assisting me to complete the dissertation. He was always there to meet with me and talk about my ideas, proofread my papers and chapters, and give me constructive criticism. I am forever grateful for his generous sharing of his resources and time, which has enabled me to move forward.

For assistance in completing my research project, I am indebted to James Holcroft, Dave Walsh, Jana Kangas, Virginia Haslett, Trina de Monyé, Brandi Jenkins, Amanda Glickman, Henry Yu, Ken Sojonky, Tao Jiang, Jian Chen, Cindy Ruttan, and Gordon Cooney. I am grateful for their active involvement, excellent technical support, and for providing a pleasant environment in which to work. I also wish to acknowledge the staff of the Animal Care Unit of the University of Victoria, in particular Wendy Lin and Ralph Scheurle, for their dedication and assistance. I would also like to thank Drs. Paul Kuttori, Shulin Zhang, and Gregory Stuart, for stimulating discussions.

I am very grateful to Pauline Tymchuk, who proofread my dissertation, and has kindly helped me from time to time, and to Dr. Moyra Brackley, for helping me in statistical analysis.

I also wish to thank my Ph.D. supervisory and examining committees, Drs. Francis Choy, Christopher Upton, Brian Weinerman, and Gwyn Bebb, for their dedication and time.

I would like to express my appreciation to Peter Anderson. Over the past several years, Peter has witnessed the many ups and downs in my dissertation process, and always has confidence in me even when I doubted myself. Without his support, this dissertation would not have been finished.

Finally, my family, which has been a constant source of love and support, receives my deepest gratitude. My parents, Quanhui Yang and Huizhong Cai, and my brother, Fan Yang, have given me continuous support and encouragement to pursue my interests, even when those interests went beyond boundaries of language and geography.

Dedications

To my parents and my brother

Chapter 1. General Introduction

1.1. Environment, diet, and cancer

According to the National Cancer Institute's 1993 publication, "Surveillance, Epidemiology, and End Result Program", cancer caused 23% of the person-years of premature loss of life (Miller et al., 1993). Rates of cancer differ by geographic region nationally and internationally, and marked changes in the rates of some cancers in the U.S. occurred in the 20th century (Ames and Gold, 1997; Devesa et al., 1995). Geographical differences, alteration of cancer rates with migration, and the changes in rates within countries over relatively short periods of time indicate a strong contribution by environmental factors. For example, the increasing incidence of lung cancer is attributed to increased cigarette smoking (Pope et al., 1999; Sastre et al., 1999), and malignant melanoma to increased exposure to sunlight (Elwood and Jopson, 1997; Breitbart et al., 1997). Epidemiological studies indicate that genetic factors by themselves are probably responsible for only about 5-10% of cancers (Perera, 1995), while approximately 80% of human cancer is caused by tobacco smoke, diet, and exposure to carcinogens in the workplace (Harris, 1991).

Human diet consists of a series of chemicals derived from microbial, plant and animal sources. In a 1981 review, Doll and Peto (1981) estimated that approximately 35% of all cancer deaths may be attributed to diet, with a range of 10 to 70%. In a more recent review, Willett (1995) discussed findings relating to diet based on progress in cancer studies and the impact of changes in diet on several major cancers was noted. It

was suggested that 70% of colon cancers, 50% of breast cancers, and 75% of prostate cancers may be avoided by dietary modification. A large number of mutagens and carcinogens such as polycyclic aromatic hydrocarbons (PAHs), aflatoxin B_1, and nitrosamines have been identified in the human diet. PAHs have been found to contribute to the increased cancer incidence of the human respiratory tract (Boffetta et al., 1997; Mollerup et al., 2001); exposure to aflatoxin B_1 in food has been associated with the high incidence of human liver cancer in China (Wang et al., 1999b; Wang et al., 1999c); and nitrosamines may be responsible for stomach cancer (De Stefani et al., 1998).

1.2. Dietary carcinogens—heterocyclic amines

A major class of dietary carcinogens, heterocyclic amines (HCAs), was first reported by Dr. T. Sugimura in the 1977 Cold Spring Harbor Symposium entitled, "Origin of Human Cancer" (Sugimura et al., 1977). These compounds were all found to be aromatic amines having an exocyclic amino group and nitrogen atoms within the aromatic skeletal structure, thus being named HCAs. HCAs are formed when proteinaceous food such as meat and fish is cooked at a high temperature, and the precursors were found to be creatinine, phenylalanine and glucose (Shioya et al., 1987). At present, 19 HCAs have been identified in cooked food (review by Ohgaki et al., 1991; Nagao, 1999), all shown to be potent mutagens in the Ames/*Salmonella* bacterial mutagenesis assay (reviewed by Nagao, 1999), to be positive in mammalian *in vivo* mutagenesis systems (Aeschbacher and Turesky, 1991; Sasaki et al., 1992; Fan et al., 1995; Leong-Morgenthaler et al., 1998) and, where tested, to be carcinogens in rodents (Table 1.1) and in non-human primates (Ohgaki et al., 1991; Dooley et al., 1992).

Concern over the health significance of these compounds is supported by evidence that human exposure does occur. Metabolites of HCAs were detected in humans consuming cooked meat (Stillwell et al., 1999a; Stillwell et al., 1999b). Furthermore, human consumption of fried or grilled meat and meat-based gravies has been shown in epidemiological studies to be correlated with colorectal and breast cancer (Lang et al., 1994; Roberts-Thomson et al., 1999). The overall cancer risk to the U.S population from exposure to HCAs was estimated to be one in ten thousand, based on the content of HCAs present in common food in the U.S. diet, the average intake of these foods, and cancer potencies derived from the results of animal bioassays (Layton et al., 1995). Turteltaub et al. (1999) studied the metabolism and adduct formation of HCAs in humans at well-defined dietary-relevant doses, and compared them with findings from rodent models. HCAs such as PhIP and MeIQ are rapidly absorbed and eliminated by humans when administrated orally at low dose and are bioavailable to colon tissues. Compared with rodents, humans may have a greater capacity to bioactivate (Davis et al., 1993a; Turteltaub et al., 1999), and a lower capacity to ring hydroxylate (a detoxification pathway) these compounds (Lin et al., 1995). Consequently, greater levels of both blood protein adducts and DNA adducts may be produced in humans, compared to rodents. Results from human studies indicated that man forms approximately 10 times more DNA adducts from HCAs per unit than laboratory animals (Garner et al., 1999). Several experiments indicated that simultaneous treatment of rats with low doses of different HCAs, which more closely parallels the HCA exposure in humans, increases the tumor yield and preneoplastic lesions in a synergistic manner (Takayama et al., 1987; Ito et al.,

1991b; Ito et al., 1995). Thus, the contribution of HCAs to human cancers extrapolated from laboratory animals may be underestimated.

1.3. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

1.3.1. PhIP and cancer

PhIP (Figure 1.1) is potentially the most important among the HCAs due to its mass-abundance and mutagenic potential in mammalian test systems. Approximately 15 μg of PhIP is produced per kilogram of cooked beef, accounting for approximately 75% of the total HCAs present in cooked meat (Felton et al., 1986). It is estimated that the average daily intake of PhIP in people in the U.S. is 16.6 ng/kg (Layton et al., 1995). The mutagenicity of PhIP, as determined in *in vivo* mammalian assays, is 5-time higher than that of any other HCAs (Thompson et al., 1987), although its mutagenicity in the Ames/*Salmonella* assay is the weakest. Unlike other HCAs, which primarily induce liver tumors in rodents (Adamson et al., 1994; Hasegawa et al., 1996; Ryu et al., 1999), PhIP has been found to induce tumors in the colon and prostate in male rats and mammary gland in female rats, respectively (Hasegawa et al., 1993; Ito et al., 1997; Shirai et al., 1999), these being the most common sites of cancer incidence in humans. Thus, PhIP is a suspected risk factor in the etiology of these cancers in human.

1.3.2. PhIP metabolism

The mutagenic effect of PhIP has been the subject of considerable interest. DNA lesions, inducible DNA repair, and cell division, are the three key factors in mutagenesis and carcinogenesis. The formation of DNA adducts is crucially important for the

induction of mutation and cancer. PhIP is not mutagenic *per se* but must be metabolized to a mutagenic form (Figure 1.2). The initiation of the mutagenicity and, presumably, the carcinogenicity of PhIP is a result of DNA adduct formation occurring after the metabolic activation of the parent amines. The first step in the activation involves the oxidation of the exocyclic amino group to its corresponding *N*-hydroxylated derivatives, catalyzed primarily by hepatic cytochrome P4501A2 and, to a lesser extent, by P4501A1 (Wallin et al., 1990). The resulting *N*-OH-PhIP is further *O*-acetylated by hepatic *N*-acetyltransferase (NAT) to form *N*-acetoxy-PhIP. Both *N*-OH-PhIP and *N*-acetoxy-PhIP can then be transported to extrahepatic tissues, in which *N*-OH-PhIP is further converted to *N*-acetoxy-PhIP by phase II esterification enzymes (Malfatti et al., 1996; Wang et al., 1999a). Cytosolic acetyltransferase, sulfotransferase, aminoacyl-tRNA synthetase and phosphatase may all participate in the esterification step (Davis et al., 1993b) and the contribution of each enzyme may be tissue- and species-specific (Buonarati et al., 1990; Ghoshal et al., 1995). *In vitro* experiments suggested that the esterification of *N*-OH-PhIP is more dependent on acetyltransferase in rats and sulfotransferase in monkeys (Davis et al., 1993b). In humans, an elevated risk for colorectal cancer was observed in meat consumers with rapid NAT2 phenotype (Lang et al., 1994; Kampman et al., 1999). The capability of the liver to esterify *N*-OH-PhIP is relatively low, when compared with extrahepatic tissues such as the mammary gland, prostate, and colon. Ghoshal et al. (1995) has shown that the activation of *N*-OH-PhIP mediated by mammary cytosolic *O*-acetyltransferase was ~16- to 17-fold higher than observed with hepatic cytosol. Thus, it is not surprising that PhIP does not induce tumors in the liver. Hydrolysis of the

esterified intermediates has been proposed to give rise to nitrenium ions, reactive electrophilic intermediates, which bind DNA covalently (Lin et al., 1992). The relative binding levels of the ultimate PhIP metabolite to genomic DNA appear to be determined by pharmacodynamic considerations. The major detoxification pathway for PhIP in the rat is hydroxylation in the 4' position on the phenyl ring of PhIP by P4501A1 to yield 4'-hydroxy-PhIP (Wallin et al., 1990). 4'-hydroxy-PhIP can be further converted to 4'-PhIP-sulfate by sulfotransferase. The detoxification of PhIP also occurs via glucuronidation of *N*-OH-PhIP (Malfatti et al., 1996) or through the formation of a glutathione (GSH) conjugate (Alexander et al., 1991; Lin et al., 1994). In the intestine, *N*-OH-gluc-PhIP is hydrolyzed by different strains of bacteria and reduced to PhIP (Alexander et al., 1991).

1.3.3. PhIP-DNA adducts

The covalent binding of the active PhIP metabolite with DNA was investigated by incubating 2'-deoxyribonucleosides with *N*-acetoxy-PhIP. A reaction was observed only between *N*-acetoxy-PhIP and dG, suggesting that PhIP may form DNA adducts principally at guanine sites (Lin et al., 1992). This PhIP-2'-deoxyguanosine adduct was characterized by mass spectrometry and ^{1}H nuclear magnetic resonance (NMR) spectroscopy analysis, showing that PhIP, like the cooked food mutagen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), reacts with the C-8 of guanine forming N2-(2'-deoxyguanosin-8-yl)-PhIP (dG-C8-PhIP) (Nagaoka et al., 1992) (Figure 1.3.). Using ^{32}P-postlabelling, one major and several minor DNA adducts, all at guanine sites, have been found when *N*-acetoxy-PhIP was incubated with calf thymus DNA. The major DNA

adduct, which comprised ~65-90% of total DNA adducts, has been identified as dG-C8-PhIP while the structures of minor adducts have not yet been clarified (Snyderwine et al., 1993). Turesky et al. (1992) has shown that, in addition to binding to guanine at the C-8 atom, IQ and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) also bound to guanine at the N^2 atom to form dG- N^2 adducts after *in vitro* incubation of *N*-acetoxy derivatives of *N*-OH-IQ and *N*-OH-MeIQx with dG or DNA. It is suspected that one of the minor PhIP-DNA adducts may correspond to an N^2-guanine derivative. Another possibility is that the minor adducts may be an artifact from undigested dinucleotides or oligonucleotides (Fukutome et al., 1994). Those possibilities remain to be clarified. *In vivo* experiments produced similar profiles of PhIP-DNA adducts as those shown by *in vitro* studies, although the proportion of each adduct varies (Takayama et al., 1989; Schut and Herzog, 1992). The major PhIP-DNA adduct, dG-C8-PhIP, accounted for 35-40% of the total detectable adducts in every tissue examined (Lin et al., 1992). A PhIP-adenine adduct was formed in *in vitro* reactions of *N*-acetoxy-PhIP with adenine-containing polynucleotides including poly(dA), poly(dA).poly(dT) and poly(dA-dT).poly(dA-dT). However, this adenine adduct was not present in any *in vitro* or *in vivo* adduct profiles from genomic DNA treated with PhIP or PhIP metabolites (Snyderwine et al., 1993).

The formation of PhIP-induced DNA adducts is tissue- and species-specific. When PhIP is given to F344 rats through diet, relatively high DNA adduct levels were produced in the lung, pancreas, and heart, followed by the prostate, colon, spleen, stomach, small intestine and kidney. The DNA adduct level in the liver was the lowest (Takayama et al., 1989; Kerdar et al., 1993). In CDF_1 mice given the same diet, PhIP

induced DNA adducts in a different manner, *viz.* liver, small intestine, colon, cecum> kidney, spleen, lung> stomach. The distribution of PhIP-DNA adduct is also found to depend on the mode of administration. When male rats were given a single dose of PhIP, the highest level of PhIP-DNA adducts was detected in the colon, followed by spleen, cecum, small intestine and stomach. The PhIP-DNA adduct levels in the liver, lung, kidneys, and heart were much lower (Cummings and Schut, 1994). Compared with chronic dietary supplementation, the removal of PhIP-DNA adducts was much faster after a single oral dose in both rats and mice (Schut et al., 1997). PhIP-DNA adducts accumulated mainly in the kidney and pancreas after chronic exposure (Friesen et al., 1996).

1.3.4. Effect of PhIP on cell proliferation

In addition to forming bulky adducts with DNA, exposure to PhIP also increases cell proliferation in carcinogenic target tissues in rats. In the mammary gland, PhIP exposure increases proliferation in the epithelial cells of the terminal end buds (TEBs), putative sites of origin of mammary carcinomas (Snyderwine et al., 1998; Snyderwine, 1999). It is plausible that the increase in proliferation in TEBs may facilitate the fixation of mutations from PhIP-DNA adducts, enhancing the likelihood of tumor initiation. Ochiai et al. (1996) found that after administration of 400 ppm PhIP in the diet for 8 weeks, the bromodeoxyuridine labeling index in the male colon increased by 42%. The increased proliferation by PhIP in the colon of male rats, but not female rats, is consistent with the observed sex-specific carcinogenicity of PhIP in the colon, indicating a central role of induced cell proliferation in targeting cancer tissues. The contribution of cell

proliferation to carcinogenicity has been well demonstrated in the kidneys of rats after exposure to tris(2,3-dibromopropyl)phosphate (de Boer et al., 2000). A gradual increase in mutation frequency was seen from inner and outer medullas, to the renal cortex. However, induced proliferation was seen only in the outer medullas where tumors were found. Furthermore, a comparison of mutagenic analogs of carcinogenic and non-carcinogenic chemicals showed that carcinogenic analogs induced cell proliferation in the target tissue while the non-carcinogenic ones did not (Cunningham and Matthews, 1995). These observations suggest that cell proliferation, especially induced cell proliferation, may be a requisite for expression of carcinogenicity.

1.3.5. PhIP mutation specificity

Different mutagens may have distinct mutational specificity, as a consequence of the sequence dependent induction and repair of specific DNA adducts and cell division. In the house-keeping gene *dhfr*, the majority of PhIP-induced mutations were found at guanines on the nontranscribed strand and 75% were G:C→T:A single and tandem double transversions (Carothers et al., 1994), consistent with the primary formation of PhIP-DNA adducts at guanine sites. The mutagenic specificity of PhIP in simian kidney (COS-7) cells, as determined by site-specific mutagenesis (modified oligodeoxynucleotides containing a single modified guanine), consisted mostly of single base substitutions, in which G:C→T:A transversions predominate, along with lesser amounts of G:C→A:T transitions and G:C→C:G transversions. Minus one frameshifts were also detected (Shibutani et al., 1999). Similar mutational spectra were recovered in the *hprt* gene in human lymphoblastoid cells (Morgenthaler and Holzhauser, 1995) and in

supF shuttle vectors (Endo et al., 1994). One specific mutation, deletions of G at 5'-GGGA-3' sequences were frequently recovered in both *in vitro* and *in vivo* studies (Kakiuchi et al., 1993; Endo et al., 1994). In the *lacI* transgenic rats, these deletions at 5'-GGGA-3' sequences, were detected in several tissues including colon, mammary gland, and prostate, ranging from 6-10% of all recovered mutants (Okochi et al., 1999; Yang et al., in press). This type of mutation was also induced at a frequency of 3% in human fibroblasts harbouring the *E. coli supF* gene (Endo et al., 1994), in the *lacZ* gene of Muta Mouse colon mucosa (5%) (Lynch et al., 1998) and at the endogenous *hprt* gene of Chinese hamster fibroblasts (10%) (Yadollahi-Farsani et al., 1996). Nagao (1999) suggested that PhIP induces deletions of G from 5'-GGGA-3' sequences at a frequency of 3-10% of all mutations, independent of the gene. The G deletion at the 5'-GGGA-3' motif is considered to be the mutational fingerprint of PhIP and may serve as a biomarker for PhIP-induced carcinogenesis. In F344 rats, 4/8 PhIP-induced colon tumors shared such an identical mutation, deletions of a G from 5'-GGGA-3' sequences, in the adenomatous polyposis coli (*Apc*) gene. This gene is the rat homologue of *APC*, a human suppressor gene frequently mutated in colon cancer (Kakiuchi et al., 1995). More recently, Burnouf and Fuchs (2000) were able to detect this specific mutation in the *Apc* gene from rat colon after exposure to 400 ppm PhIP for only one week. Although the G deletions from 5'-GGGA-3' sequences have not been observed in any tumor-related gene in the mammary gland, frequent mutations (8/12) containing base substitution at 5'-GGA-3' or 5'-GGC-3' sites in the H-*ras* gene were detected in mammary tumors induced by PhIP. In contrast, the *ras* gene was not found to be frequently mutated in colon tumors

induced by PhIP (Kakiuchi et al., 1993). The findings support the view that specific mutations appear to be critical for the activation of selected oncogenes and inactivation of tumor suppressor genes; these molecular fingerprints also strengthen the association between human exposure to these agents and neoplasia (Harris, 1995).

PhIP exposure also induces several other mutational events. Using a human-hamster A_L activated by chick embryo liver co-culture, Waldren et al. (1999) found that PhIP induced a substantial amount of large deletions in the S1 locus, ranging from 4.2 to 133 Mbp. PhIP also induced structural chromosomal aberrations (CAs) and sister-chromatid exchanges (SCEs) in human lymphocytes and human diploid fibroblasts (TIG-7) (Otsuka et al., 1996).

1.4. Antimutagens

1.4.1. Antimutagenesis and the mechanisms involved

Antimutagenesis, the process of reducing the frequency or rate of spontaneous or induced mutation, is an important part of the overall strategy of cancer prevention. Mechanistic studies of multistage carcinogenesis revealed that carcinogenic agents acted via mutagenesis (Ashendel, 1995). De Flora (1988) pointed out that "genotoxic effects are not merely the *primum movens* in carcinogenesis, but that multiple genetic alterations occur along sequential stages of the whole process." This view clearly illustrates the comparable and often overlapping nature of antimutagenesis and anticarcinogenesis, and the potential for preventing cancers through antimutagenesis.

The pathways or mechanisms involved in antimutagenesis may be ascribed to: 1) altering the metabolism of mutagens/carcinogens by inhibiting the activation and/or increasing the detoxification of the chemicals; 2) scavenging mutagens or their metabolites through binding or adsorption; 3) acting on DNA repair processes via enhancing error-free DNA repair, and blocking error-prone DNA repair (Hartman and Shankel, 1990); 4) inhibiting cell proliferation. Several excellent reviews of antimutagenic mechanisms have been presented (e.g. De Flora and Ramel, 1988). Many antimutagens may act via more than one pathway.

1.4.2. Diet and antimutagenesis

Similar to its contribution to carcinogenesis, diet is equally effective in preventing mutagenesis and carcinogenesis. Compelling evidence has indicated the importance of protective factors present in human diet. For example, a reverse correlation has been established between the consumption of fruits and vegetables and risk of cancer incidence (Van Duyn and Pivonka, 2000; Feskanich et al., 2000; Terry et al., 2001), and much effort has been made to identify dietary compounds with cancer preventive potential. The knowledge of dietary compounds has been collected over many generations (which helps gauge the incidence of unwanted effects in humans more easily than in the case for novel synthetic compounds), therefore, they represent readily available candidates for chemoprevention. A typical example is green tea, the chemopreventive properties of which have been consistently demonstrated in a number of experimental models. Schut and Yao (2000) reported the inhibition of PhIP-DNA adducts by green tea in F344 rats and a decrease of B(*a*)P-induced mutation by green tea was observed in Big Blue® rats

(Jiang et al., in press). Epidemiological studies also suggested beneficial effects of tea consumption on human health and cancer prevention. Other dietary components such as dietary fiber, vitamins C and E, chlorophyllin, and certain unsaturated fatty acids have also been shown to be effective antimutagens in animal models. The chemopreventive activities of several dietary antimutagens are outlined in Table 1.2.

1.4.3. Chemopreventive properties of conjugated linoleic acid

Conjugated linoleic acid (CLA) is a collective term that refers to a mixture of positional and geometric isomers of linoleic acid (Figure 1.4). CLA is normally a minor constituent in the lipid fraction of many different kinds of food, primarily from bacterial isomerization of linoleic acid in the rumen. Relatively rich sources of CLA in our diet include dairy products and meat products, especially those from ruminant animals. Daily consumption of CLA is estimated to be ~1 g/person in the USA (Ha et al., 1989). A shift from a dairy product-rich to a dairy product-free diet has been associated with a significant effect on colon cancer risk (Glinghammar et al., 1997).

The antimutagenic activity of CLA was first noted by Pariza et al. (1979) who found that grilled ground beef inhibited dimethylbenz[a]anthracene (DMBA)-induced epidermal tumors. The effective compound was subsequently identified to be CLA. CLA has been shown to inhibit the formation of mammary tumors, forestomach tumors, and skin tumors in rodents (Ha et al., 1990; Ip et al., 1995; Belury et al., 1996). One unique characteristic of CLA is that exposure to CLA during the window of active mammary gland morphogenesis (from weaning to pubescence) can offer protection against subsequent tumorigenesis in adult life. A significant decrease in mammary tumor

incidence was observed in DMBA- or N-methyl-N-nitrosourea (MNU)-treated female rats when 1% CLA was added to the diet from the time of weaning until the administration of carcinogens (Ip et al., 1994b; Ip et al., 1995). Continuous supply of CLA in the diet did not provide additional protection (Thompson et al., 1997). These observations are in accordance with the dose-dependent suppression by CLA of the population of cancer sensitive target sites in the mammary gland (terminal end buds) during pubescence. The mechanism may rely on the competitive inhibition of linoleic acid metabolism by CLA, which results in a decrease of arachidonic acid formation (Banni et al., 1999). The products of arachidonic acid metabolism such as prostaglandin E_2 have been associated with an enhancement of tumor induction (Tang et al., 1996).

CLA is also capable of inhibiting tumor promotion/progression. In this case, a continuous supply of CLA is required, which suggests that different protective mechanisms may be involved in this stage. Supplementation with 1% and 1.5% CLA significantly reduced 12-O-tetradecanoylphorbol-13-acetate-promoted skin tumors in mice (Belury et al., 1996). In immunodeficient (*SCID*) mice, Visonneau et al. (1997) showed that CLA inhibited local tumor growth in the mammary gland induced by subcutaneous inoculation with MDA-MB468 cells and completely abrogated the spread of breast cancer cells to other tissues. These results suggested that inhibition of tumor promotion/progression by CLA is independent of the host immune system. Studies with tumor cell lines indicated that CLA significantly reduced cancer cell proliferation (Shultz et al., 1992a), especially estrogen receptor-positive tumor cells, through inhibiting expression of *c-myc* or interfering with the hormone regulated mitogenic pathway

(Durgam and Fernandes, 1997). Some studies also suggested that the inhibition may be related to the elevated lipid peroxidation in tumor cells after exposure to CLA (Schonberg and Krokan, 1995).

Chemoprevention by CLA is both sex- and tissue-specific. CLA caused a dose-dependent decrease of PhIP-DNA adduct in the liver of female rats, but not in colon and mammary epithelium cells (Josyula and Schut, 1998). In IQ-treated male rats, both aberrant crypt foci (ACF) and DNA adducts were reduced in the colon by CLA, while the DNA adducts were not inhibited in the liver (Liew et al., 1995). CLA supplementation also decreased IQ-adduct formation in the kidney in female rats, but not in male rats (Zu and Schut, 1992). Although the underlying mechanism is not clear, it may relate to the effect of CLA on the expression of P450 1A1/1A2 in the liver of experimental animals (Liew et al., 1995).

1.5. Assays

A general testing strategy was proposed by Ferguson (1994) for the detection of antimutagens. Ferguson suggested that antigenotoxic compounds should be initially identified in time- and cost-effective screening trials *in vitro*, particularly in bacterial mutagenicity tests. I*n vivo* experiments should subsequently be carried out to elucidate if protective effects take place in mammals and humans as well.

1.5.1. *In vitro* and *in vivo* assays used for detection of antimutagens

Based on the pathways involved in antimutagenesis, many *in vitro* and *in vivo* assays have been employed to identify antimutagens. The basic assumption has been that

every method that is useful for the detection of mutagens should also be able to detect antimutagens. However, in the case of antimutagens against HCAs, some important mechanisms are not adequately represented in a number of test systems. A survey by Schwab et al. (2000) has shown that protective effects against HCAs *in vitro* are almost entirely based on bacterial gene mutation assays, while measurement of DNA adducts is the method most frequently used *in vivo*. The suitability of using these methods to identify antimutagens is debatable. HCAs require metabolic activation by phase I and II enzymes, thus *in vitro* assays with metabolically incompetent cells are obviously not suitable for the detection of compounds that interfere with the metabolism of HCAs. When an exogenous activation mix is introduced, false-negative or false-positive results may be obtained. An experiment with *Salmonella* strain TA98 and IQ showed that any compound causing a pronounced deviation from the pH optimum, or of the ideal salt concentration of the enzyme homogenate, will reduce the mutagenicity of the HCAs in a bacterial assay and would be classified as an antimutagen (Schwab et al., 2000). Moreover, traditional *in vitro* methods have proven to be ineffective in elucidating the role of cell proliferation on mutagenesis. The usage of DNA adducts as a biomarker to identify antimutagens is weakened by the lack of association between DNA adduct levels and cancer incidence. On the other hand, mutations induced by HCAs, e.g. PhIP, correlate relatively well with the occurrence of tumors. Therefore, transgenic rodent mutagenesis assays, such as the Big Blue® transgenic assay, may be a better candidate for short-term screening systems, while *in vitro* short-term assays may be helpful in clarifying the mechanisms involved.

1.5.2. The Big Blue® transgenic system

The development of transgenic rodent models met the requirement for test assays that allow analysis of gene mutations in virtually every tissue or organ following exposure *in vivo* to chemical agents (reviewed in Gossen and Vijg, 1993; Dycaico et al., 1994; Nohmi et al., 2000). In addition, mutational spectra reflect the specific deposition and handling of DNA lesions, thus providing an opportunity to examine the mechanisms by which the mutations occur. The efficacy of chemoprevention can be elucidated by decreases in mutation frequency or modification of the specific mutational fingerprint of the mutagens.

Big Blue® *lacI* transgenic rodents are derived from microinjection of constructs based on bacteriophage λ containing a bacterial *lacI* gene (Figure 1.5), into fertilized oocytes of rats or mice. These transgenic rats and mice carry 30-40 copies of the λLIZ/*lacI* transgene integrated in a linear tandem array at a single locus on chromosome 4 (Dycaico et al., 1994). The λLIZ/*lacI* transgenes can be rescued from the genome by *in vitro* bacteriophage lambda packaging reactions and introduced into *E. coli* SCS-8 cells. The infected *E. coli* cells are then plated on agar media containing the chromogenic substance 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The phages harboring a mutant *lacI* gene give rise to blue plaques while a wild type gene result in a colorless plaque. The *lacI* gene codes for the Lac repressor protein, which binds to the operator of the *lacZ* gene and thus blocks its expression. The *lacZ* gene codes for β-galactosidase which can cleave X-gal, resulting in a blue color (Figure 1.6). The blue

plaques containing a mutant *lacI* gene are cored and stored in buffer at 4°C. Pure mutant plaques obtained by re-streaking the phages are amplified by PCR, and sequenced by cycle sequencing to establish mutational spectra.

1.6. Hypothesis

PhIP induces tumors in a tissue specific manner, and CLA has been shown to provide chemoprotection against PhIP-induced carcinogenesis. I hypothesize that CLA can provide chemoprotection through changes in PhIP-DNA adducts and mutagenicity. I also expect that CLA provides this protection in a tissue specific manner. The possible involvement of DNA repair pathways in the modification effect of CLA will be explored. In addition, the experiments will also provide formal controls to establish whether CLA has a mutagenic property of its own.

1.7. Outline of the thesis

Chapter 2 describes how Big Blue® transgenic rats were used to study the antimutagenic potential of two dietary chemopreventive agents, CLA and 1,2- dithiole-3-thione (DTT). This chapter provides preliminary data for design of the subsequent experiments. The suitability of using the Big Blue® system as a screening model, the effective dose of CLA, and a suitable dose of the carcinogen, PhIP, were initially determined. Chapter 3 is an extension of Chapter 2, and addresses the chemopreventive potential of CLA using a different treatment schedule. Concerning the sex-specific carcinogenicity of PhIP in the colon, I compared the antimutagenic effect of CLA between male and female rats. Chapter 4 describes the effect of CLA on the prostate, a

target tissue of PhIP-induced mutagenesis and carcinogenesis. Chapter 5, for the first time, describes the mutagenicity of PhIP in the kidney of male and female rats. Sex-specific induction of mutations was observed. The effect of CLA is also shown to be sex-related. Chapter 6 describes the formation and removal of DNA adducts in a R2λLIZ rat cell line exposed to UV or benzo(*a*)pyrene diol epoxide (BPDE). A major concern in using the Big Blue® mutagenesis assay is that λLIZ/*lacI* is transcriptionally inactive, and also is a target for endogenous methylation at CpG dinucleotides (Kohler et al., 1990), which may affect the induction and/or removal of induced DNA damage. This chapter shows that the global DNA repair pathway can efficiently repair DNA adducts formed in the λLIZ transgene, supporting the suitability of the Big Blue® transgenic assay as an *in vivo* test system of mutagenesis as well as a chemopreventive screening system. Chapter 7 describes our attempt to understand the chemopreventive mechanisms of CLA. The effect of CLA on mismatch repair pathways was determined in *E. coli* strains, which are proficient or deficient in mismatch repair. Chapter 8 provides a general discussion.

Table 1.1. Induction of tumors in mice and rats by HCAs

HCA[a]	CDF_1 mice	F344 rats	Reference
IQ[a]	Liver, forestomach, lung	Liver, small and large intestines, Zymbal gland, clitoral gland, skin	Ohgaki et al., 1991
MeIQ	Liver, forestomach	Large intestines, Zymbal gland, skin, oral cavity, mammary gland	
MeIQx	Liver, lung, hematopoietic system	Large intestines, Zymbal gland, clitoral gland, skin	
PhIP	Lymphoid	Large intestines, mammary gland, prostate gland, lymphoid tissue, hematopoietic system	
Trp-P-1	Liver	Liver	
Trp-P-2	Liver	Lymphoid tissue, bladder	
Glu-P-1	Liver, blood vessels	Liver, small and large intestines, Zymbal gland, clitoral gland, skin	
Glu-P-2	Liver, blood vessels	Liver, small and large intestines, Zymbal gland, clitoral gland, skin	
AαC	Liver, blood vessels		
MeAαC	Liver, blood vessels	Liver	

a. Abbreviations: AαC, 2-amino-9H-pyrido[2,3-b]indole; Glu-P-1, 2-amino-6-methyl-dipyrido[1,2-a:3',2'-d]imidazole; Glu-P-2, 2-aminodipyrido[1,2-a:3',2'-d]imidazole; IQ, 2-amino-3-methylimidazo 4,5-f]quinoline; MeIQ, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole; MeAαC, 2-amino-3-methyl-9H-pyrido[2,3-b]indole

Table 1.2. Chemopreventive properties of several dietary compounds

Mechanisms of chemoprevention	Examples from the diet
Blocking agent	
Inhibit carcinogen formation	Vitamin C, vitamin E, caffeic acid, ferulic acid, garlic acid, proline, thioproline, phenols, fermented dairy products
Inhibit phase I enzymes	Dithiocarbamate, ellagic acid, diallyl sulfide, isothiocyanates
Induct phase II enzymes	Allyl sulfide, dithiolethiones, isothiocyanates, polyphenols, selenium,
Increase level or fidelity of DNA repair	Vanillin, protease inhibitor
Antioxidant	
Scavenge reactive electrophiles	Chlorophyllin, ellagic acid
Scavenge oxygen radicals	Polyphenols, vitamin E, vitamin C
Inhibit arachidonic acid metabolism	Polyphenols, vitamin E, retinoid
Antiproliferation/ antiprogression agents	
Modulate signal transduction	Retinoid, protease inhibitor
Reverse abnormal proliferation	Retinoid, CLA, calcium, vitamin D
Inhibit oncogene activity	Monoterpenes, polyphenols

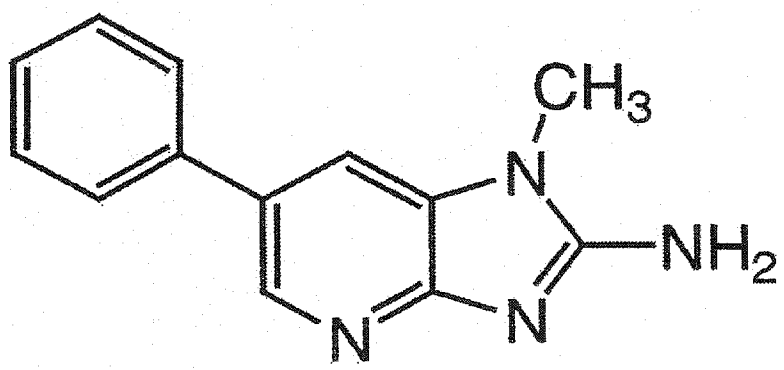

Figure 1.1. Structure of PhIP

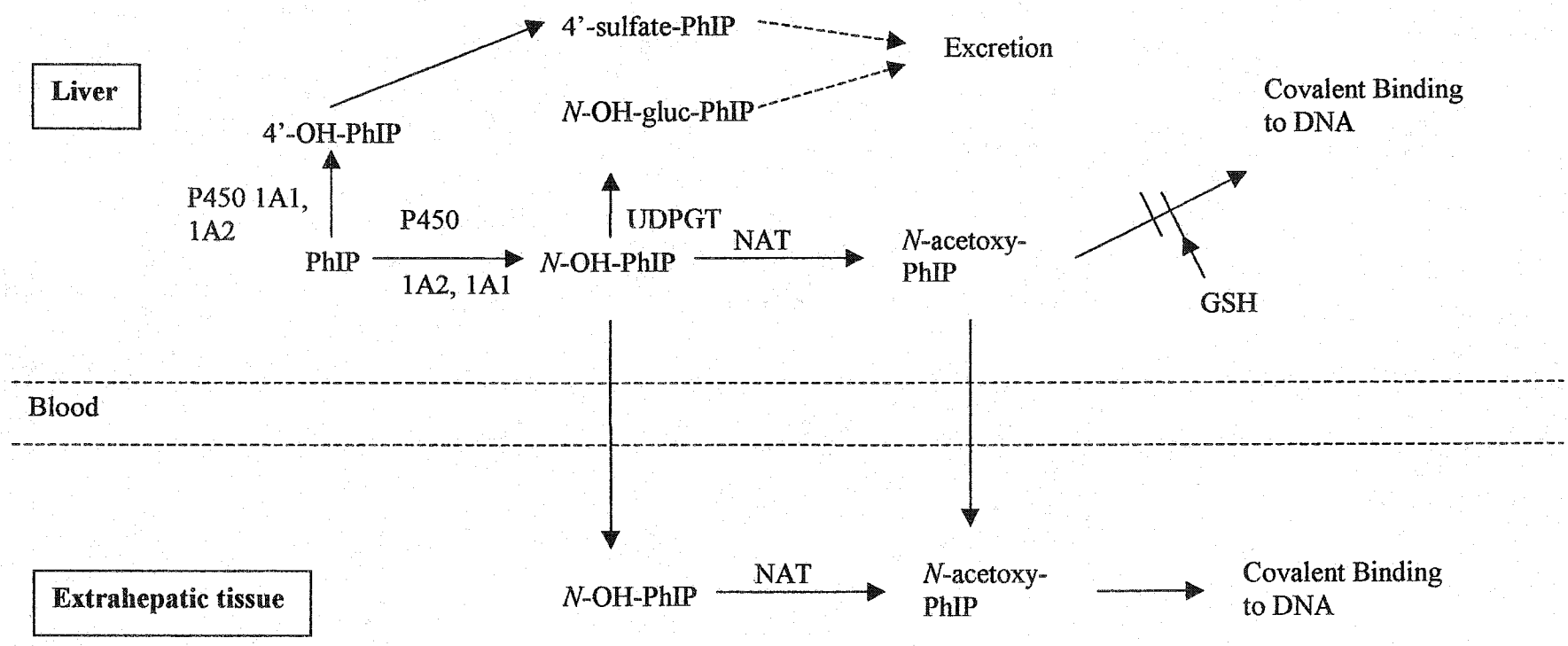

Figure 1.2. Hypothesis for the metabolic activation of PhIP leading to colon carcinogenesis. PhIP is first oxidated to its *N*-hydroxylated derivatives, catalyzed primarily by hepatic cytochrome P4501A2 and P4501A1. The resulting *N*-OH-PhIP is further *O*-acetylated by hepatic *N*-acetyltransferase (NAT) to form *N*-acetoxy-PhIP. Both

N-OH-PhIP and *N*-acetoxy-PhIP can then be transported to extrahepatic tissues, in which *N*-OH-PhIP is further converted to *N*-acetoxy-PhIP by phase II esterification enzymes. The major detoxification pathway for PhIP in the rat involves 4'-hydroxylation of PhIP by P4501A1, glucuronidation of *N*-OH-PhIP, or the formation of a glutathione (GSH) conjugate.

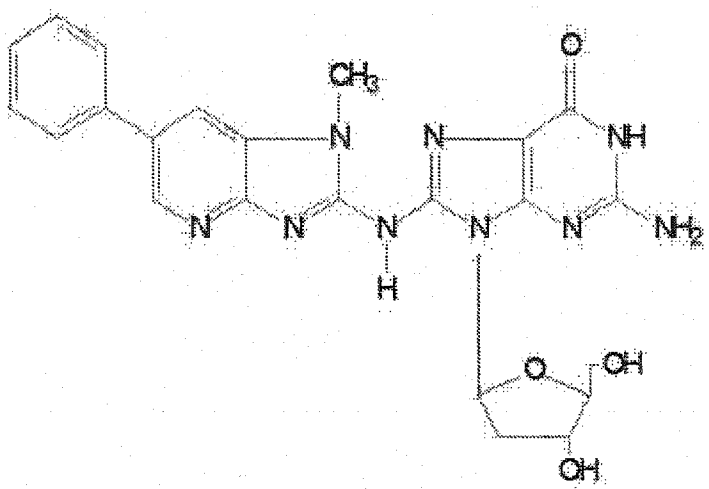

Figure 1.3. Structure of the primary PhIP-DNA adduct, N2-(2'-deoxyguanosin-8-yl)-PhIP (dG-C8-PhIP)

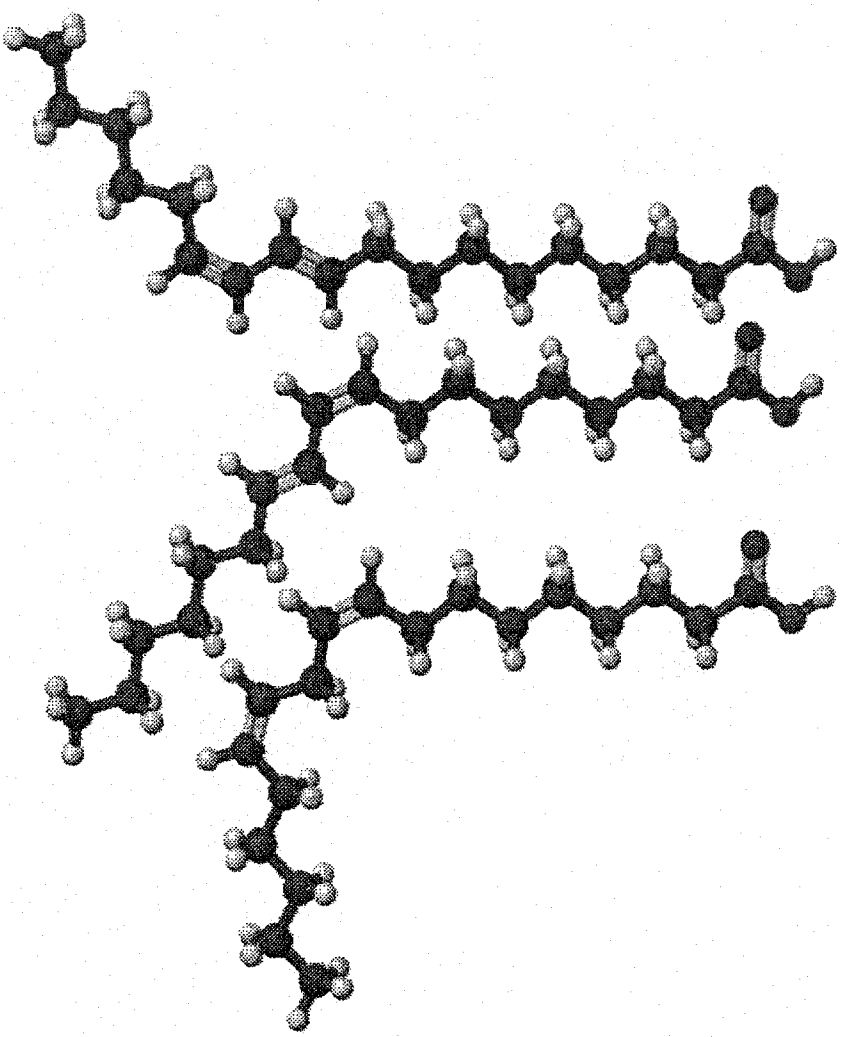

Figure 1.4. Structures of t-10, c-12-CLA (top), c-9, t-11-CLA (center), and ordinary linoleic acid, c-9, c-12-octadecadienoic acid (bottom).

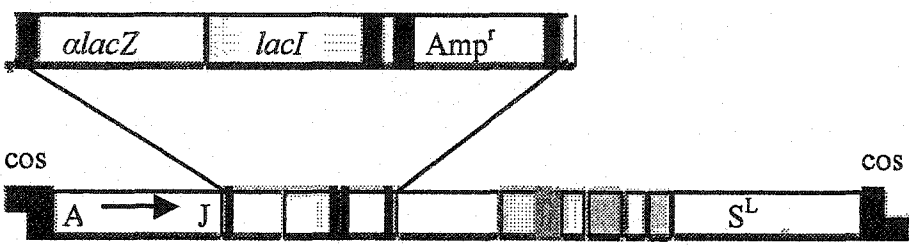

Figure 1.5. λLIZ shuttle vector used to generate Big Blue® transgenic mice and rats. This shuttle vector is produced by insertion of a plasmid containing a *lacI* and *αlacZ* gene into λ DNA.

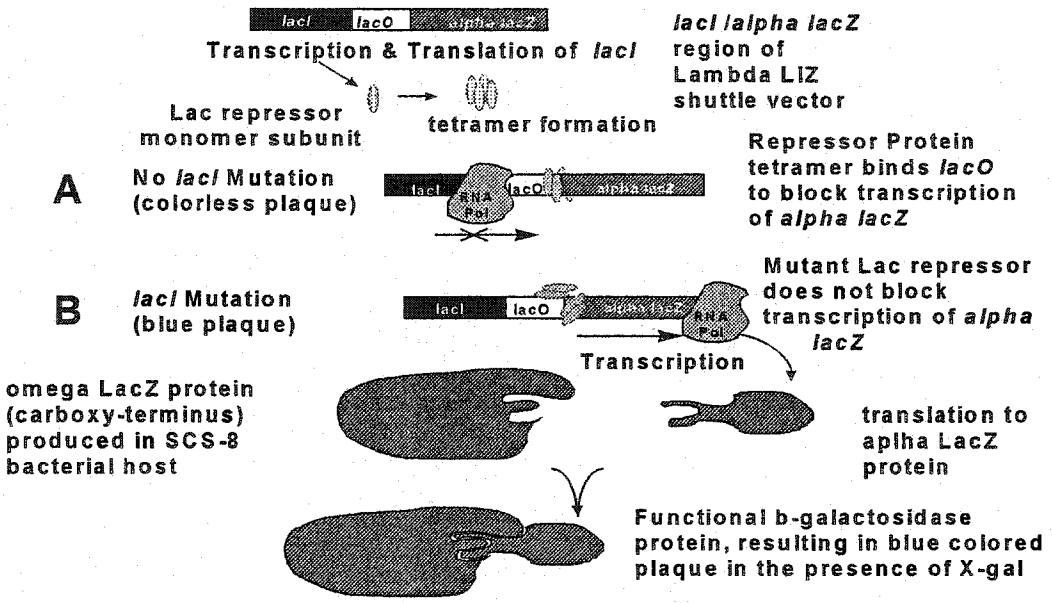

Figure 1.6. The regulation of *lacZ* gene expression by the Lac repressor. The Lac repressor synthesized by the *lacI* gene forms a tetramer, which binds to the lacO sequence. **A)** A colourless plaque is generated by an intact Lac repressor. **B)** A mutant Lac repressor leads to a blue plaque in the presence of the chromogenic substrate, X-gal.

Chapter 2. Modulation of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-induced Mutation in the Cecum and Colon of Big Blue® Rats by Conjugated Linoleic Acid and 1,2- Dithiole-3-thione

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Nutrition and Cancer (In press)

Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a potent mutagen and suspected human carcinogen present in cooked protein-rich food. It preferentially induced colon tumors in male and mammary tumors in female rats. In the present study, the *in vivo* antimutagenic efficacy of two dietary compounds, conjugated linoleic acid (CLA) and 1,2-dithiole-3-thione (DTT), against PhIP was explored using *lacI* transgenic Big Blue® rats. Five- or six-week old male Big Blue® rats were fed a diet containing CLA (0.5%, w/w) or DTT (0.005%, w/w) starting one week prior to exposure to 200 ppm PhIP for 61 days. PhIP treatment induced a ~17- to 19-fold increase in the mutation frequency (MF) in the colon. The induced MF observed in the cecum was significantly lower than that in the proximal and distal colon (DC) (~ 52 $x10^{-5}$ vs. 100 $x10^{-5}$, $p<0.008$). CLA and DTT significantly reduced the PhIP-induced MF in DC ($p<0.05$) by 14% and 24%, respectively. Notably, the frequency of minus one (–1) frameshift mutations was lower in the DC of CLA- or DTT-treated rats. This protective effect was not observed in the cecum or in the proximal colon (PC). In contrast, the PhIP-induced MF in the cecum (specifically, the frequency of –1 frameshifts and G:C→T:A transversions) was elevated by 43% after treated with CLA. In conclusion, CLA and DTT modulate PhIP-induced mutagenesis in a tissue-specific manner and different modulation pathways are employed by CLA and DTT.

2.1. Introduction

Humans are constantly exposed to low levels of carcinogens that are naturally present in the diet. Doll and Peto (1981) estimated that diet is responsible for up to 70% of all avoidable cancers. On the other hand, many dietary compounds have also been found to have anticarcinogenic potential. The identification of anticarcinogens in food as

well as the understanding of responsible mechanisms are increasingly important aspects of an overall strategy for cancer prevention (Stoner et al., 1997).

Colon cancer is the second leading cause of death from cancer in males and the third in females in the U.S. (Boring et al., 1994), with an annual incidence in the United States of about 155,000 new cases (Krishnan et al., 1998). Epidemiological studies suggest that the incidence of colon cancer is related to the consumption of well-cooked meat (Hsing et al., 1998; Bingham, 1999). Heterocyclic amines (HCAs) are present in cooked proteinaceous food in the ppb (μg/kg) range. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant HCA in the human diet and is present in human food at 0.56-69.2 ng/g (Nagao and Sugimura, 1993). PhIP predominantly causes colon and prostate tumors in male rats (Ito et al., 1991a; Shirai et al., 1999), mammary gland tumors in female rats and lymphomas in mice (Esumi et al., 1989; Okonogi et al., 1997a). PhIP may also be relevant to human colon cancer (Kadlubar et al., 1995).

PhIP is not mutagenic per se but undergoes metabolic activation thus forming active metabolites. The activation of PhIP is catalyzed by cytochrome P450 (CYP) 1A2-mediated N2-hydroxylation to form 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine, which can be acetylated or sulfated to produce DNA-binding PhIP metabolites, or deactivated by glutathione-S-transferases (GST) (Huber et al., 1997) and UDP-glucuronosyltransferases (UDPGT) (Malfatti et al., 1999, Nowell et al., 1999). Detoxification of PhIP also occurs via CYP1A1 dependent carbon-4-ring hydroxylation

(Alexander et al., 1995). It is expected that chemopreventive agents that interfere with the metabolism of PhIP could modulate mutagenesis/carcinogenesis induced by PhIP.

Conjugated linoleic acid (CLA) is a collective term that refers to a mixture of positional and geometric isomers of linoleic acid (Ip et al., 1994b). Dietary sources of CLA include dairy products and meat products, especially those from ruminant animals. The daily consumption of CLA is estimated to be ~1 g/person in the USA (Ha et al., 1989). *In vivo* and *in vitro* experiments have indicated that CLA can effectively inhibit mutagenicity/carcinogenicity of HCA and dimethylbenz[a]anthracene (DMBA) (Liew et al., 1995; Thompson et al., 1997). For example, in 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)-treated male rats, both aberrant crypt foci (ACF) and DNA adducts were reduced in the colon by CLA (Liew et al., 1995). Supplementation with 0.05-0.5% CLA inhibited DMBA-induced mammary gland tumors in a dose-dependent manner (Ip et al., 1994b). Several epidemiological studies have suggested that dairy product intake is associated with a decreased incidence of colon cancer (McMichael et al., 1980; Rosen et al., 1988). As a compound present in dairy products, CLA may play a role in preventing colon cancer. Several chemopreventive mechanisms of CLA have been proposed, including decreased formation of arachidonic acid by competitive inhibition of linoleic acid metabolism (Banni et al., 1999); reduced cancer cell proliferation (Shultz et al., 1992b); interference with hormone regulated mitogenic pathways (Durgam and Fernandes, 1997); and decreased hepatic CYP1A1/1A2 in experimental animals (Liew et al., 1995; Josyula and Schut, 1998). However, no *in vivo* studies of the antimutagenic effect of CLA have been reported.

1,2- Dithiole-3-thione (DTT), a constituent of cruciferous vegetables, can provide numerous target organs with protection from structurally diverse carcinogens (Kensler et al., 1992; Zhu et al., 1995). Dietary supplementation with DTT effectively reduced aflatoxin B_1-induced hepatic DNA adduct levels and the focal area of preneoplastic lesions in male F344 rats, presumably due to elevated GST activities in the liver (Bolton et al., 1993). Oltipraz, a synthetic derivative of DTT, has been reported to suppress PhIP-induced lymphomas in rats (Rao et al., 1996). The efficacy of DTT in inhibiting the carcinogenic process has been attributed to its ability to induce phase II detoxification enzymes, including GST and NAD(P)H:quinone reductase (Ansher et al., 1986). DTT would thus be expected to enhance the detoxification of PhIP metabolites, resulting in the modulation of its mutagenic potency.

The long-term nature of animal experiments required for cancer studies and the relatively low incidence of tumors make screening for chemopreventive agents a challenge, which makes the use of *in vivo* animal models attractive as an alternative biomarker. The Big Blue® *lacI* rodent system (Kohler et al., 1991) has proven to be suitable for evaluating the susceptibility of different tissues to mutagenic environmental carcinogens. Tissue- or species-specific responses to selected mutagens are reflected by changes in MF or mutational spectrum (MS) (de Boer et al., 1996b; Dycaico et al., 1996). Thus we expect that the antimutagenic efficacy of a compound can be demonstrated either by a decrease in induced MF or by a reduction of a specific class of induced mutation. In the present study, the antimutagenic properties of the two dietary compounds, CLA and DTT, against the dietary mutagen, PhIP, were examined in the *lacI*

transgenic Big Blue® rat model. The purposes of this study were: 1) to evaluate the usage of the Big Blue® *lacI* transgenic assay as a screening tool for anti-mutagenic/carcinogenic compounds; 2) to provide a better understanding of the chemopreventive mechanisms of CLA and DTT; and 3) to provide information for human cancer prevention through dietary supplementation, as PhIP, CLA, and DTT are all present in the human diet.

2.2. Materials and Methods

2.2.1. Chemicals

PhIP (>98% pure as determined by TLC) was obtained from Toronto Research Chemicals (Toronto, ON, Canada). CLA (>94% pure as determined by GLC and TLC; the composition of CLA is: ~41% *cis*-9,*tran*-11 CLA, ~44% *trans*-10,cis-12 CLA, ~10% *cis*-10,12 CLA, ~5% *cis*-9,12 linoleate) was purchased from Nu-Chek-Prep (Elysian, MN). DTT (>97% pure as determined by NMR) was a generous gift from Dr. Kent S. Gates, Department of Chemistry, University of Missouri-Columbia.

2.2.2. Animals and treatments

Male F344 Big Blue® rats (4-5 weeks old) were obtained from Stratagene (La Jolla, CA), and housed individually. Food and water were provided *ad libitum*. The rats were weighed weekly and the food consumed was measured twice weekly. After one week of acclimatization to the powdered basal diet, AIN-93G without the *tert*-butylhydroquinone antioxidant (Dyets Inc., Bethlehem, PA), the rats were divided into 4 groups consisting of 5 rats each, with supplementation to the basal diet as follows: 1)

None; 2) PhIP; 3) PhIP + CLA; and 4) PhIP + DTT. PhIP, CLA and DTT were mixed into the powdered diet as required, at a concentration of 200 ppm for PhIP, 0.5% (w/w) for CLA, and 0.005% (w/w) for DTT. Two percent (w/w) tocopherol-stripped corn oil was incorporated into the powdered diet throughout the whole experiment in order to reduce the formation of dust. CLA or DTT treatment began one week prior to supplementation with PhIP. After 61 days of PhIP treatment, the rats were returned to the basal diet and sacrificed one week later. The cecum and the colon (divided into PC and DC) were removed immediately, frozen in liquid nitrogen, and stored at -80°C until DNA isolation.

2.2.3. Mutation assay

High molecular weight genomic DNA was isolated from tissues using a modification of the RecoverEase™ DNA dialysis method. Briefly, the colon or the cecum was cut into small pieces, and dounced in cell lysis solution (10 mM Tris-HCl, pH 8.3, 140 mM NaCl, 3 mM KCl, 0.35 M sucrose, 1 mM EDTA, 1% Triton X-100) using a Kontes™ dounce tissue grinder (Kontes Glass Co.), followed by filtration to remove underlying tissues. The remaining mucosa cells were digested with proteinase K/SDS (2 mg/ml proteinase K, 2 % SDS, 100 mM EDTA) and dialyzed against TE (10 mM Tris-HCl, 1 mM EDTA, pH7.5) for 48-72 hours. The DNA was then added to a λ phage packaging extract (Transpack, Stratagene) and the resulting phages were plated on *E.coli* SCS-8 bacteria on trays of NZY media containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside. The mutant frequency was determined by the ratio of the number of blue mutant plaques to the total number of plaques. The *lacI* gene from mutants was

amplified by PCR (Erfle et al., 1996), purified and sequenced on a LICOR automated fluorescent sequencer, and corrected for clonal expansion, thus establishing a mutational spectrum (MS) for each specific treatment.

2.2.4. Statistical analyses

Statistical analyses of mutation frequency (MF, generated from the mutant frequency after corrected for clonal expansion) were performed using COCHARM (created by Troy Johnson, Procter and Gamble, Cincinnati, OH), a computer program that executes the Generalized Cochran-Armitage test (Carr and Gorelick, 1995). Statistical comparisons of MS were made using the Monte Carlo method of Adams and Skopek (1987). The MF of each class of mutation was calculated by multiplying the fraction of each class by the overall MF.

2.3. Results

2.3.1. Animal growth

No significant difference in food consumption nor animal weight gain was observed among the treatment groups, indicating that the addition of PhIP, CLA, or DTT did not affect the growth and food intake of the animals (data not shown).

2.3.2. Mutation frequencies

The *lacI* MF in the cecum, PC, and DC in male rats treated with PhIP, PhIP+CLA, and PhIP+DTT are summarized in Table 2.1. The PhIP-induced MF ($\times 10^{-5}$, $\pm$ SD) in the cecum, PC, and DC were 51.5 ± 6.1, 97.5 ± 6.8, 104.3 ± 5.2, compared with

the untreated control values of 6.4 ± 1.6, 4.9 ± 0.7, and 6.1 ± 1.4 (reported previously by Stuart et al., in press), respectively. The MF following PhIP was thus 17- to 19-fold greater than the control MF. The PhIP-induced MF in PC and DC are similar (p=0.52). Interestingly, the PhIP-induced MF in the cecum is only about half of those in PC and DC ($p < 0.008$).

Mutation frequencies from rats treated with chemopreventive agents were next examined. Statistical analysis of the treatment data suggests that the protection of CLA and DTT against PhIP may only take place in DC, in which a statistically significant reduction of MF of 14% and 24% was observed in CLA and DTT-treated groups, respectively ($p < 0.05$). No effect of CLA or DTT was observed in PC. The addition of DTT in the diet also did not change the PhIP-induced MF in the cecum. However, CLA supplementation unexpectedly increased the PhIP-induced MF in the cecum by 43% ($74.4 \pm 3.6 \times 10^{-5}$ *vs.* $51.5 \pm 6.1 \times 10^{-5}$, p=0.018).

2.3.3. Mutation Spectra

A total of 832 *lacI* genes of mutants from the cecum and the colon (including the PC and DC) of different treatment groups were sequenced and 574, 146, and 95 independent mutants were identified from PhIP, PhIP+CLA, and PhIP+DTT treatment groups, respectively. The treatment with PhIP resulted in large increases in the frequencies of G:C→T:A transversions and –1frameshifts at G:C base pairs (Figure 2.1). This PhIP-induced MS was consistent with the PhIP spectrum reported by Okonogi et al. (1997a), and significantly different from the spontaneous spectrum ($p<0.0001$) in the

cecum and the colon (Table 2.2, from Stuart et al., in press). The signature mutation of PhIP, the deletion of a G:C base pair at 5'-GGGA-3' sites (Okonogi et al., 1997a), was recovered in all three tissues. The addition of CLA or DTT to the diet did not result in significant changes in the overall spectrum as judged by the Adams and Skopek test ($p>0.1$). To better understand the nature of changes of mutations upon exposure to PhIP, CLA, and DTT, the frequencies of several major classes of mutations in the cecum, PC, and DC, were calculated and presented in Figure 2.1. In DC, the addition of CLA reduced the frequency of –1 frameshifts by 27%, accounting for the overall decrease in MF. DTT appeared to decrease the frequency of both –1 frameshifts and G:C→C:G transversions. In PC, DTT reduced the MF of G:C→T:A transversions induced by PhIP by 42% although the overall MF induced by PhIP was not affected by this treatment. In the cecum, 2- to 2.5-fold increases in the frequency of both G:C→T:A transversions and –1 frameshifts were observed in CLA treated rats, reflecting the overall increase in mutation frequency.

2.4. Discussion

In the current study, we investigated whether the consumption of the two dietary components, CLA and DTT, can modulate the colorectal mutagenesis in male rats treated with PhIP. As anticipated, our mutation assay results showed that supplementation with CLA or DTT significantly changed the PhIP-induced MF in certain sites of the large intestine while some trends of MS alteration were also observed. Our results also support the use of the Big Blue® system to screen chemopreventive agents and to provide

information for tissue-, sex-, and species-specific chemoprevention as well as for protective mechanism studies.

In our study, dietary supplementation with 200 ppm PhIP for 61 days induced a MF of ~$100x10^{-5}$ in the colon of male rats. This result is about 1.5-fold of that reported by Okonogi et al. (1997a) who found that dietary supplementation with 400 ppm PhIP in a CE-2 basal diet for 60 days resulted in a *lacI* MF of 66×10^{-5} in male rat colon. To address the difference in the PhIP-induced MF between the two studies, the MF from the colon treated with a lower PhIP concentration (100 ppm) was determined in a follow-up experiment, in which a MF of 43.2×10^{-5} was induced (Yang , manuscript in preparation). This finding shows a dose-dependent increase in the PhIP-induced MF when using the AIN-93G diet. The lower MF when using the CE-2 diet may be attributed to the differences in the composition of the basal diets. The energy provided by two diets is similar (AIN-93G:CE-2=1.12:1), while in general, the CE-2 diet has higher contents of vitamins than AIN-93G. For example, the content of vitamin C is 280 mg/kg in CE-2 and absent in AIN-93G, and vitamin A is 10,000 IU/kg in CE-2 and 4,000 IU/kg in AIN-93G. Meanwhile the antioxidant, *tert*-butylhydroquinone, was omitted in the AIN-93G diet we used. Thus the total amount of antioxidants in the basal diet we used may be less than that in the CE-2 diet. Antioxidants may prevent oxidative DNA damage and inhibit the bioactivation of PhIP (Hammons et al., 1999), thus may decrease the MF induced by PhIP. Another possibility may be associated with the incorporation of 2% corn oil in the AIN-93G diet since corn oil can increase the cell proliferation rate (Pell et al., 1992; Lee et al., 1993), enhance mutagenic activation (Wade et al., 1982), as well as

promote tumor formation (reviewed by Woutersen et al., 1999). Both enhanced cell proliferation and mutagenic activation directly contribute to an elevated MF. Corn oil was absent in the CE-2 diet used by Okonogi et al. (1997a).

As demonstrated by our data, there is no significant difference between the PhIP-induced MF in the PC and DC, while the induced MF in the cecum is significantly lower. This difference may be attributed to the difference in PhIP-DNA adduct formation, in which a higher PhIP-DNA adduct level was detected in the colon than in the cecum (Josyula and Schut, 1999; Schut and Yao, 2000). However, the observed regional distribution of PhIP-induced mutations along the large intestine is not directly proportional to the appearance of ACF or tumors induced by PhIP. The majority of PhIP-induced ACF were initially located in DC (Ochiai et al., 1996), with a shift towards the cecum and PC at a later time point, while PhIP-induced tumors were localized mainly in the cecum and PC (Tsukamoto et al., 1999). Cell proliferation may be the key factor in this process, based on two observations: first, rates of PhIP-adduct removal from the cecum and colon are very fast and are more likely to be related to cell turnover of epithelial cells than to enzymatic repair (Cummings and Schut, 1994). In other words, most of these DNA adducts are fixed into mutations through cell replication instead of being repaired. Second, the cell proliferation rate increases from the rectum to cecum in rats, with the cecum having a higher cell turnover rate than elsewhere in the colon (Hall et al., 1992). The effect of PhIP on cell proliferation along the cecum and colon should be studied.

The modulation of PhIP-induced mutation by CLA and DTT also varied along the large intestine. The protective effect of CLA and DTT against PhIP was only observed in DC. These findings are not surprising, considering the regional differences in morphology, cell proliferation, and proliferative responses existing among these tissue parts (reviewed by Stuart et al., in press). A few other chemopreventive agents have also been reported to show regional anticarcinogenic effects on the colon. For example, piroxicam reduced the incidence of azoxymethane-induced tumors in PC, while oltipraz tended to reduce the incidence of tumors in DC (Liu et al., 1995). Dietary maltitol decreased the incidence of 1,2-dimethylhydrazine-induced tumors in cecum and PC in rats (Tsukamura et al., 1998). Although the mechanisms of this regional protection by CLA and DTT were not clear, we suspected that it may be due to the inhibition of DNA adduct formation by CLA and DTT in DC. Both CLA and DTT have been reported to inhibit chemical-induced DNA adducts in various tissues (Liew et al., 1995; Josyula et al., 1998, Izzotti et al., 1999). Moreover, the trend observed in the MS, the selective reduction of –1 frameshifts by CLA and DTT, indicated that inhibition of a certain PhIP-DNA adduct may be involved.

As *in vivo* studies have not shown any evidence suggesting that CLA or DTT is mutagenic or carcinogenic, control groups supplemented with only CLA or only DTT were omitted from the experiment. However, PhIP-induced MF in the cecum was unexpectedly enhanced by CLA, raising the question whether CLA is mutagenic in the cecum. To clarify this point, a higher concentration of CLA (1%) was employed in a follow-up study. The preliminary results (Yang, manuscript in preparation) indicated that

supplementation with CLA did not increase the spontaneous MF in the cecum. Thus we ascribe the enhancement of the PhIP-induced mutations by CLA as a comutagenic effect, similar to the increase of the mutagenicity of 2-aminofluorene in bacteria by CLA when incubated with S-9 from Aroclor-treated rats (Pariza et al., 1983). The mechanism of this comutagenic effect is not clear. It is worth mentioning that the increase of PhIP-induced MF by CLA was accompanied by an increase of the frequency of G:C→T:A transversions.

In conclusion, we have demonstrated that regional differences exist within the colon with respect to PhIP-induced mutagenesis. Similarly, the effects of CLA and DTT are also dependent on the location in the intestine. The different effects of CLA and DTT in the cecum suggest that CLA and DTT interfere with the mutagenesis of PhIP through different pathways. To our knowledge, this is the first report to show the modulation of CLA and DTT on *in vivo* mutagenesis. Our mutation data helps to fill a gap in understanding the chemopreventive mechanisms of these agents. Further studies on DNA adduct formation and cell proliferation along the large intestine may shed light on the mechanisms of colon carcinogenesis as well as pathways of chemoprevention.

Acknowledgements

We would like to thank Dr. Kent S. Gates (Department of Chemistry, University of Missouri-Columbia) for providing DTT. We also appreciate the technical support, including mutant screening and sample sequencing, from Jana Kangas, Dave Walsh, Ken Sojonky, James Holcroft, Amanda Glickman, and Tao Jiang.

Table 2.1. The effect of CLA and DTT on the PhIP-induced MF ($\times 10^{-5}$, ± SD) in the cecum, proximal, and distal part of the colon

Tissue	Control[a]	PhIP	PhIP+CLA	PhIP+DTT
Cecum	6.4 ± 1.6	51.5 ±13.7	74.4 ± 7.3[b]	58.8 ± 3.9
Proximal part of the colon	4.9 ± 0.6	97.5 ± 15.2	102.9 ± 13.2	93.0 ± 3.9
Distal part of the colon	6.1 ± 1.4	104.3 ± 11.6	87.0 ± 10.1[b]	78.3 ± 8.5[b]

a. Previously reported by Stuart et al. (2001)

b. Significantly different from the PhIP-treated groups ($p<0.05$)

Table 2.2. Major classes of mutations recovered in the cecum, PC, and DC in Big Blue® rats treated with PhIP, PhIP+CLA, and PhIP+DTT

Treatment	Control[a]			PhIP			PhIP+CLA			PhIP+DTT		
	Cecum (60)[b]	PC (70)	DC (95)	Cecum (222)	PC (106)	DC (246)	Cecum (23)	PC (63)	DC (60)	Cecum (24)	PC (13)	DC (58)
Transitions												
G:C→A:T	44.1[c]	38.9	41.9	16.2	7.5	8.5	4.3	11.1	8.3	20.8	15.4	10.3
A:T→G:C	5.9	2.8	0.0	1.4	0.9	1.2	0.0	0.0	0.0	0.0	0.0	0.0
Transversions												
G:C→T:A	29.4	47.2	39.5	27.5	35.8	27.2	30.4	31.7	33.3	20.8	15.4	31.0
G:C→C:G	2.9	2.8	0.0	17.1	10.4	16.3	17.4	12.7	15.0	16.7	7.7	12.1
-1 frameshfts	5.9	2.8	0.0	26.6	34.9	35.8	34.8	30.2	31.7	25	46.2	34.5

a. Previously reported by Stuart et al. (2001)

b. Number of characterized mutants

c. Values in percent

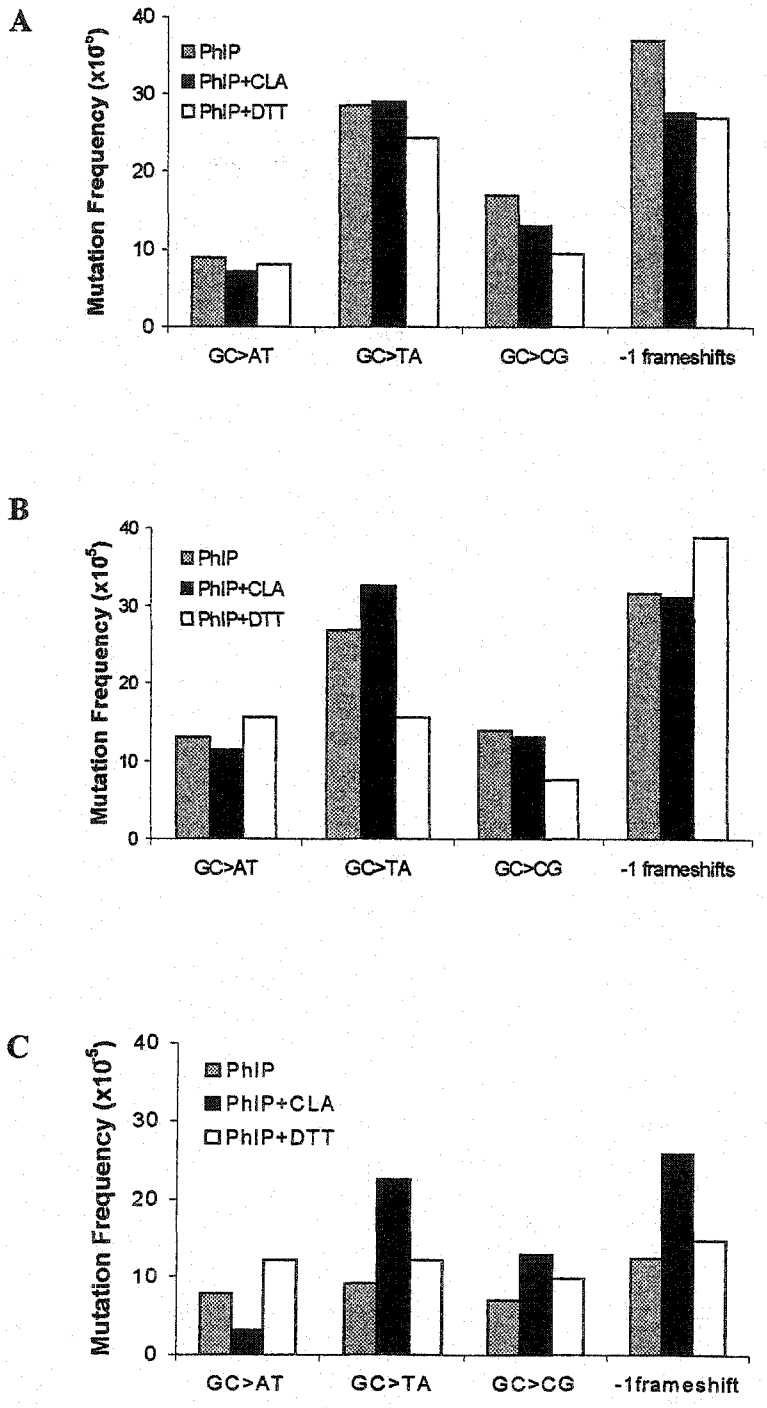

Figure 2.1. The individual MF of several classes of mutations induced by the treatment of PhIP, PhIP+CLA or PhIP+DTT in: A) distal colon, B) proximal colon, and C) cecum.

Chapter 3. Effect of Conjugated Linoleic Acid on the Formation of Spontanoues and PhIP-induced Mutation in the Colon and Cecum of Rats

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To be submitted to **Mutation Research**

Abstract

Conjugated linoleic acid (CLA), a mixture of positional and geometric isomers of linoleic acid, has been reported to inhibit chemically induced mammary and colon carcinogenesis in rodents. In a preliminary experiment, we found that CLA significantly reduced the induction of mutations by the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in the distal part of the colon (DC) in male rats. Here, the chemopreventive properties of CLA were further evaluated by assessing its effect on PhIP-induced mutation and aberrant crypt foci (ACF) in both male and female rats. CLA (1%, w/w) was added into the diet (1) from weaning to fifty-day old, or (2) starting one week prior to exposure to PhIP. The fifty-day old Big Blue® and F344 rats were then exposed to 100 ppm PhIP for 47 days. No sex differences were observed in mutagenic response to the various treatments in either DC or cecum. The mutation frequency (MF) in the cecum and DC from control animals are 4.3 ± 1.3 and $5.3 \pm 1.4 \times 10^{-5}$, respectively, showing no statistically significant difference. Administration of PhIP induced a 4- to 5-fold increase in the MF in the cecum ($19.1\text{-}20.9 \times 10^{-5}$) and a 6- to 8-fold increase in DC ($36.9\text{-}43.2 \times 10^{-5}$) compared to the corresponding controls. Supplementation with CLA lowered the PhIP-induced MF in the DC by 25% ($p=0.03$) and 22% ($p=0.02$) in male and female rats, respectively, whereas having no effect in the cecum. The PhIP-induced ACF, determined 9 weeks after the termination of PhIP treatment, was 0.75 ACF/rat, 1.7 aberrant crypts/ACF in the colon of male rats, all located in DC. This induction was completely inhibited by the addition of CLA.

3.1. Introduction

Conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid (Parodi, 1997) that are formed from linoleic acid by bacteria present in the rumen. Although first identified as an antimutagenic fraction in ground beef (Ha et al., 1987), CLA is present in several dietary sources, including milk and

cheese (Pariza and Ha, 1990). CLA has been reported to inhibit mammary tumors induced by dimethylbenz[a]anthracene (DMBA) (Ip et al., 1994b) and N-methyl-N-nitrosourea (MNU) (Ip et al., 1995), skin tumors induced by DMBA (Ha et al., 1987), and forestomach cancer induced by B(*a*)P (Ha et al., 1990). As a natural component of human diet, CLA has sparked increased attention due to its remarkable protective effect against a broad range of chemically induced tumors in rodents, and low effective concentration (0.1-1%) which is close to the levels consumed by humans in the diet (Ip et al., 1994b; Ip and Scimeca, 1997). Since mutagenesis and carcinogenesis share some common pathways, understanding the effect of CLA on mutagenesis would provide useful information concerning its anticarcinogenic mechanisms. In a previous study, we showed that CLA modulates PhIP-induced mutagenesis in a tissue- and region-specific manner (Yang et al., in press). We reported an inhibitory effect in the distal part of the colon (DC), no effect in the proximal colon, and a comutagenic effect in the cecum. In the current study, we examined the efficacy of CLA supplemented at a higher concentration in modulating PhIP-induced mutagenesis in DC and cecum. The mutagenicity of CLA itself was also assessed.

One unique characteristic of the anticarcinogenic activities of CLA is that supplementation with CLA from weaning to 50 days of age can effectively suppress the development of mammary tumors induced by various chemical carcinogens such as DMBA and MNU later in life (Ip et al., 1994b; Ip et al., 1995). The mechanism that was proposed involved the inhibition of proliferation of terminal end bud cells (target cell population for carcinogenesis) during the maturation of the mammary gland, thus

conceivably resulting in a smaller target cell population susceptible to carcinogen-induced neoplastic transformation (Thompson et al., 1997). Whether similar protection is offered to other tissues not undergoing extensive morphological changes during the pubertal period has not been studied. Therefore, we proposed to determine whether short term feeding of CLA from weaning to 50 days of age was able to offer protection against subsequent mutagenicity of PhIP in the colon and cecum.

Aberrant crypt foci (ACF) are early preneoplastic lesions in the process of colon carcinogenesis. The formation of ACF has been detected in human colon resections and in animals treated with azoxymethane, 1,2-dimethylhydrazine, and PhIP (Vivona et al., 1993; Guo et al., 1995; Tsukamura et al., 1998) Consistent with the sex-specific carcinogenicity of PhIP in the colon, the induction of ACF by PhIP is preferentially observed in the colon of male rats compared to female rats. The development of ACF can be inhibited by compounds such as chlorophyllin and indole-3-carbinol, and is recognized as an intermediate biomarker of colon cancer (Guo et al., 1995). In the current study, the induction of ACF by dietary PhIP and its possible inhibition by CLA were determined in male F344 and compared with the mutational data.

3.2. Materials and Methods

3.2.1. PhIP and CLA administration

Male and female F344 and Big Blue® rats were produced through in-house breeding and were housed 2 per cage after weaning. Food and water were provided *ad libitum*. The experimental schedule is shown in Figure 3.1. One percent (w/w) CLA was

added to the basal diet, AIN-93G minus the *tert*-butylhydroquinone antioxidant (Dyets Inc., Bethlehem, PA), starting from weaning to 50 days of age to female rats (assigned as CLA + PhIP), or one week prior to the beginning of PhIP treatment to the end of PhIP exposure to both male and female rats (assigned as PhIP + CLA). PhIP was given to 50-day-old rats at a concentration of 100 ppm in the basal diet for 47 days. Two percent (w/w) tocopherol-stripped corn oil was added into the basal diet during the PhIP supplementation period to decrease the formation of dust. After PhIP treatment, the rats were returned to the basal diet. PhIP (>98% pure as determined by TLC) was obtained from Toronto Research Chemicals (Toronto, ON). CLA (>94% pure as determined by GLC and TLC; the composition of CLA is: ~41% *cis*-9,*trans*-11 CLA, ~44% *trans*-10,cis-12 CLA, ~10% *cis*-10,12 CLA, ~5% *cis*-9,12 linoleate) was purchased from Nu-Chek-Prep (Elysian, MN). Tocopherol-stripped corn oil was from ICN (Costa Mesa, CA).

3.2.2. DNA isolation and packaging reaction

Big Blue® rats were sacrificed one week after being returned to the basal diet. Tissues including the colon (divided into the distal and proximal parts) and cecum were removed immediately, rinsed with PBS, snap-frozen in liquid nitrogen, and stored at -80°C until DNA isolation (Yang et al., in press). Packaging of the lambda vector was performed by mixing 8 µl of genomic DNA with 10 µl of packaging extract (Transpack, Stratagene) and incubating at 30°C for 90 minutes. Twelve µl of packaging extract was added into the mixture and continued to incubate at 30°C for another 90 minutes. The

reaction was stopped by diluting the mixture to 1 ml with SM buffer (0.1 M NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, and 0.01% gelatin).

3.2.3. Screening for mutants

An appropriate aliquot of phage solution resulting from a packaging reaction was added to 2.0 ml of *E. coli SCS-8* cells (suspended in 10 mM $MgSO_4$, OD_{600} = 0.5) and incubated at 37°C for 15 minutes. Thirty-five ml of molten NZY top agarose with 1.5 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was added to the bacterial cell / phage mixture and dispersed evenly over a 200 ml NZY agar bottom layer in a 25 × 25 cm assay tray. Plates were incubated overnight at 37°C and screened over a light box the next day for blue mutant plaques. A control (SCS-8 alone) and all four-color standards (CM0-3, Stratagene) were plated in parallel to ensure the standard color selection conditions were maintained. The total number of plaque forming units (pfu), ranging from 8,000 – 20, 000 per plate, was estimated by counting the phage plaques in five 2.5 × 2.5 cm squares on each plate. Mutant frequency was determined by the ratio of the number of blue mutant plaques to the total number of plaques. Sectored plaques and pinpoint mutant plaques were not considered for calculating mutant frequency. The blue mutant plaques were cored and stored at 4°C in 0.5 ml of SM buffer with 50 µl chloroform. To verify and isolate the mutant plaques, NZY plates (9 cm petri dishes) were prepared and covered with 3 ml of a mixture of NZY agarose and *E. coli SCS-8* cells. The mutant plaque in SM buffer was streaked on the plate with a toothpick. After incubating overnight at 37°C, isolated blue mutants on the plates were collected for PCR

and sequencing (Erfle et al., 1996). Mutational spectrum (MS) of each treatment was established according to previous protocol (de Boer, 1995).

3.2.4. Detection of aberrant crypt foci

Male F344 rats treated with PhIP or PhIP + CLA were sacrificed 9 weeks after the termination of PhIP supplementation. The cecum and colon (divided into the distal and proximal parts) were removed, rinsed with ice-cold PBS, slit open along the longitudinal axis, and fixed flat with 10% formalin. Following the procedure described by Bird (1987), the colon and cecum were stained in 0.025% methylene blue for 10 to 20 minutes and examined under a microscope at 100x magnification. The number and localization of ACF and the number of aberrant crypts (ACs) in each focus were recorded.

3.2.5. Statistical analyses

Statistical analysis of MF was performed using the computer program, COCHARM (written by Troy Johnson, Procter and Gamble, Cincinnati, OH) which executes the Generalized Cochran-Armitage test (GCA) (Carr and Gorelick, 1995). Since multiple comparisons of MFs were involved, analysis of variance (ANOVA) testing the differences of means of MFs was used in addition to the GCA test. Statistical comparison of MS was made using the Monte Carlo method of Adams and Skopek (1987) and the Fisher's exact test (Instat, GraphPad Software). Class-specific mutation frequencies were analyzed by the method described by Carr and Gorelick (1996).

3.3. Results

3.3.1. Food intake and animal growth

Administration of PhIP had an inhibitory effect on the food intake of male rats, which became significant after 17 days of PhIP supplementation (Figure 3.2A). At the end of the PhIP treatment, the food intake in the male PhIP-group or PhIP + CLA-group was only ~70-80% of that in control groups. The inhibition of food intake was accompanied by a slower gain of body weight compared with the control (Figure 3.2B). In contrast, supplementation with PhIP alone in the female rats did not cause significant changes in the amount of food consumed and did not appear to suppress the animal growth (Figure 3.3). However, the administration of PhIP + CLA did significantly slow down the growth of female rats without reducing food consumption, when compared with control rats. Female rats receiving CLA from weaning to 50 days of age consumed less food than the control animals. Although some differences exist between the control groups and some treatment groups with respect to food intake and animal growth, the rats treated with PhIP-alone showed no differences from those treated with PhIP/CLA (includes PhIP + CLA and CLA + PhIP groups). The total intake of PhIP per animal of male rats is higher than that of female rats. However, if PhIP intake per g body weight is compared, there was no difference. The suppression of food intake and animal growth by PhIP and a slightly inhibitory effect of CLA on body weight gain are in agreement with observations in other studies (Belury et al., 1996; Hirose et al., 2000).

3.3.2. Mutation frequencies

Mutation frequency (MF) was defined as the mutant frequency corrected for clonal expansion which can be estimated by correcting for "jackpot" mutations, i.e. mutations occurring more than once in a given tissue from a given animal (Nishino et al., 1996). The "jackpot" mutations were identified by sequencing the mutated *lacI* genes. The MFs of DC and cecum samples from control, CLA-, PhIP-, CLA + PhIP- and PhIP + CLA-treated male and female rats are summarized in Table 3.1 and 3.2. No significant differences existed between the MF in male and female rats in either the control or the PhIP-treated groups, confirming the observation by Stuart et al. (2001). Likewise, the effect of CLA on control and PhIP-induced mutations in the cecum and DC is similar between male and female rats. Therefore, the MFs from male and female animals in the same treatment were pooled together to achieve a higher statistic power.

The GCA test was used primarily to analyze the MF data. In order to avoid false significant results due to multiple comparisons, an ANOVA test was performed additionally. In general, the statistical results from the two methods agree with each other, with the exception that the effect of CLA on the spontaneous MF in DC was found to be significant as determined by the GCA test but not significant by the ANOVA test.

The MFs in the cecum and DC from the control animals showed no significant differences, *viz.* $4.3 \pm 1.3 \times 10^{-5}$ in the cecum and $5.3 \pm 1.0 \times 10^{-5}$ in DC. However, these two tissues responded differently to PhIP. In DC, PhIP treatment resulted in a MF of 39.9 $\pm 5.9 \times 10^{-5}$, twice as high as that induced in the cecum ($20.1 \pm 2.3 \times 10^{-5}$). Compared with the corresponding controls, PhIP administration increased the MF by 6- to 8-fold in

DC and by 4- to 5-fold in the cecum. In our previous study, administration of PhIP at 200 ppm also induced twice as much mutation in DC as in the cecum. These data from the two studies clearly indicate a regional difference in PhIP-induced mutation along the large intestine. Supplementation with CLA starting from 50 days of age did not influence mutagenesis in the cecum of control or PhIP-treated animals, whereas in DC, it inhibited the PhIP-induced MF by 23%. Supplementation of the diet with CLA from weaning to 50 days of age did not provide any protection against subsequent mutagenesis by PhIP. The MF in DC of the CLA + PhIP group is $42.8 \pm 9.6 \times 10^{-5}$, not different from that of the PhIP-treated group ($39.9 \pm 5.9 \times 10^{-5}$).

3.3.3. Mutation spectra

Randomly selected *lacI* mutants recovered from the cecum and DC of male and female rats supplemented with corn oil (control), PhIP, CLA, and PhIP + CLA were sequenced and corrected for clonal expansion. Since there was no difference between the MF recovered from PhIP + CLA and CLA + PhIP groups, mutants from the CLA + PhIP group were not further analyzed. Sex differences have not been observed in the control nor in the PhIP-induced MS from the cecum and DC as reported by Stuart et al. (2001). Our data also shows that the MS obtained from different treatments, including control, CLA, PhIP, and PhIP + CLA, are similar between male and female rats ($p>0.1$, data not shown). Therefore, mutants recovered from the same tissue of male rats and female rats from the same treatment were combined to generate a MS for a specific treatment (Table 3.3).

The MS recovered were first analyzed by the Monte Carlo method as described by Adams and Skopek (1987), with 2500 iterations. These tests of significance consisted of pairwise comparison of MS, using the 10 mutational classes shown in Table 3.3. The spectra in the cecum and DC from control rats showed no significant difference, with G:C→A:T transitions comprising about 50% of all mutations, followed by G:C→T:A transversions. These spectra are similar to previously reported control spectra (de Boer et al., 1998). CLA treatment did not change the specificity of mutations recovered in the cecum and DC compared with the corresponding controls. The addition of PhIP to the diet significantly altered the MS in both DC and the cecum ($p<0.001$). In the cecum, the differences were demonstrated by a significantly lower percentage of G:C→A:T transitions (10.9% *vs.* 53.1%, $p<0.0001$), and a higher percentage of G:C→T:A (43.6% *vs.* 18.8%, $p<0.03$) and G:C→C:G (18.2% *vs.* 3.1%, $p<0.05$) transversions. In DC, G:C→A:T transitions decreased from 48.8% in the control group to 13.1% in the PhIP-treatment group, representing the biggest spectral change produced by PhIP ($p<0.0001$). The proportions of -1 frameshifts (31.5% *vs.* 7.5%, $p<0.0001$), G:C→T:A (31.1% *vs.* 15.0%, $p<0.01$) and G:C→C:G (14.9 % *vs.* 2.5%, $p<0.01$) transversions were significantly elevated in the PhIP-treatment group. Additional supplementation with CLA did not produce significant changes in the PhIP-induced MS in either DC or cecum as judged by the Monte Carlo methods described by Adam and Skopek (1987).

However, using the method recommended by Adam and Skopek (1987) to analyze the MS overlooks the contribution of changes in overall MF to the induction of specific classes of mutations. Carr and Gorelick (1996) described a method for analysis

of spectra data in terms of mutation frequency. This method provides an improved means of identifying those classes or sites of mutations that have treatment-related induction. The spectra resulting from PhIP treatment or PhIP + CLA treatment were compared by this method and presented in Table 3.4. The effective size (ES) was calculated as:

ES = [number of mutants sequenced / number of mutants recovered] × [number of transgenes screened]

Contingency tables were generated according to the number of mutants in individual mutational class and ES, and were analyzed by the two-sided Fisher's exact test. The resulting p values were corrected by Bonferroni and step-down Boferroni adjustment. These comparisons showed that among all the classes of PhIP-induced mutations, the occurrence of G:C→T:A transversions was significantly inhibited by CLA treatment in DC despite the fact that analysis of those spectra by the Adams and Skopek test did not display any difference between the PhIP- and PhIP + CLA-treatment groups. To better visualize the differences resulting from addition of CLA, the frequencies of several individual classes of mutations were calculated and listed in Figure 3.4.

3.3.4. Aberrant crypt foci

In the absence of the carcinogen PhIP, none of the animals given CLA or corn oil developed ACF (Table 3.5). After exposure to PhIP, fifty percent (2/4) of male F344 rats developed ACF, at a level of 0.75 ACF/rat, 1.7 ACs/ACF, although this was not statistically significant due to the small number of animals used. All the ACF identified were located in DC. The aberrant crypts were larger than normal crypts, had an irregular

shape, showed a thickened cell layer with dilated or slit-shaped lumina (Figure 3.5). No ACF was observed in the PhIP + CLA-treated rats.

3.4. Discussion

Both PhIP and CLA are present in a typical Western diet and may thus have a direct effect on human cancer risk. Several studies have shown CLA capable of modulating the formation of PhIP-DNA adducts in animal models, however, the extent of this modulation depends on the route of administration (Josyula et al., 1998; Josyula and Schut, 1998). While rodents are substitutes for the human situation, it is important to attempt to mimic human dietary supplementation in order to be able to best extrapolate data obtained from animal studies to humans. In the current study, rats were given PhIP and CLA through chronic dietary supplementation. Compared with other administration protocols such as i.p. injection and bolus administration, this method represents a more realistic approach to evaluate the involvement of PhIP and CLA in human cancers. The effect of CLA in the colon has mostly been studied on the formation of PhIP-DNA adducts; and it is not actually known whether it has an effect on carcinogenesis in the colon. Therefore, we chose to examine the effect of CLA in other biomarkers in the colon, *viz.*, the induction of mutation and preneoplastic lesions. In an earlier experiment, we observed that 0.5% CLA decreased the PhIP-induced MF by 14% in DC. This protective effect of CLA was modest and was possibly saturated by the high dose of PhIP used (200 ppm), which in that study induced a 17-fold increase of MF over the control (Yang et al., in press). Thus, a lower PhIP dose (100 ppm) and a higher CLA

concentration (1%) were employed in the current study to better demonstrate an effect of CLA.

Dietary supplementation with PhIP at 25-400 ppm has been found to induce colon tumors in a dose-dependent manner in male rats (Hasegawa et al., 1993; Shirai et al., 1995). The formation of PhIP-DNA adducts showed a linear dose response. In the current study, the average food intake of male rats was about 15 g/rat/day, thus the total intake of PhIP during 47 days exposure is 0.071 g per rat, which produced a MF of 43.2×10^{-5}. We previously reported that supplementation with PhIP at 200 ppm for 61 days, corresponding to a total PhIP intake of 0.183 g per rat, resulted in a MF of 104×10^{-5} in DC of male rats (Yang et al., in press). Regression analysis of the mutational dose response of PhIP (0, 0.071 g, and 0.183 g) showed a linear regression with correlation of 1 ($R^2 = 1$). Thus our results are consistent with a linear dose response to PhIP in the DC of male rats. Likewise, a linear mutational dose response to PhIP was also observed in the cecum of male rats ($R^2 = 1$).

The PhIP-induced MF in the DC is twice as high as that in the cecum, which has been attributed, at least partially, to the differences between DC and cecum in morphology, cell proliferation and response to mutagens (Stuart et al., in press). In a previous study, we have shown that modulation of PhIP-induced mutations by CLA varied along the intestine, *viz.* inhibition in DC, no effect in PC, and a comutagenic effect in the cecum. The current study confirmed that simultaneous administration of CLA with PhIP suppressed PhIP-induced mutations in DC, and possibly in a dose-dependent manner since CLA supplemented at 1% decreased the PhIP-induced MF by 25%, while

0.5% CLA inhibited the PhIP-induced MF by 14%. However, the possibility that the more pronounced inhibition is due to the lower dose of PhIP employed in the current study can not be excluded, considering that a lower dose of PhIP may not saturate the preventive capacity of CLA. When CLA was given from weaning to 50 days of age, we did not see any protection against subsequent mutagenesis, unlike that reported in the mammary gland (Ip et al., 1994b; Ip et al., 1995). This result clearly suggests that CLA interferes with mutagenesis in the colon via a different pathway from that in the mammary gland.

The previously reported comutagenic effect of 0.5% CLA in the cecum (Yang et al., in press) was not observed. However, it is interesting to note that when CLA was given at 0.1-1% to PhIP-treated rats, the level of PhIP-DNA adducts in the colon and mammary epithelium cells appeared to be higher in rats supplemented with 0.5% CLA compared to those supplemented at other concentrations (Josyula et al., 1998). A recent report by Leung and Liu (2000) pointed out that different isomers of CLA display antioxidant property or pro-oxidant property depending on the concentration. At all concentrations tested (2-200 μM), t10,c12-CLA performed as an antioxidant, more effective than c9,t11-CLA and alpha-tocopherol at lower concentrations (2 and 20 μM). On the other hand, c9,t11-CLA possessed weak antioxidant activity at 2 and 20 μM, whereas at 200 μM it acted as a strong pro-oxidant. CLA used in our study consists of both t10,c12-CLA (~44%) and c9,t11-CLA (~41%). Although it is not clear how these two isomers functioned in the two studies, the different effects of CLA on PhIP-induced

mutations in the cecum may be partially attributed to the dose-dependent antioxidant or pro-oxidant properties of CLA isomers.

Activated PhIP is known to react almost exclusively with guanines, at the C8-position. The mutagenicity of dG-C8-PhIP was investigated using a site-specifically modified oligodeoxynucleotides (Shibutani et al., 1999). This adduct induced mainly G→A transversions, along with lesser amounts of G → A transitions and G → C transversions. Mutations were produced most frequently when the 5'-flanking sequence of dG-C8-PhIP was dG or dC, followed by dT > dA. In our study, among 222 independent mutants scored in DC of PhIP-treated rats, 132 are base substitutions at G:C base pairs. Analyzing the sequence context of these substitutions showed a preference for a 5'-flanking base of G (75/132) > C (38/132) > T (12/132) > A (7/132), and a 3'-flanking of C (48/132) > G = T = A (28/132). The mutational data we recovered agree reasonably well with the context preference of dG-C8-PhIP observed *in vitro* (Shibutani et al., 1999). In DC from PhIP + CLA-treated rats, the base substitution mutations (103/208) occurring at G:C base pairs showed preference of a 5'-flanking base of C (49/103) > G (31/103) > T (16/103) > A (7/103), and a 3'-flanking base of C (39/103) > G (24/103) = A (22/103) = T (18/103). Using the Fisher's exact test, we found that the occurrence of substitutions at G:C sites with a 5'-flanking base of G was significantly lower in the PhIP + CLA-group than the PhIP-group ($p < 0.0001$). It is likely that the decrease of PhIP-induced mutations by CLA in the DC is associated with sequence context and that the incidence of mutations at 5'-G<u>G</u>-3' (<u>G</u> is the mutated base) is preferentially inhibited. In the cecum, substitutions at G:C base pairs consisted 75% of

PhIP-induced mutations (55/73). Those mutations showed a preference of a 5'-flanking sequence of C (31/55) > G (13/55) > T (6/55) > A (5/55), and a 3'-flanking sequence of C (20/55) > T (18/55) > A (11/55) > G (6/55). CLA did not alter the preference of sequence context of those PhIP-induced mutations in the cecum ($p > 0.2$).

ACF were proposed as putative preneoplastic lesions of colorectal carcinomas and the earliest recognizable changes produced in the colon by carcinogens such as 1,2-dimethlhydrazine and azoxymethane (Paulsen et al., 2000; Liu et al., 2001; Tanaka et al., 2001). HCAs such as IQ and PhIP, are also able to induce ACF in the colon and the majority of chemically induced ACF were located in DC. The level of ACF induced by PhIP in the current study is compatible with those reported in other studies (Takahashi et al., 1991; Hasegawa et al., 1993; Ochiai et al., 1996), when we assume that PhIP induces ACF in the colon in a linear dose-dependent manner (Hasegawa et al., 1993). Although the level of induction was not statistically significantly higher than that in the controls, the biological significance seems real because spontaneous formation of ACF in untreated animals is rare. Rats treated with PhIP + CLA did not develop ACF, suggesting an inhibition effect of CLA on PhIP-induced ACF. This effect is consistent with the inhibition of PhIP-induced MF in DC. Together, the inhibition of MF and ACF by CLA reflects its cancer preventive potential.

In conclusion, we have shown that CLA modulates PhIP-induced mutagenesis in a region-specific manner. The inhibitory effect of CLA on PhIP-induced mutations in DC is consistent with its inhibition of the development of ACF after PhIP exposure.

Acknowledgements

We would like to thank James Holcroft, Virginia Haslett, Trina de Monyé, Brandi Jenkins, Amanda Glickman, Gregory Stuart, Henry Yu, Dave Walsh, Jana Kangas, Ken Sojonky, Shulin Zhang, Cindy Ruttan, and the Animal Care Unit staff of University of Victoria for their excellent technical support in the experiment.

Table 3.1. Mutation frequencies in the distal colon

Treatment	Sex	No. of animals	Total pfu	MF ± SD ($\times 10^{-5}$)	Group MF ± SD ($\times 10^{-5}$)
Control	Male	4	1,370,910	5.6 ± 1.0	5.3 ± 1.4
	Female	2	715,000	4.6 ± 1.6	
CLA	Male	2	676,587	4.4 ± 0.6	3.1 ±1.2
	Female	3	1,036,100	2.2 ± 0.2	
PhIP	Male	5	1,677,000	43.2 ± 6.4	39.9 ± 5.9[a]
	Female	4	1,378,700	36.9 ± 3.7	
PhIP + CLA	Male	5	1,629,900	32.4 ± 4.8	30.7 ±3.9[b]
	Female	4	1,211,100	28.8 ± 1.7	
CLA + PhIP	Female	5	560,300	42.8 ± 9.6	42.8 ± 9.6

[a] Significantly different from the corresponding control MF ($p<0.001$ as determined by the ANOVA test).

[b] Significantly different from the corresponding PhIP-induced MF ($p<0.01$ as determined by the ANOVA test).

Table 3.2. Mutation frequencies in the cecum

Treatment [a]	Sex	No. of animals	Total pfu	MF ± SD ($\times 10^{-5}$)	Group MF ± SD ($\times 10^{-5}$)
Control	Male	4	1,211,010	4.7 ± 1.3	4.3 ±1.3
	Female	2	674,960	3.6 ± 0.5	
CLA	Male	5	1,130,230	5.6 ± 1.3	4.9 ± 1.7
	Female	2	574,960	3.3 ± 0.8	
PhIP	Male	5	1,677,000	20.9 ± 2.2	20.1 ± 2.3[b]
	Female	3	953,030	19.1 ± 1.8	
PhIP + CLA	Male	5	1,629,900	18.9 ± 3.7	19.3 ± 3.6[b]
	Female	4	1,003,230	18.4 ± 3.1	

a. The MF in the cecum of female rats treated with CLA+PhIP was not examined due to an absence of effect by this treatement in DC (compared with PhIP treatment), where the effect of CLA appeared to be most pronounced.

b. Significantly different from the control MF ($p<0.001$ as determined by the ANOVA test).

Table 3.3. Classes of mutations recovered in the distal part of the colon in Big Blue® rats treated with PhIP and PhIP+CLA

Treatment	Control (%)		CLA (%)		PhIP (%)		PhIP+CLA (%)	
	Cecum (43)[a]	DC (81)	Cecum (40)	DC (38)	Cecum[b] (73)	DC[b] (222)	Cecum (79)	DC (208)
G:C→A:T	21 (48.8)	39 (48.1)	13 (32.5)	18 (47.4)	13 (15.1)	29 (13.1)	19 (24.1)	33 (15.9)
A:T→G:C	5 (11.6)	5 (6.2)	0 (0.0)	0 (0.0)	1 (1.4)	1 (0.5)	1 (1.3)	3 (1.4)
G:C→T:A	7 (16.3)	12 (14.8)	12 (30.0)	6 (15.8)	31 (42.5)	69 (31.1)	17 (21.5)	48 (23.1)
G:C→C:G	1 (2.3)	2 (2.5)	2 (5.0)	7 (18.4)	13 (17.8)	33 (14.9)	9 (11.4)	22 (10.6)
A:T→T:A	0 (0.0)	4 (4.9)	1 (2.5)	2 (5.3)	1 (1.4)	1 (0.5)	2 (2.5)	6 (2.9)
A:T→C:G	2 (4.6)	1 (1.2)	1 (2.5)	0 (0.0)	0 (0.0)	2 (0.9)	0 (0.0)	4 (1.9)
+1 frameshifts	0 (0.0)	1 (1.2)	1 (2.5)	0 (0.0)	1 (1.4)	2 (0.9)	1 (1.3)	1 (0.5)
-1 frameshifts	5 (11.6)	6 (8.6)	6 (15.0)	3 (7.9)	10 (13.7)	70 (31.5)	24 (30.4)	74 (35.6)
Deletions	1 (2.3)	3 (3.7)	2 (5.0)	0 (0.0)	3 (4.1)	9 (4.1)	5 (6.3)	10 (4.8)
Others	1 (2.3)	7 (8.6)	2 (5.0)	2 (5.2)	2 (2.7)	6 (2.8)	1 (1.3)	7 (3.4)

a. Number of independent mutants

b. Significantly different than the corresponding control mutation spectrum ($P < 0.05$), as determined by the Monte Carlo method recommended by Adams and Skopek (1987).

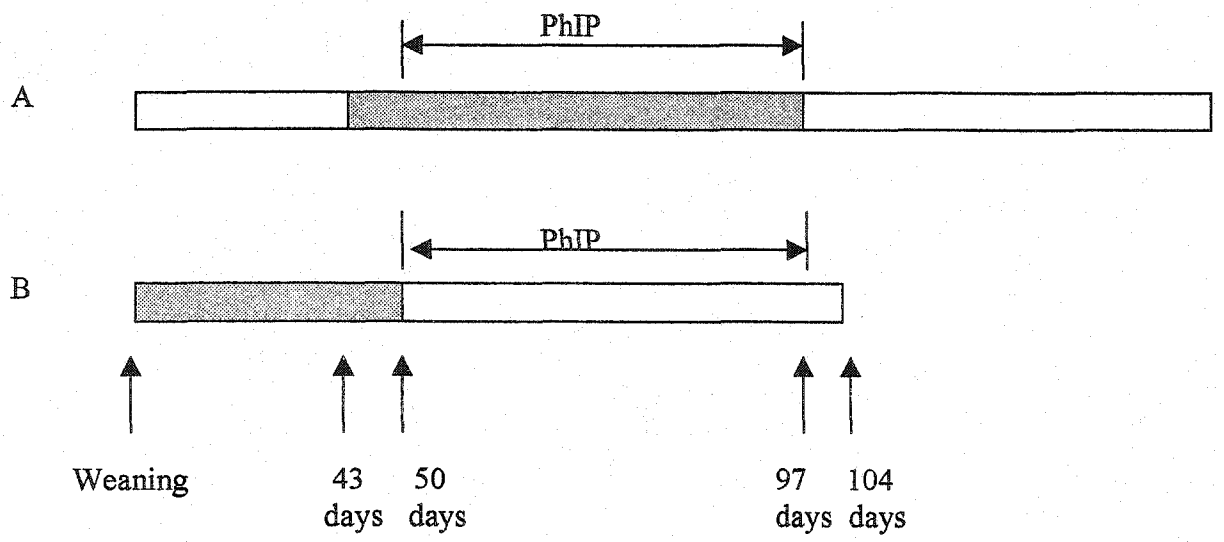
Table 3.4. Statistical analysis of spectra data in terms of mutation frequency using the method recommended by Carr and Gorelick (1996)

Treatment	PhIP	PhIP+CLA	Unadjusted	Adjusted[a]	PhIP	PhIP+CLA	Unadjusted	Adjusted
	DC	DC	*P* value	*P* value	Cecum	Cecum	*P* value	*P* value
G:C→A:T	29	33	0.8	1	11	19	0.4	1
A:T→G:C	1	3	0.78	1	1	1	0.8	1
G:C→T:A	69	48	0.002	0.02	31	17	0.01	0.09
G:C→C:G	33	22	0.028	0.28	13	9	0.2	1
A:T→T:A	1	6	0.22	1	1	2	0.68	1
A:T→C:G	2	4	0.89	1	0	0	N/A	N/A
+1 frameshifts	2	1	0.84	1	1	1	0.68	1
-1 frameshifts	70	74	0.35	1	10	24	0.1	0.9
Deletions	9	10	0.97	1	3	5	0.94	1
Others	6	7	0.9	1	2	1	0.86	1
Mutants sequenced	239	228			76	83		
Total Mutants	1233	871			552	493		
Total pfu	3055700	2841000			2630030	2633130		
ES	592309	742830			362106	443306		

[a] The p values obtained after adjusted by Bonferroni and step-down Bonferrni correction are similar. Therefore, only one adjusted p value (Bonferroni correction) is shown in the table.

Table 3.5. The effect of CLA on PhIP-induced aberrant crypt foci formation in male rats

Treatment	Sex	No. of rats	ACF/rat	Aberrant crypts/ ACF	Location of ACF
Control	Male	4	0	0	N/A
PhIP	Male	4	0.75	1.7	DC
PhIP+CLA	Male	4	0	0	N/A



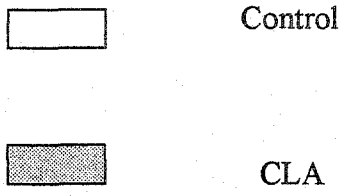


Figure 3.1. Experimental schedule. **A.** Male and female rats were given CLA starting at the age of 43 days. PhIP was incorporated into the diet one week later and continued for 47 days. **B.** Female rats were given CLA from weaning to the age of 50 days and then subjected to PhIP treatment for 47 days. Proper control groups were included.

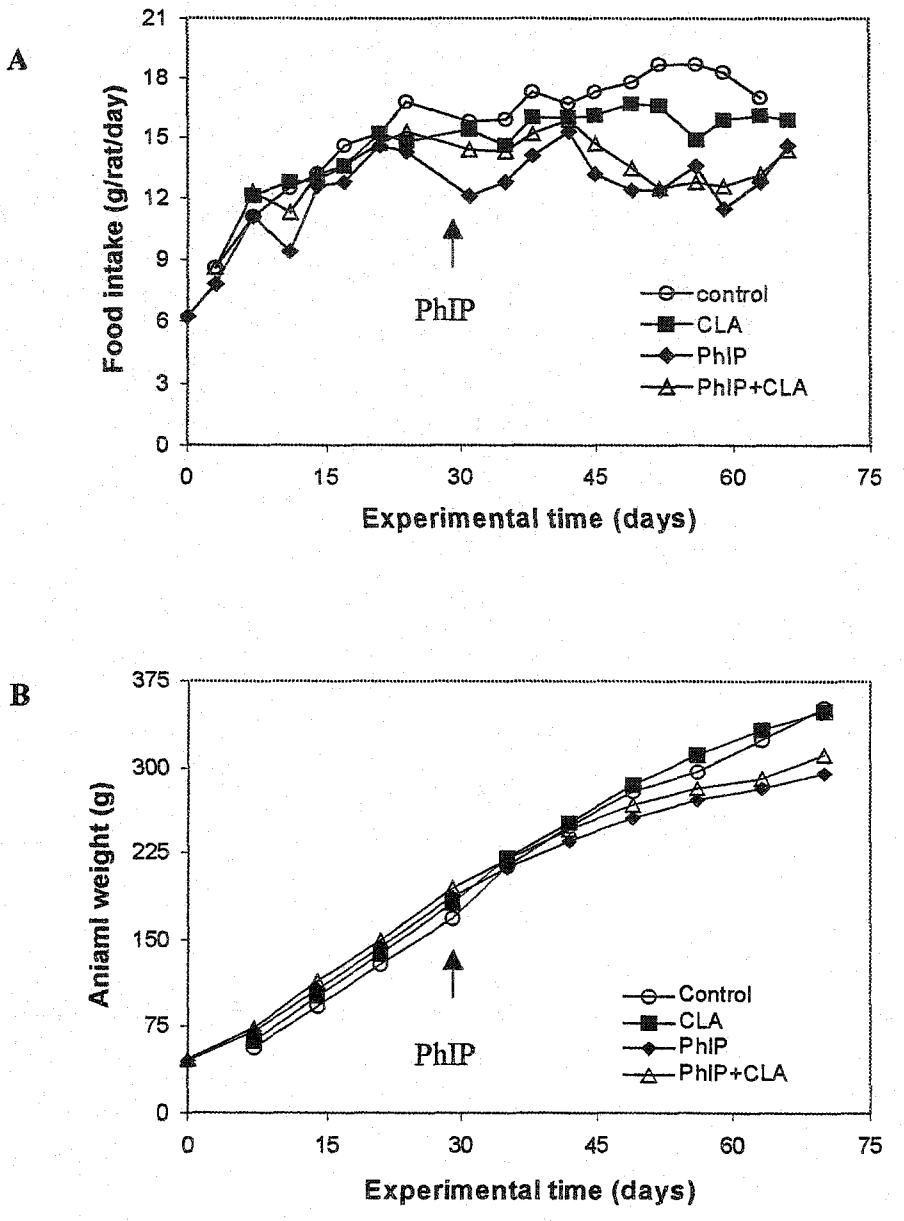


Figure 3.2. The effect of CLA and PhIP on the growth (A) and food intake (B) of male Big Blue® rats. At the beginning of the experiment, no significant differences were observed in the growth and food intake of rats among various treatment groups. After PhIP was added into the diet for 17 days, the food intake in the rats exposed to PhIP and PhIP + CLA was significantly inhibited, accompanied by a slower weight gain in a later stage. The arrow points to the time when PhIP was added into the diet.

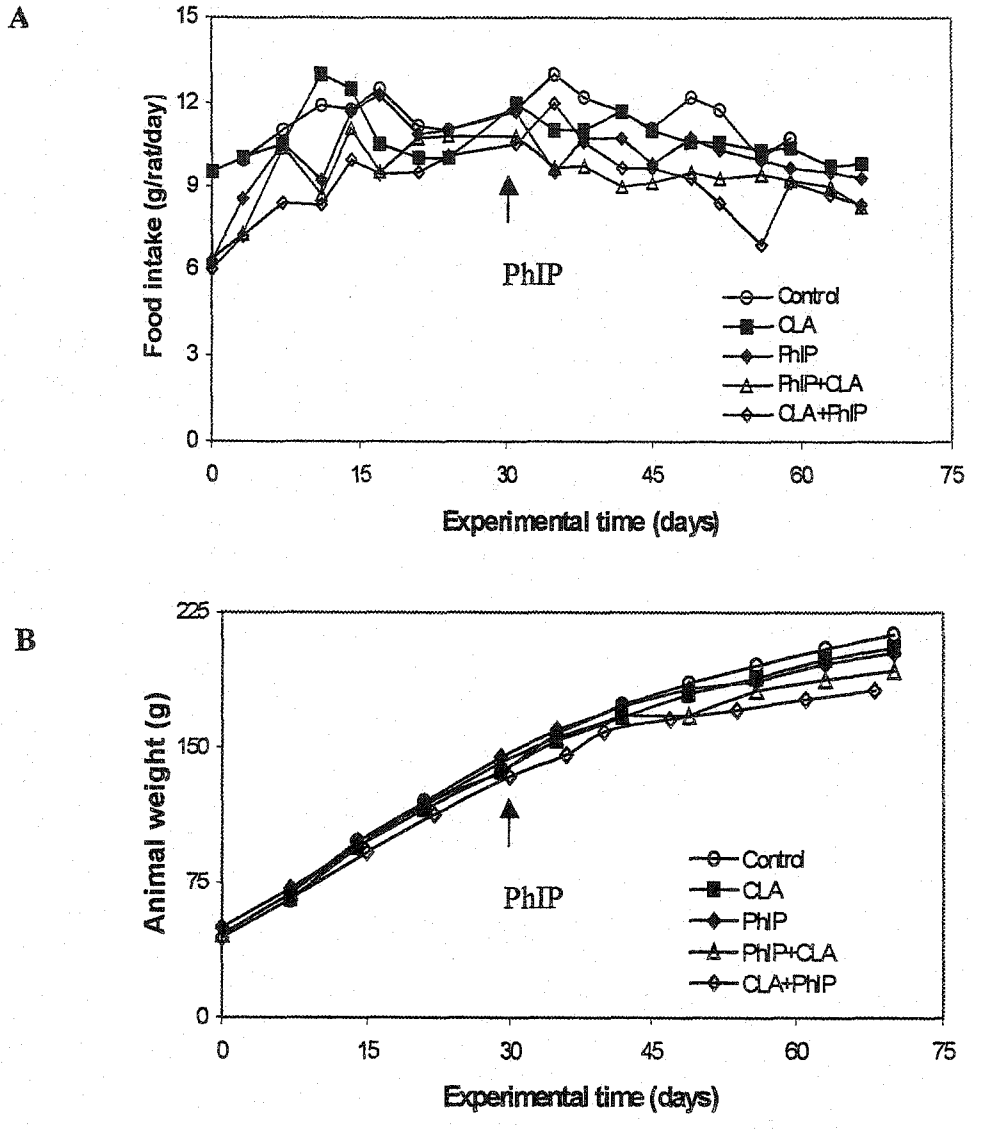


Figure 3.3. The effect of CLA and PhIP on the growth (A) and food intake (B) of female Big Blue® rats. Supplementation with CLA from weaning but not from 50 days of age suppressed the food intake of rats. A slower animal weight gain of rats in the PhIP + CLA and CLA + PhIP groups was observed. The arrow points to the time when PhIP was added into the diet.

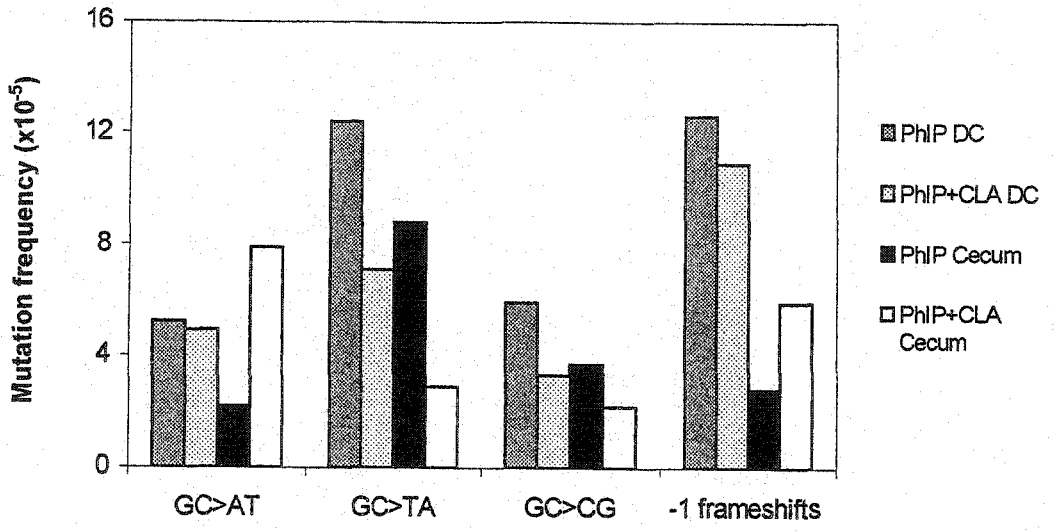


Figure 3.4. The effect of CLA on the mutation frequencies of major classes of mutations induced by PhIP in DC and cecum of Big Blue® rats

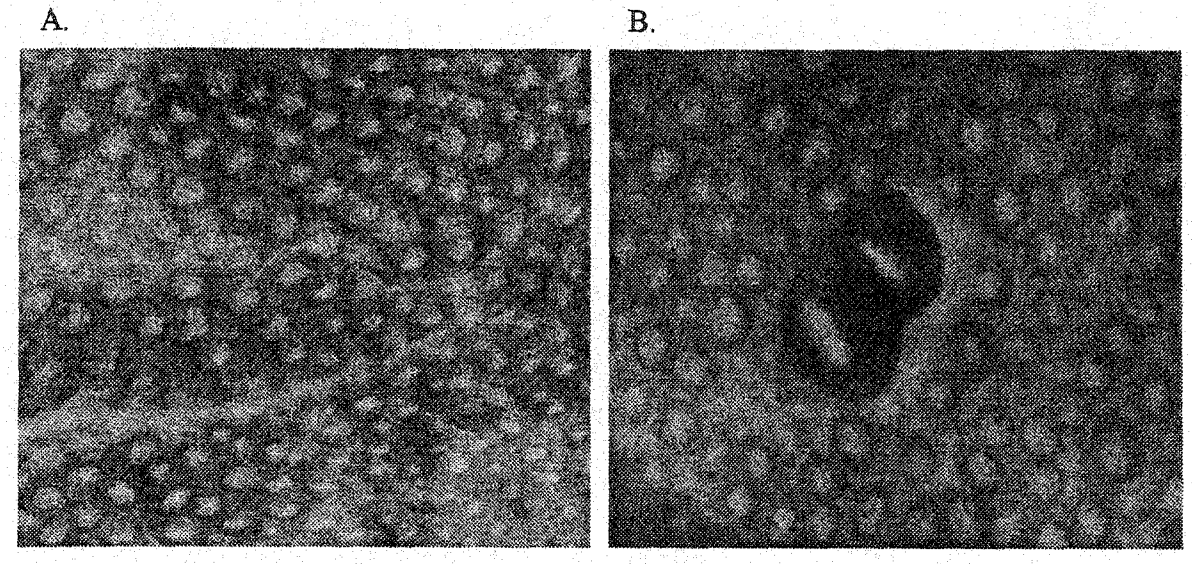


Figure 3.5. The appearance of aberrant crypt foci under a dissecting microscope (×100). **A.** normal colon mucosa; **B.** a single aberrant crypt focus containing two aberrant crypts (from http://www.ahf.org/research/research07.html). The aberrant crypts were larger than normal crypts, had an irregular shape, showed a thickened cell layer with dilated or slit-shaped lumina, and exhibited an increased pericryptal zone.

Chapter 4. Conjugated Linoleic Acid Inhibits Mutagenesis of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in the Prostate of Rats

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Submitted to **Cancer Research**

Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a potent mutagen and carcinogen formed at high temperature during the cooking of meat. PhIP induces tumors in the colon and prostate of male and in the mammary gland of female rats and has been associated with the etiology of human cancers. We recently have demonstrated that PhIP induces mutations in the prostate in Big Blue® transgenic rats. In the current study, we examined the effect of a dietary anticarcinogen, conjugated linoleic acid (CLA), on PhIP-induced mutagenesis in the prostate. CLA is a mixture of positional and geometric isomers of linoleic acid and has been reported to inhibit various chemical-induced cancers in rodent models. Fifty-day old male Big Blue® rats were fed a standard diet containing 100 ppm PhIP for 47 days, which induced a mutation frequency of 14.6×10^{-5} in the prostates, 5.1-fold over that of controls. The addition of 1% CLA (w/w) in the diet starting one week prior to exposure to PhIP decreased PhIP-induced mutagenesis by 38% ($p=0.03$). The predominant class of mutations induced by PhIP is –1 frameshifts involving the loss of G:C base pairs, followed by G:C→T:A transversions and G:C→A:T transitions. Addition of CLA to the diet significantly changed the PhIP-induced mutation spectrum, notably, -1 frameshifts and G:C→A:T transitions were selectively inhibited, suggesting involvement of mismatch repair. This is the first report to show the protective effect of CLA against PhIP-induced mutagenesis in the prostate in both mutation frequency and mutational spectrum. The inhibitory effect of CLA against PhIP-induced mutagenicity suggests a possibility for its application in human chemoprevention studies.

4.1. Introduction

Prostate cancer is the second leading cause of cancer deaths for men in the United States with African-Americans having the highest rate in the world, immediately followed by White Americans (Pienta and Esper, 1993). The etiology of human prostate

cancer is essentially unknown, although certain dietary factors, such as the consumption of red meat and saturated fat, have been implicated in this disease (Kolonel, 1996). The identification of mutagenic and carcinogenic heterocyclic amines (HCAs) in cooked meat has raised the possibility that these dietary HCAs may play a role in the etiology of human prostate cancer.

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a pyrolysis product in meat and fish and is currently recognized as the principal mutagenic HCA in the human diet (Layton et al., 1995). The finding of PhIP in the urine of healthy volunteers eating a normal diet and that of PhIP-related DNA adducts in the urine of smokers of black tobacco suggests that humans are continuously exposed to PhIP (Lynch et al., 1992; Felton et al., 1997). The possible association of PhIP with several dietary related cancers, including those of mammary gland, colon, and prostate, has attracted increasing interest, especially after carcinogenicity of PhIP was demonstrated in rodent models, in which colon and prostate tumors were predominately induced in male rats (Ito et al., 1991a; Shirai et al., 1999) and mammary gland tumors (Ito et al., 1991a) in female rats.

Similar to many chemical carcinogens, PhIP requires metabolic activation for DNA adduct formation and genotoxicity. PhIP is first metabolized to *N*-hydroxy-PhIP by cytochrome P4501A2 in the liver and is then further activated to reactive ester derivatives by phase II esterification enzymes in the liver or the target tissues (Wallin et al., 1990; Alexander et al., 1995). The major PhIP-DNA adduct has been identified as *N*-(deoxyguanosin-8-yl)-PhIP (dG-C8-PhIP) (Lin et al., 1992), while others remain uncharacterized. Mutational events generated by PhIP have been observed primarily at

G:C base pairs, consistent with the predominant formation of PhIP-DNA adducts at guanines (Carothers et al., 1994; Morgenthaler and Holzhauser, 1995). In the *lacI* gene of Big Blue® transgenic rats, G:C→ T:A transversions and –1 frameshifts of G:C base pairs are the primary mutational classes induced by PhIP, followed by G:C→ C:G, and G:C→ A:T (Okonogi et al., 1997a).

CLA is a mixture of positional and geometric isomers of linoleic acid (LA). The major dietary source of CLA is animal food, especially foods derived from ruminant animals (e.g. dairy products and beef) (Ip et al., 1994a). CLA has shown remarkable cancer prevention properties, including inhibiting chemically induced carcinogenesis (Ip et al., 1994b); inhibiting the growth of transplanted tumors (Visonneau et al., 1997); and suppressing the growth of cancer cells (Durgam and Fernandes, 1997). In a recent review paper, Pariza et al. (1999) proposed that CLA may influence the development and progression of cancer in three ways: 1) by directly affecting the process of carcinogenesis (e.g. inhibiting the activation of carcinogens (Liew et al., 1995; Josyula and Schut, 1998); inhibiting cell proliferation (Shultz et al., 1992b); decreasing the formation of arachidonic acid by competitive inhibition of LA metabolism (Banni et al., 1999)); 2) by reducing excessive body fat accumulation which indirectly influences cancer risk; and 3) by reducing cachexia which is associated with advanced cancer and with certain cancer treatment strategies. The effective concentration of CLA is 0.1-1% of the diet (Ip et al., 1991; Ip et al., 1994b), representing concentrations close to human consumption levels. This may imply that CLA has direct implications for human health.

In a previous study, we have shown that CLA inhibited PhIP-induced mutagenesis in the distal colon of Big Blue® rats (Yang et al., in press). In this paper, we examined the modification of PhIP-induced mutagenesis by CLA in the prostate.

4.2. Materials and Methods

4.2.1. Chemicals

PhIP (>98% pure as determined by TLC) was obtained from Toronto Research Chemicals (Toronto, ON, Canada). CLA (>94% pure as determined by GLC and TLC; the composition of CLA is: ~41% *cis*-9,*tran*-11 CLA, ~44% *trans*-10,cis-12 CLA, ~10% *cis*-10,12 CLA, ~5% *cis*-9,12 linoleate) was purchased from Nu-Chek-Prep (Elysian, MN). Tocopherol-stripped corn oil was from ICN (Costa Mesa , CA).

4.2.2. Treatment of rats

Male and female F344 Big Blue® rats (10 weeks old) obtained from Stratagene (La Jolla, CA) were used for in-house breeding. The offspring male rats were weaned at 21-days old and were given the powdered basal diet, AIN-93G without the *tert*-butylhydroquinone antioxidant (Dyets Inc., Bethlehem, PA). Food and water were provided *ad libitum*. The rats were weighed weekly and the food consumed was measured twice weekly. The rats were divided into 5 groups consisting of 4-6 rats each, with the following supplementation to the basal diet: 1) None; 2) corn oil; 3) corn oil + PhIP; 4) corn oil + CLA; and 5) corn oil + PhIP + CLA. The number of animals per group was chosen as recommended by a statistical analysis of mutation studies in transgenic mice by Carr and Gorelick (1995). When the rats were 50-days old, PhIP (100

ppm) was incorporated into the diet. CLA (1%, w/w) was added one week prior to supplementation with PhIP and continued till the end of PhIP treatment. Corn oil (2%, w/w) was incorporated into the powdered diet of groups 2-5 during the PhIP-exposure period to reduce the formation of dust. After 47 days of PhIP treatment, the rats were returned to the basal diet and euthanized one week later. Prostate tissues were removed immediately, frozen in liquid nitrogen, and stored at -80°C until DNA isolation.

4.2.3. *lacI* mutational assay and statistical analyses

High molecular weight genomic DNA was isolated from the prostate using a modification of the RecoverEase™ DNA dialysis method (Yang et al., in press). The genomic DNA was added to a λ phage packaging extract (Transpack, Stratagene) and the resulting phages were plated on *E. coli* SCS-8 bacteria on trays of NZY media containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside. The mutant frequency was determined by the ratio of the number of blue mutant plaques to the total number of plaques. The *lacI* gene from mutants was amplified by PCR (Erfle et al., 1996), purified using a Qiagen column, and sequenced on a LICOR automated fluorescent sequencer, thus establishing a mutational spectrum (MS) for each treatment.

MFs were compared using the computer program COCHARM (created by Troy Johnson, Procter and Gamble, Cincinnati, OH) that executes the Generalized Cochran-Armitage test (Carr and Gorelick, 1995). Statistical comparisons of MS were made using the Monte Carlo method of Adams and Skopek (1987). The comparison of individual mutation classes was performed by the Fisher's exact test. The MF of each class of mutation was calculated by multiplying the overall MF by the fraction of that class.

4.3. Results

4.3.1. Animal growth

The body weight and food consumption over the course of the treatments are shown in Figure 3.2. In comparison with the non-PhIP-exposed groups, the PhIP- and PhIP+CLA-treated animals exhibited significantly reduced food intake after exposure to PhIP for 17 days ($p=0.01$), accompanied by a significant reduction in body weight gain at the later stage of treatment ($p<0.05$). The differences tend to be more significant with longer PhIP exposure. CLA had no significant effect on the body weight and food intake.

4.3.2. Mutation frequencies

The MFs of the *lacI* gene in the prostate from various treatment groups are summarized in Table 4.1. The spontaneous MF in the prostate is not significantly different from the MF of the corn oil- or CLA-supplemented control group ($p>0.6$). Supplementation with 100 ppm PhIP for 47 days induced a *lacI* MF of $14.6\pm3.2\times10^{-5}$, a 5.1-fold increase over the control group (2.4×10^{-5}). Addition of CLA into PhIP-containing diet resulted in a MF of $9.1 \pm 1.2 \times 10^{-5}$, significantly decreasing the PhIP-induced MF by 38% ($p=0.03$).

4.3.3. Mutation spectra

Randomly selected *lacI* mutants recovered from each prostate sample were sequenced to establish individual MS for each treatment (Table 4.2). A total of 65, 32, 41, 142, and 97 independent mutants (identical mutations occurring at a single nucleotide position in the same organ of a single rat were scored only once) were identified from the

spontaneous, control (corn oil), CLA, PhIP, and PhIP + CLA groups, respectively. The predominant spontaneous mutation was G:C→ A:T transition , followed by G:C→ T:A transversion, and deletion in descending order. A significantly different MS ($p<0.01$) was obtained from the control rats supplemented with corn oil, in which -1 frameshifts were recovered at a surprisingly high proportion (25.0%), similar to those of G:C→ T:A transversions and G:C→ A:T transitions. In the CLA-treatment group, similar proportions of G:C→ A:T transitions, G:C→ T:A transversions and –1 frameshifts were seen. The MS recovered from CLA exposure was not significantly different from the control nor from the spontaneous MS. The leading class of mutation induced by PhIP was –1 frameshifts (51.4%), followed by G:C→ T:A transversions (17.6%), G:C→ A:T transitions, and G:C→ C:G transversions. CLA supplementation significantly changed the PhIP-induced MS ($p=0.016$), with –1 frameshifts (38.1%) and G:C→ T:A transversions (29.9%) as the major mutational types, followed by deletions and G:C→ A:T transitions.

The frequencies of major classes of mutations were calculated and presented in Figure 4.1. The addition of CLA resulted in a 50% decrease of the frequency of G:C→A:T transitions in the control group as well as the frequencies of G:C→A:T transitions and -1 frameshifts in the PhIP-treated group.

4.4. Discussion

The effect of CLA on DNA adduct formation or on carcinogenesis in the mammary gland, colon, and liver in rodents (especially in female rats) has been

extensively studied (Josyula et al., 1998; Snyderwine et al., 1998; Josyula and Schut, 1998) while research on the prostate is relatively rare. The first *in vivo* study about the inhibitory effect of CLA on prostate tumors was performed by Cesano et al. (1998), who demonstrated opposite effects of CLA and LA on inoculated human prostatic cancer cells in severe combined immunodeficient mice. Addition of CLA to the diet reduced the local prostatic tumor growth and the systemic spread of the prostatic tumor. Although there is no direct evidence showing that CLA is protective against human prostate cancer, epidemiological studies do suggest that the non-fat portion of milk is associated with an increased risk of prostate cancer (Grant, 1999). Since CLA is a fatty acid in the fat of milk, the promotional effect of non-fat milk may be related to the absence of CLA. The effect of CLA on chemically induced mutagenesis/carcinogenesis in the prostate has not been reported hitherto. Results from the present investigation show for the first time that CLA protects against mutagenesis induced by a dietary HCA in the prostate.

The daily food consumption and weight gain of experimental animals were dependent on the dietary supplementation. PhIP appears to be toxic, as was evident from the lower food intake and slower gain in body weight. CLA has no obvious effect on animal growth and food intake. Thus we may conclude that any differences in mutation between PhIP- and PhIP + CLA -treated animals are not the result of an effect of CLA on PhIP intake.

PhIP induced a 5.1-fold increase of MF in the prostate, confirming the finding by Stuart et al. (2000a) that PhIP is mutagenic in the prostate. Two factors directly contribute to the induction of mutations by PhIP: formation of DNA adducts and

elevation of DNA synthesis in the epithelial cells in the prostate (Shirai et al., 1997). Purewal et al. (2000) reported that supplementation with 100 ppm PhIP for one week induced an adduct level of $3.39 \pm 0.25 \times 10^{-7}$, $1.98 \pm 0.59 \times 10^{-7}$, and $0.46 \pm 0.10 \times 10^{-7}$, in the prostate, colon, and liver, respectively. The higher level of PhIP-DNA adduct in the prostate may be related to its capacity to *N*-hydroxylate PhIP. Unlike the mammary gland and colon, which showed a low capacity to metabolically activate PhIP (Fan et al., 1995, Malfatti et al., 1996), the prostate was found to have a relatively high level of P4501A2 and thus be able to convert PhIP to its hydroxy form. Although the formation of DNA adducts is considered an initiation event of mutagenesis and carcinogenesis, the level of PhIP-DNA adduct does not reflect the tissue-specific mutagenicity nor carcinogenicity of PhIP. Lower MF and fewer tumors were recovered from the prostate than from the colon after exposure to the same PhIP doses (Hasegawa et al., 1993). Clearly, factors other than the amount of PhIP-adduct take part in determining the susceptibility to mutagenicity/carcinogenicity of PhIP. One possibility relies on the different rates of cell proliferation. The results from proliferating cell nuclear antigen (PCNA) assay showed that in the colon crypts the PCNA labeling index was over 20% while in the prostate, it was only ~0.87-2.69% (Yao et al., 1996), suggesting a much higher cell dividing rate in the colon. Using immunohistochemistry, Takahashi et al. (1998) measured the kinetics of PhIP-DNA adducts in various tissues after a single dose of PhIP. The PhIP-DNA adducts in the prostate appeared to be removed slower than that in the colon, which may be indicative of faster cell division in the colon compared with prostate after PhIP treatment.

The carcinogenicity and mutagenicity of PhIP in the prostate have been determined at several doses. It was found that PhIP given at 400 ppm but not at 100 ppm or 25 ppm induced prostate tumors in rats, suggesting that a relatively high PhIP dose is required for carcinogenicity in the prostate (Hasegawa et al., 1993) compared with the mammary gland and colon. Since PhIP induces DNA adducts in a linear dose-dependent manner (Purewal et al., 2000), which can not explain the absence of tumors at lower PhIP doses, we determined the dose-response of PhIP-induced mutagenesis in the prostate (Figure 4.2). An exponential dose-response of PhIP-induced mutation (R^2=0.9714) was observed. From this curve, we can see that the MF in the prostate increases relatively slowly when PhIP is given at low doses (e.g. at the current regimen schedule). Thus, tumors correlate with mutation response but not with adducts.

The spontaneous MS in the prostate is similar to that in the liver (de Boer et al., 1996a). However, the MS from the control animals (supplemented with basal diet and corn oil) were significantly different from that recovered from animals on the basal diet alone. A surprisingly high proportion of –1 frameshifts was recovered from the prostate samples of these rats. In a previous study by Stuart et al. (2000a), -1 frameshifts were only recovered at 9% of total mutations from the prostates of control rats. However, the sample size (11 mutants) in that study was very small. The high percentage of –1 frameshifts in the control samples may be due to the supplementation with corn oil but the mechanism is unknown. The high prevalence of –1 frameshifts was not observed in the colon or cecum of these rats, in which only 8.6 – 11.6% (Yang, manuscript in preparation) of the *lacI* mutants were identified as –1 frameshifts. It is possible that the

formation of –1 frameshifts is tissue-specific. Further analysis of the MS showed that in the prostate samples from spontaneous, control, or CLA group, minus frameshifts occurred at G:C base pairs and A:T base pairs in similar proportions, with the majority at runs of G:C or A:T base pairs.

A high percentage of –1 frameshifts was induced by PhIP as expected. PhIP has been reported to induce –1 frameshifts, specifically G:C deletions, in the target tissues of rodents (Kakiuchi et al., 1995; Okonogi et al., 1997a). In the current study, seventy-one out of the seventy-three –1 frameshifts are G:C deletions, significantly different from that observed in control samples, in which the frequency of A:T deletions is close to that of G:C deletions. This observation confirmed that guanine is the primary binding target of PhIP metabolites (Lin et al., 1992; Endo et al., 1995).

The PhIP-induced spectrum in the current study was then compared with that reported by Stuart et al. (2000a). Significant differences exist between the two spectra, notably, a higher proportion of –1 frameshifts and a lower proportion of G:C→T:A transversions were recovered in the current study. The differences may possibly be due to different PhIP doses. The MF of each individual class of mutation was plotted against different PhIP doses and shown in Figure 4.3. The frequencies of –1 frameshifts and G:C→ T:A transversions almost doubled when the total PhIP intake increased from 0.071 g/rat to 0.183 g/rat, while insignificant increases in the MF of G:C→ C:G, G:C→ A:T, and deletions were observed. This observation is compatible with the preferential induction of –1 frameshifts and G:C→ T:A transversions by PhIP, and is also indicative of a dose-dependent effect of PhIP on the induction of MS.

In the current study, CLA was found to inhibit prostate mutagenesis induced by PhIP. CLA has been reported to inhibit the formation of PhIP-DNA and IQ-DNA adducts in tumor target organs such as colon (Josyula and Schut, 1998). It is, therefore, possible that CLA inhibits the formation of PhIP-DNA adducts in the prostate. On the other hand, PhIP was also observed to induce oxidative DNA damage in the mammary gland (El Bayoumy et al., 2000). CLA is an antioxidant, reducing the oxidation of LA more efficiently than alpha-tocopherol (Ha et al., 1990). Thus, the inhibition of PhIP-induced mutagenesis by CLA may result from inhibiting the formation of PhIP-DNA adducts or of oxidative DNA damage. The inhibition of one or several specific DNA adducts by CLA would subsequently alter the MS induced by PhIP. Indeed, significant differences exist between the MS in the prostate recovered from PhIP- and PhIP + CLA-treated rats. The comparison of MS from PhIP- and PhIP + CLA-treated groups using the Fisher's exact test revealed that the PhIP + CLA-treatment group had a significantly higher percentage of G:C→ T:A transversions, as compared to the PhIP-treated group ($p=0.0068$). However, when the MF is considered, the frequencies of G:C→A:T transitions and –1 frameshifts were selectively decreased by the addition of CLA (Figure 4.1). Interestingly, this reduction in the frequencies of these two classes of mutations by CLA is opposite to the effect of the loss of function of the mismatch repair (MMR) gene MSH2 on spontaneous and PhIP-induced mutagenesis (Zhang et al., in press) as determined in an MSH2 knock-out *lacI* transgenic mouse model. The similar effect of CLA and MMR suggests that the antimutagenic effect of CLA in the prostate may be due to an enhancement of MMR. Some bulky aromatic amine adducts at the C8 position of

guanine are bound by the mismatch recognition complex hMutSα (hMSH2/hMSH6) (Li et al., 1996), indeed implicating MMR in the processing of such damage. The MMR gene hMSH2 was found mutated in the human prostate cancer cell line LNCaP (Leach et al., 2000), suggesting a possible association of the impairment of the MMR pathway with the prostate cancer occurrences, and *vice versa*, that the enhancement of MMR may reduce prostate carcinogenesis. The involvement of MMR in the prevention of prostate mutagenesis by PhIP should be further addressed.

Since CLA significantly changed the PhIP-induced MS, further analyses were performed to examine whether CLA has any effect on the induction of the fingerprint mutation of PhIP, a deletion of G:C base pairs at 5'–GGGA-3' sequences. This deletion has been considered as the mutational fingerprint of PhIP and has been found to occur at a frequency of 3-10% of total mutations induced by PhIP, independent of the gene and species (Nagao, 1999). This characteristic G:C deletion was identified in the tumor suppressor gene adenomatous polyposis coli (APC) in PhIP-induced tumors and in sporadic human colorectal cancer (Huang et al., 1996). In the prostate, this fingerprint mutation constituted 9.9% (14/142) of the total mutations induced by PhIP. Out of the 97 mutants from the PhIP + CLA-treatment group, nine G:C deletions at 5'–GGGA-3' were identified. The recovery of the G:C deletions at 5'–GGGA-3' sequences from PhIP- and PhIP + CLA-treated rats is similar as judged by the Fisher's exact test. The G:C deletions at other sequences were also compared and no sequence-preferential inhibition was detected with CLA supplementation ($p>0.6$).

In conclusion, we have shown that CLA inhibited PhIP-induced mutagenesis in the rat prostate, which suggests that CLA inhibits the initiation of PhIP-induced prostate carcinogenesis. The alteration in control and PhIP-induced MS by CLA may reflect an influence of CLA on the formation and removal of oxidative DNA damage and PhIP-DNA adducts. Two classes of mutations, G:C→A:T transitions and –1 frameshifts, were selectively inhibited by CLA, possibly via an enhancement of mismatch repair.

Acknowledgements

We would like to thank Trina de Monyé, Brandi Jenkins, Amanda Glickman, Greg Stuart, Dave Walsh, Jana Kangas, Ken Sojonky, Shulin Zhang and the Animal Care Unit staff at the University of Victoria for their assistance in the experiment.

Table 4.1. Mutation frequencies in the prostates of Big Blue® rats with different supplementation. Big Blue® rats were exposed to different supplementation as indicated in the table. PhIP (100 ppm) was incorporated into the diet for 47 days. Corn oil was mixed into the diet during the period of PhIP exposure. CLA (1%, w/w) was added one week prior to supplementation with PhIP and continued till the end of PhIP treatment. The *lacI* MF of each group was determined by standard Big Blue® transgenic assay.

Group	Supplementation	No. of rats	Total pfu	MF ± SD ($\times 10^{-5}$)
Spontaneous	None	6	3,049,437	2.5 ± 0.3
Control	Corn oil	4	1,434,840	2.4 ± 0.5
CLA	CLA and corn oil	5	2,680,211	1.8 ± 0.5
PhIP	PhIP and corn oil	5	2,390,060	14.6 ± 3.2[a]
PhIP+CLA	PhIP, CLA, and corn oil	5	1,878,600	9.1 ± 1.2[a,b]

a. Significantly different from the control MF ($P<0.05$).

b. Significantly different from the PhIP-induced MF ($p<0.05$)

Table 4.2. Mutation spectra recovered from the prostate of Big Blue® transgenic rats after various treatments

Mutation type	Spontaneous (65)[a]	Control[b] (43)	CLA (41)	PhIP[c,d] (142)	PhIP+CLA[c,d,e] (97)
G:C→ A:T	35.4	32.6	22.2	12.0	10.3
A:T→ G:C	4.6	2.3	9.8	0.0	1.0
G:C→ T:A	20.0	25.6	19.5	17.6	29.9
G:C→ C:G	4.6	0.0	0.0	5.6	1.0
A:T→ T:A	1.5	4.7	4.9	2.1	0.0
A:T→C:G	3.1	4.7	7.3	0.7	0.0
+1 frameshifts	7.7	0.0	0.0	1.4	2.1
-1 frameshifts	9.2	20.9	19.5	51.4	38.1
A:T deletions	4.6	7.0	9.8	1.4	1.0
G:C deletions	4.6	14.9	9.8	50.0	37.1
Deletions	12.3	2.3	7.3	5.6	13.4
Insertions	0.0	0.0	0.0	0.0	0.0
Complex changes	1.5	0.0	2.4	2.8	2.1
Double mutants	0.0	7.0	4.9	0.7	2.1

a. The number of independent mutants.

b. Significantly different from the spontaneous MS ($p<0.05$).

c. Significantly different from the control MS ($p<0.001$).

d. Significantly different from the CLA-induced MS ($p<0.01$).

e. Significantly different from the PhIP-induced MS ($p<0.007$).

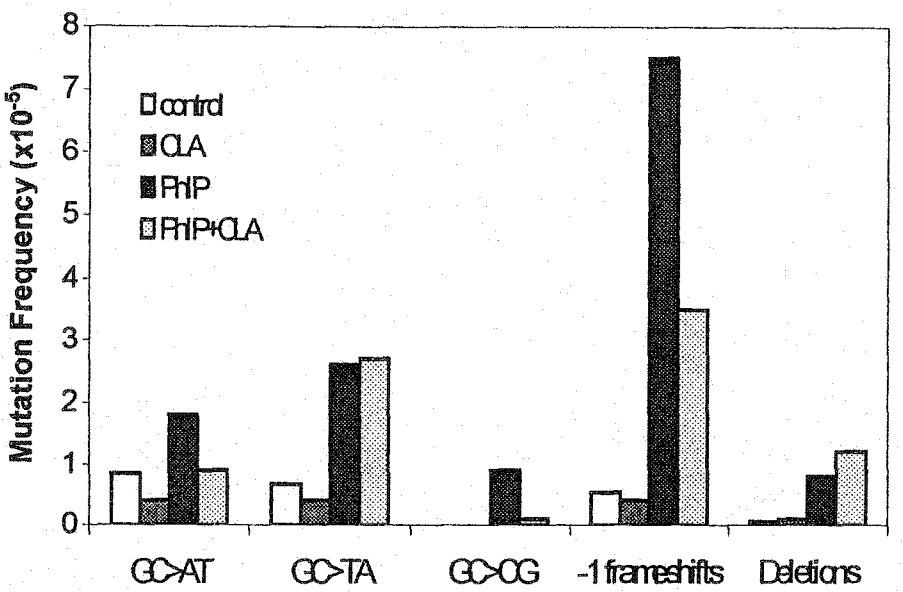

Figure 4.1. The frequencies of several classes of mutations recovered from different treatments.

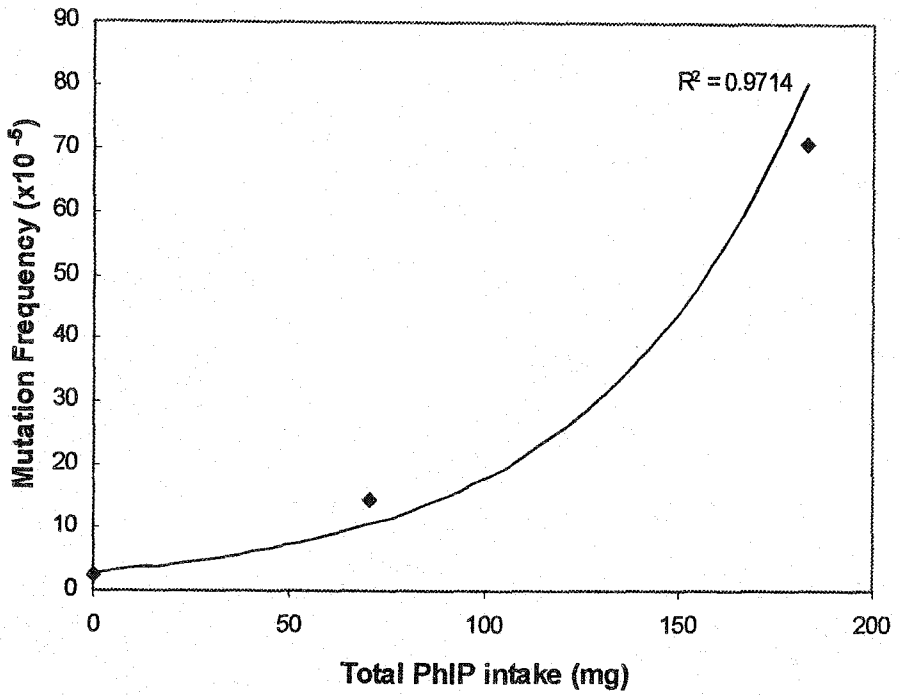

Figure 4.2. The mutation frequencies induced by different doses of PhIP in the prostate. The intake of PhIP was calculated as: PhIP concentration × average food intake per day (15 g) × PhIP exposure duration.

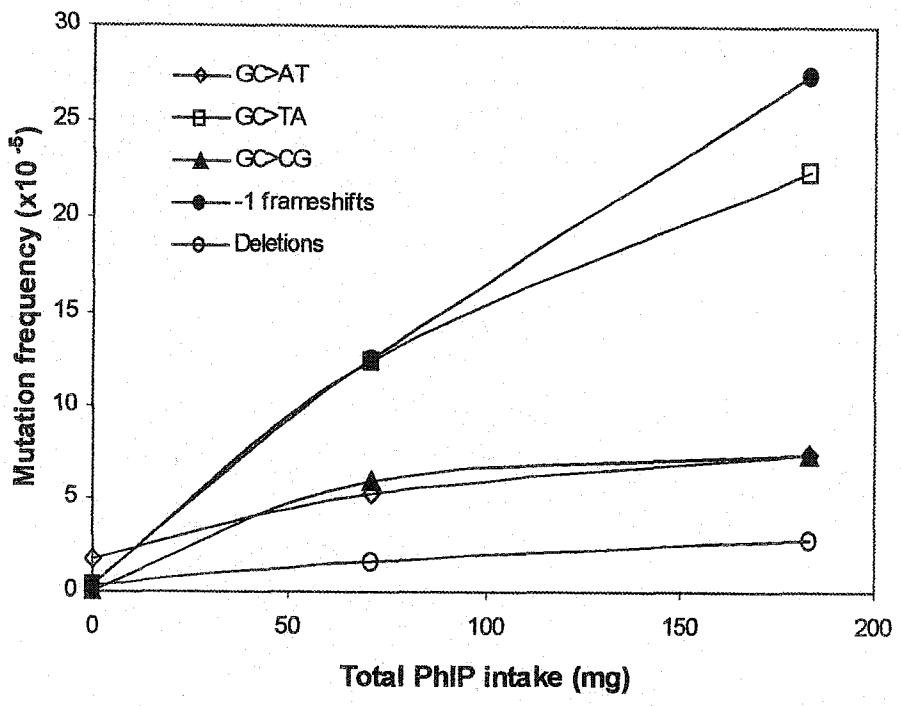

Figure 4.3. The effect of different doses of PhIP on the induction of mutation types. The intake of PhIP was calculated as: PhIP concentration × average food intake per day (15 g) × PhIP exposure duration.

Chapter 5. Sex-specific Induction of Mutations by PhIP in the Kidney of Rats and Its Modulation by Conjugated Linoleic Acid

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To be submitted to **Mutation Research**

Abstract

The heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a recognized mutagen and carcinogen in the colon and prostate in male, and in the mammary gland in female rats. In the current study, we examined the mutagenicity of PhIP in the kidney of male and female *lacI* transgenic rats and its modulation by a dietary chemopreventive agent, conjugated linoleic acid (CLA). Sex-specific induction of mutation by PhIP and CLA was observed. Exposure to 100 ppm PhIP through diet supplementation for 47 days induced a *lacI* mutation frequency (MF) of $7.7 \pm 0.3 \times 10^{-5}$ and $4.7 \pm 1.0 \times 10^{-5}$ in the kidney of male and female rats, respectively. The PhIP-induced MFs in the kidney of male and female rats were significantly different from each other and were 3-fold ($p<0.001$) and 0.6-fold ($p<0.05$) higher than the corresponding controls, respectively. However, when rats were given CLA along with PhIP, CLA completely inhibited the formation of PhIP-induced mutations in the kidney of female rats, but not in male rats. Comparison of mutational spectra (MS) did not detect significant differences between male rats treated with PhIP and PhIP + CLA. However, unlike the –1 frameshifts induced by PhIP in the colon and prostate, which consist primarily of G:C deletions, –1 frameshifts in the kidney involved the loss of both G:C and A:T base pairs. Our data indicates that the kidney of the rats responds in a sex-dependent way to mutagenesis and antimutagenesis by PhIP and CLA. These differences may be related to hormonally regulated P450 enzyme induction or cell proliferation.

5.1. Introduction

PhIP is a potential mutagen and carcinogen. It has been shown to induce colon and prostate tumors in male rats (Shirai et al., 1999), mammary gland tumors in female rats (Ito et al., 1991a), and lymphomas in mice (Esumi et al., 1989). Concomitantly, the mutagenicity of PhIP in those tissues has been confirmed, mostly by using transgenic rodent models (Masumura et al., 2000). The mutagenesis and carcinogenesis of PhIP are attributed partially to the initial formation of PhIP-DNA adduct in those tissues. For

example, feeding of rats with 400 ppm PhIP in their diet for one week produced 5.6×10^{-7} and 12.3×10^{-7} DNA adducts per nucleotide in the colon and prostate, respectively, compared to 0.80×10^{-7} in the non-target tissue liver (Purewal et al., 2000). However, exposure to PhIP also induces relatively high levels of DNA adducts in tissues such as pancreas and lung in which tumors were rarely induced. Like other heterocyclic amines (HCAs), the carcinogenicity of PhIP is sex-specific. In CDF_1 mice fed a diet containing 0.04% PhIP for 83 weeks, the incidence of lymphomas was 31% in male and 68% in female mice (Esumi et al., 1989). In F344 rats, PhIP induced colon tumors in male rats, but not in females (Ito et al., 1991a). Thus, a comparison of the mutagenicity of PhIP between target (e.g. colon, prostate, and mammary gland) and non-target tissues (kidney, pancreas) may provide explanations other than the PhIP-DNA adduct level for the tissue- and sex-specific carcinogenicity of PhIP.

Conjugated linoleic acid is a mixture of isomeric heat-generated derivatives of linoleic acid, each with a conjugated double bond system (Ha et al., 1987). A number of studies have reported a protective activity of CLA against HCA-induced carcinogenesis by measuring changes in HCA-DNA adduct levels. CLA was found to reduce the formation of PhIP-DNA adducts in the colon, IQ-DNA adducts in the mammary gland of female rats (Josyula and Schut, 1998), and DNA adducts and aberrant crypt foci induced by IQ in the colon of male rats (Liew et al., 1995). Of particular interest is that the protective efficacy of CLA is sex-related, as shown by Zu and Schut (1992). About 74% of the IQ-DNA adducts in the lung and 39% in the large intestine of female mice were inhibited by CLA. No inhibition was observed in males.

We previously demonstrated the antimutagenic effect of CLA in the colon and prostate, both target tissues of PhIP-induced carcinogenicity (Yang et al., in press; Yang et al., submitted). The present paper describes the mutagenicity of PhIP in the kidney , a non-tumor-target tissue of male and female rats and the modulation of the mutagenicity by CLA.

5.2. Materials and Methods

5.2.1. Animal treatment

Male and female F344 Big Blue® rats (10 weeks old) obtained from Stratagene (La Jolla, CA) were used for in-house breeding. The offspring rats were weaned at 21-days and were given a powered basal diet, AIN-93G without the *tert*-butylhydroquinone antioxidant (Dyets Inc., Bethlehem, PA). Food and water were provided *ad libitum*. The rats were weighed weekly and the food consumed was measured twice weekly. Male and female rats were divided into 8 groups, each consisting of 4-6 rats, consistent with statistical recommendation by Carr and Gorelick (1995). When the rats were 50 days old, one-hundred ppm PhIP (>98% HPLC pure, Toronto Research Chemicals, Toronto, Canada) were incorporated into the diet. One percent CLA (~41% *cis*-9,*trans*-11 CLA, ~44% *trans*-10,*cis*-12 CLA, ~10% *cis*-10,12 CLA, ~5% *cis*-9,12 linoleate, Nu-Chek-Prep, Elysian, MN) was added one week prior to supplementation with PhIP and continued till the end of the PhIP treatment. Two percent tocopherol-stripped corn oil (ICN, Costa Mesa, CA) was incorporated into the powdered diet during the PhIP-exposure period to reduce the formation of dust. Food was prepared weekly and purged

with argon gas and stored at 4°C. After 47 days of PhIP treatment, the rats were returned to the basal diet and euthanized one week later (Figure 3.1A). Kidney tissues were removed immediately, frozen in liquid nitrogen, and stored at -80°C until DNA isolation.

5.2.2. Determination of mutant frequency

High molecular weight genomic DNA was isolated from the kidney using a modification (Yang et al., in press) of the RecoverEase™ DNA dialysis method. The genomic DNA was added to a λ phage packaging extract (Transpack, Stratagene) and the resulting phages were plated on *E. coli* SCS-8 bacteria on trays of NZY media containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The mutant frequency was determined by the ratio of the number of blue mutant plaques to the total number of plaques.

5.2.3. Amplification of mutated *lacI* gene and sequencing

A small aliquot of phage stock (5 μl) was used as a template to amplify the *lacI* gene from mutants (Erfle et al., 1996). The PCR reaction mixture (100 μl) contained 10 μl of 10×PCR buffer (150 mM Tris-HCl, pH 8.5, 15 mM $MgCl_2$, and 600 mM KCl), 25 mM of each dNTP (Pharmacia Biotech), 20 μmol forward primer (5'-GCGTCGATT TTTGTGATGCT-3'), 20 μmol reverse primer (5'- TCCGCTCACAATTCCACACAA – 3'), and 10 units *Taq* polymerase. Samples were subjected to 30 PCR cycles consisting of 95°C for 36 seconds, 59°C for 36 seconds, and 72°C for 90 seconds. The resulting PCR products were purified using a QIAquick™ PCR purification kit (Qiagen, Mississauga, Ontario). Sequencing reactions were performed using a Thermo sequenase DYEnamic

direct cycle sequencing kit (Amersham) with a template concentration (i.e. PCR DNA product) of 30-90 ng/μl. The sequencing solution was loaded and sequenced on a Licor automated fluorescent sequencer (Licor, Lincoln, Nebraska) and the DNA sequence was determined by the DNA sequence analysis software (Licor) and Seqman II sequence assembler (DNAStar, Madison, USA). Mutant sequence data were managed and analyzed by using custom software (de Boer, 1995) to establish MS for each treatment. Statistical comparisons of MS were made using the Monte Carlo method of Adams and Skopek (1987). The comparison of individual mutation classes was performed using the Fisher's exact test.

5.2.4. Clonal expansion and mutation frequency

Clonal expansion events are defined as identical mutations occurring more than once in a given tissue from a given animal (Nishino et al., 1996), and are considered to originate from an independent mutation. Therefore, clonal expansion mutations were scored only once as independent mutation. The ratio of the number of independent mutations / the number of total mutations was used to correct mutant frequency to mutation frequency (MF). Statistical comparisons of MFs were performed using the computer program COCHARM (created by Troy Johnson, Procter and Gamble, Cincinnati, OH) that executes the Generalized Cochran-Armitage test (GCA) (Carr and Gorelick, 1995). An analysis of variance (ANOVA) with Bonferroni correction as the multiple comparison post test (Instat, GraphPad Software) was used as alternative method to analyze the trends in the MFs.

5.3. Results

5.3.1. Mutation frequencies and sex-related difference

The MFs in the *lacI* gene of kidney genomic DNA from Big Blue® rats are summarized in Figure 5.1, and were analyzed by the GCA test first as recommended by Carr and Gorelick (1995). The MFs in the kidney of male and female control rats were $1.9 \pm 0.1 \times 10^{-5}$ and $2.9 \pm 1.0 \times 10^{-5}$, respectively, showing no significant difference between the males and females. PhIP treatment increased the MF significantly in male rats. A 3-fold increase of MF was observed compared to the control MF ($p<0.01$), while a marginal 60% increase was seen in female rats ($p=0.058$), suggesting that the kidneys of male rats are more sensitive to PhIP-induced mutagenesis. Following treatment with CLA, the MF also increased by approximately 80% in the kidney of male rats, but not in female rats. CLA did not influence the PhIP-induced MF in male rats, however, significant inhibition was observed in female rats, reducing the MF from $4.7 \pm 1.0 \times 10^{-5}$ to $2.5 \pm 0.7 \times 10^{-5}$ ($p<0.05$).

The GCA test is a suitable method to compare MFs in two treatment groups and when a small number of animals are used. However, analysis of variance (ANOVA) is required when multiple groups are compared. The result of this analysis is shown in Table 5.1. Significant differences in MFs were detected between the control and the PhIP-treatment group for both male and female rats, between male and female rats treated with PhIP, and between female rats treated with PhIP and PhIP + CLA. These results are consistent with those determined by the GCA test, with the exception that CLA was not found to increase the spontaneous MF significantly in the male kidney.

5.3.2. Mutation spectra

The mutants recovered from each group were sequenced and, after correction for clonal expansion, used to establish the mutational spectra (MS) for each individual treatment (Table 5.2). However, due to the limited number of mutants recovered in some treatment groups (<40), only the MS from PhIP- and PhIP + CLA-treated male rats were compared using the Adams and Skopek Monte Carlo analysis (1987).

The MS recovered from male and female rats of control groups appear to be similar, with more than half of the mutations being base substitutions. Most interestingly, -1 frameshifts were common in the kidney of both male and female rats (29 and 17%, respectively). This is higher than generally found in other background MS for the kidney (Bol et al., 2000), or other tissues (de Boer et al., 1998). However, it is similar to the fraction of –1 frameshifts recovered from the prostate in a previous study (Yang et al., submitted). In both studies, corn oil was added to the food. Minus one frameshifts include deletions at both G:C and A:T base pairs, consistent with the general trend seen in background spectra from various tissues[1]. The addition of PhIP to the diet results in a spectrum dominated by G:C→A:T transitions, G:C→T:A transversions, and -1 frameshifts. Again, -1 frameshifts consist of deletions of both G:C and A:T base pairs. The MS in the kidney of the PhIP + CLA group appears to be similar to that seen in the PhIP-only group. Statistical analysis of the two groups of male rats reveals no difference between the MS. The spectrum in the CLA-alone group did not appear to differ from that of the control group.

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[1] Internet address: http://eden.ceh.uvic.ca/cgi-win/laci1.exe

5.4. Discussion

PhIP is a well-recognized mutagen and carcinogen in rodents. The mammary gland in female rats, and the colon and prostate in male rats have been identified as target tissues for carcinogenicity (Hasegawa et al., 1993; Ito et al., 1997; Shirai et al., 1999). Correspondingly, the mutagenicity of PhIP in those tissues was also confirmed (Okochi et al., 1999; Masumura et al., 2000; Stuart et al., 2000a). In the current study, we examined the mutagenicity of PhIP in the kidney of male and female Big Blue® rats, and its possible inhibition by CLA.

Two statistical methods, the GCA and ANOVA test, were used to compare the MFs following different treatments. Data from both analyses showed that PhIP significantly increased the mutation frequencies in the kidney, and this induction was sex-dependent. The modulation of PhIP-induced mutagenesis by CLA is also found to be sex-specific. The effect of CLA on the spontaneous frequency is small. Although the GCA test shows this effect is significant in the male rats ($p<0.05$), the difference disappears when the ANOVA test is applied with multiple comparisons.

The initiation of PhIP-induced mutagenesis and carcinogenesis involves the formation of PhIP-DNA adducts. The reactive metabolites of PhIP bind to C8 of guanine and the resulting DNA adducts, primarily dG-C8-PhIP (Nagaoka et al., 1992), have been detected in various tissues of rats in the general order of pancreas, heart>lung, prostate>colon, stomach, kidney, small intestine, spleen, mammary gland>liver (Takayama et al., 1989; Kaderlik et al., 1994). The kidney, a non-target organ for tumor induction, showed a surprisingly high level of adducts, approaching that found in the

colon (Takayama et al., 1989). It is likely correlated with the high uptake of PhIP or its metabolites in the kidney. Indeed, PhIP is found to remain mainly in liver and kidney as a cell-bound form (Watkins et al., 1991). The PhIP metabolite, *N*-hydroxy-PhIP, formed in the liver is believed to be distributed through blood circulation, which may be responsible for the formation of adducts in a high-perfusion organ such as the kidney. The lack of association between DNA adduct level and carcinogenicity suggests that the extent of adduct formation and rate of removal are probably not major determinants of interorgan differences in susceptibility to PhIP-induced carcinogenesis. Since mutations result from DNA adducts during cell division, it is reasonable to envision that cancer incidence might be more closely related to MF. In the kidney, PhIP induced a MF of 7.6×10^{-5} in male rats, and 4.7×10^{-5} in females, which are only 10-20% of that in the colon (Yang et al., in press), and 30-50% of that in the prostate ($p < 0.05$, Yang et al., submitted). The lower MF induced by PhIP is consistent with the absence of carcinogenicity of PhIP in the kidney. Therefore, with the exception of the colon in female rats and the cecum, the PhIP-induced MF may serve as a better biomarker to predict the tissue specific risk for cancers induced by PhIP exposure. Lynch et al. (1996) found that a single dose of PhIP (20 mg/kg) failed to cause mutations in the kidney of the male Muta™ mouse. One possible reason for the different observations is lower PhIP-DNA adduct levels in mice than those in rats, given the same dose of PhIP (Josyula and Schut, 1999). In addition, organ distribution of PhIP-DNA adducts depends on the mode of PhIP administration. The removal of PhIP-DNA adducts was found to be much faster after a single oral dose than after chronic dietary supplementation in both rats and mice (Schut et al., 1997).

PhIP-DNA adducts accumulated mainly in the kidney and pancreas after chronic exposure (Friesen et al., 1996).

Sex differences have been observed in susceptibility to carcinogenicity of HCAs (reviewed by Ohgaki et al., 1991). Male F344 rats were found to be more susceptible than females to hepatocarcinogenesis induced by IQ, 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-6-methyl-dipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1), and 2-aminodipyrido[1,2-a:3',2'-d]imidazole (Glu-P-2). On the other hand, female CDF_1 mice were more susceptible than males to the hepatocarcinogenic effects of all HCAs tested. Therefore, it is not surprising to see an almost 1-fold difference in the PhIP-induced MF between male and female rats. Higher PhIP-DNA adduct levels were detected in the tissues of male rats than in those of female rats after repeated daily dosing with PhIP (Schut et al., 1997; Schut and Yao, 2000). In the kidney, the PhIP-DNA adduct level was 0.4×10^{-7} in male rats and 0.1×10^{-7} in females, which correlates well with the sex differences in the mutational response to PhIP that we observed. DNA adducts resulting from treatment with another HCA, MeIQ, were also found to be higher in male rats than in female rats in several tissues, including the liver, kidney, and white blood cells (Thorgeirsson et al., 1995). All these findings indicate that, in general, male rats are more susceptible than female rats to carcinogenesis induced by HCAs.

The MS in the kidney of male and female rats resulting from PhIP treatment are significantly different from the PhIP-induced MS in the prostate and colon. Unlike the PhIP-induced –1 frameshifts in other target tissues, which contain, almost exclusively, deletions of single G:C base pairs, about 30-40% of –1 frameshifts in the kidney of PhIP-

treated rats were A:T deletions. Consequently, the frequency of A:T deletions increased from 0.1- 0.2 $\times 10^{-5}$ to 0.7$\times 10^{-5}$ upon treatment with PhIP. In a previous study, we found that control mutations from the prostate consisted of –1 frameshifts at A:T and G:C sites, while after treatment with PhIP, -1 frameshifts occurred nearly exclusively at G:C base pairs (Yang et al., submitted). The frequency of A:T deletions in the prostate upon PhIP treatment remained the same as that in the control. Thus, the response of the kidney to PhIP is different from that of the prostate, even though background mutations, especially –1 frameshifts, are similar. Minus one frameshifts at G:C base pairs are believed to result from the PhIP-DNA adduct, dG-C8-PhIP. The discrepancies between the PhIP-induced MS from the kidney and those from other tissues suggest that the mutational mechanisms in the kidney differ from those in the prostate and colon, in which the mutagenic potential of dG-C8-PhIP is thought to have a determining impact. There is a possibility that dG-C8-PhIP may contribute less to mutagenesis in the kidney than in the prostate and colon. Factors, e.g. spontaneous mutagenesis, induction of oxidative damage, or increased cell proliferation following PhIP treatment, may be more important in shaping the overall spectra. It is interesting to note that the fingerprint mutation of PhIP, a G:C deletion at 5'-GGGA-3' sequence, was present at 4.8% and 5.7% in the kidney of male and female rats treated with PhIP, respectively, strongly indicating that part of these mutations are indeed induced by PhIP (Masumura et al., 2000).

The modulation of PhIP-induced mutation by CLA in the kidney was also found to be sex-specific. In female rats, CLA treatment inhibited the PhIP-induced mutations completely. By contrast, CLA had no effect on males. This is in agreement with the

finding by Zu and Schut (1992), in which CLA inhibited IQ-DNA adducts by >95% in the kidney of female CDF_1 mice but had no effect in males. The inhibitory effect of CLA on IQ-DNA adduct formation in all organs examined was, with the exception of liver, restricted to the female.

These sex and species differences in susceptibility to PhIP-induced mutagenesis and its inhibition by CLA are considered to be at least partly related to differences in the amount and inducibility of P450 (Degawa et al., 1987; Degawa et al., 1988). Most carcinogenic aromatic amines possess activity for induction of P4501A family isozymes (Degawa et al., 1992), especially P4501A2, responsible for the bioactivation of amines in the rat liver. The induction of hepatic P4501A2 in the liver of CDF_1 mice is sex-specific in that it is observed only in females. This induction can be blocked by pretreatment with testosterone (Degawa et al., 1987, Degawa et al., 1988), pertaining to a possible hormonal regulation of the sex differences in susceptibility. Correspondingly, the sex-specific inhibitory effect of CLA may act in part by inhibiting the induction of P4501A2 through affecting hormone status. PhIP treatment has been reported to induce P4501A1 and 1A2 enzymes in the liver, and P4501A1 in the kidney in males F344 rats but not in mice (Degawa et al., 1992). Whether P450 enzymes are induced by PhIP in female F344 rats is unknown, although PhIP failed to induce P4501A in female Wistar rats (Kleman et al., 1990). On the other hand, androgen levels have been shown to contribute to a higher susceptibility of male rats to liver cancer (Blanck et al., 1984), which helps to explain the higher PhIP-induced MF in the male kidney compared to females. CLA has been shown to inhibit cell proliferation of an estrogen-responsive human breast cancer cell line

MCF-7 (Liew et al., 1995; Durgam and Fernandes, 1997). It was suggested that CLA may be involved in the regulation of hormone mediated mitogenic pathways in estrogen-responsive positive cell lines. Therefore, it is possible that the sex-specific inhibitory effect of CLA against PhIP-induced mutagenesis in rodents may be related to hormone status, including estrogen and androgen levels.

. In conclusion, our study has shown that PhIP induced mutations in the kidney of male and female rats in a sex-specific manner. Mutation induction is higher in male rats. An inhibitory effect of CLA against the mutagenicity of PhIP was only detected in the kidney of female rats. Those sex differences in susceptibility to mutagenesis and antimutagenesis may be related to the induction of P450 enzymes or hormonal regulation.

Acknowledgements

This work was supported by the National Cancer Institute of Canada. We also appreciate the excellent technical support, including mutant screening and sample sequencing, of Trina de Monyé, Virginia Haslett, Brandi Jenkins, and James Holcroft.

Table 5.1. Analysis of variance (ANOVA) tests of the differences of the means of mutation frequencies from the kidney of Big Blue® rats following various treatments. The Bonferroni post test was used to compare pairs of means from selected treatment groups.

Group	Control-Female	CLA-Female	PhIP-Female	PhIP+CLA-Female	Control-Male	CLA-Male	PhIP-Male	PhIP+CLA-Male
Control-Female								
CLA-Female	P>0.05							
PhIP-Female	P<0.05							
PhIP+CLA-Female			P<0.01					
Control-Male	P>0.05							
CLA-Male					P>0.05			
PhIP-Male			P<0.001		P<0.001			
PhIP+CLA-Male							P>0.05	

Table 5.2. The mutational spectra (%) recovered from the kidney of male and female rats after exposure to CLA or PhIP

Mutation type	Control (%)		CLA (%)		PhIP (%)		PhIP+CLA (%)	
	Male (14)[a]	Female (24)	Male (36)	Female (24)	Male (103)	Female (35)	Male (71)[b]	Female (24)
G:C→ A:T	28.6	25.0	38.9	41.7	29.1	17.1	19.7	36.4
A:T→ G:C	7.1	0.0	2.8	4.2	2.9	5.7	5.6	4.5
G:C→ T:A	14.3	37.5	19.4	16.7	26.2	5.7	25.4	13.6
G:C→ C:G	0.0	4.2	5.6	0.0	1.0	5.7	1.4	4.5
A:T→ T:A	0.0	0.0	0.0	8.3	1.0	2.9	1.4	0.0
A:T→C:G	7.1	0.0	2.8	4.2	1.0	0.0	1.4	0.0
+1 frameshifts	7.1	0.0	2.8	0.0	6.8	2.9	1.4	0.0
-1 frameshifts	28.6	16.7	19.4	16.7	22.3	42.9	35.2	18.2
A:T deletions	7.1	8.3	11.1	8.3	9.7	14.3	12.6	0.0
G:C deletions	21.5	8.3	8.3	8.3	12.6	28.6	22.6	18.2
Deletions	0.0	12.5	5.6	8.3	4.9	11.4	2.8	9.1
Insertions	7.1	0.0	2.8	0.0	0.0	0.0	2.8	0.0
Others	0.0	4.2	0.0	0.0	4.8	5.7	2.8	13.6

[a] Total number of independent mutants identified

[b] Not significantly different from the PhIP-induced MS in male rats

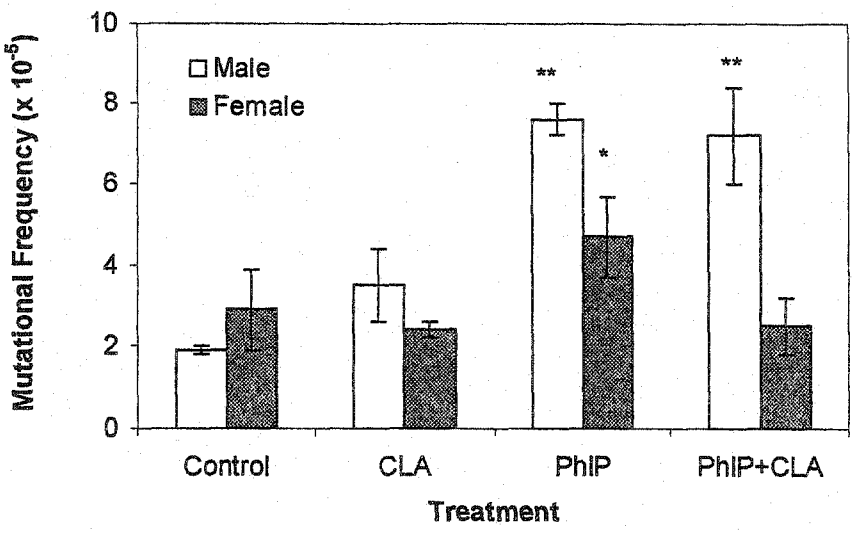

Figure 5.1. The mutation frequencies recovered from the kidney of male and female rats following exposure to PhIP and CLA. For each sample, over 800,000 pfus have been screened. * and ** indicate that the MF from that specific group is significantly different from the background MF with a $p < 0.05$ and $p<0.001$, respectively.

Chapter 6. Measurement of DNA Damage and Repair in the λLIZ Transgene in a Big Blue® Rat Cell Line by Quantitative PCR

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Environmental and Molecular Mutagenesis *33*: 21-27, 1999

Abstract

DNA damage and its subsequent repair occur heterogeneously throughout the genome, which reflects the nature of the damaging agents, gross chromosomal structure, the specific nucleotide sequence, and transcriptional status. We elected to investigate the repair of DNA damage in an artificial, transgenic situation. Here we report the repair of UV and benzo(*a*)pyrene diol epoxide (BPDE)-induced DNA damage in the non-transcribed λ construct of the Big Blue® rat-2 transgenic cell line. This was determined by Quantitative PCR (QPCR) using genomic DNA isolated at specific times following treatment with UV or BPDE. The results indicate that, despite the absence of transcription, lesions induced in the *lacI*-containing λ insert by UV and BPDE are efficiently repaired. The half life of the polymerase-blocking lesions is 4.2 and 5.5 hours for UV- and BPDE-induced lesions respectively. This is an important observation vis-a-vis the use of this transgene as a model for studies of mutational mechanism.

Keywords: transgenic, transcription-coupled nucleotide excision repair, quantitative PCR

6.1. Introduction

Mutations recovered after exposure to a mutagen reflect both the formation of DNA lesions and their subsequent repair. The development of the *lacI* transgenic rodent assay provides a unique and potential opportunity to examine mutation in a mammal *in vivo*. However, there are significant differences between the λ *lacI* transgene and normal, endogenous genes. For instance, the λ inserts are present in concatenated arrays in the DNA of Big Blue® animals. The λ inserts are also highly methylated (Dycaico et al., 1994) and not expressed (Kohler et al., 1990). As a consequence, these transgenes are not

subject to transcription-coupled excision repair (TCR). This may have consequences for the recovery of mutations in this system even though other repair systems such as global genomic excision DNA repair are expected to operate (van Hoffen et al., 1995). The mutagenic response at the *lacI* gene in the nonexpressed λ insert has been compared with those of endogenous genes, mainly the *hprt* gene. Similar dose-responses of mutation frequency were observed in the *lacI* and *hprt* genes in N-ethyl-N-nitrosourea (ENU)-treated Big Blue® mice (Skopek et al., 1995), although the mutation frequency at the *lacI* locus is considerable higher. In addition, the mutation spectra recovered from ENU-treated (Walker et al., 1996) and dimethylbenz[a]anthracene (DMBA)-treated (Mittelstaedt et al., 1998) Big Blue® rats or mice indicate that the general types of mutations in *lacI* and *hprt* are quite similar. These results would suggest that similar DNA lesions yield similar spectra, but to date no data on the repair of the λ insert has been reported. Here we report on the kinetics of DNA repair of the λ insert to provide a better understanding of DNA repair in the λLIZ transgene.

DNA damage and its repair are not uniformly distributed throughout the mammalian chromosome. They appear to be influenced by several factors, including the base composition, nucleosome structure, and gene activity (Denissenko et al., 1996; Tijsterman et al., 1996). The repair of DNA damage induced by UV and BPDE has been extensively studied (Van Houten et al., 1986; Tung et al., 1996; Venema et al., 1991). UV photoproducts (Figure 6.1) are subject to TCR. However, the role of global genome repair in the removal of UV photoproducts is not clear. In the case of BPDE-DNA adducts (Figure 6.2), Tang (1994) found neither preferential, nor significant strand-

specific repair of these adducts in the *dhfr* gene of B-11 and AT3-2 CHO cells while Chen et al. (1992) observed preferential and strand-specific repair of these lesions in the *hprt* gene, indicating a role of TCR in the repair of BPDE lesions. The different observations suggested that the preferential repair of BPDE lesions in the transcribed strand was less significant than that of UV photoproducts. In the present study, Quantitative PCR (QPCR) was used to study region-specific DNA damage and repair (McCarthy et al., 1996) of the λ insert in UV or BPDE-treated R2λLIZ cells. This method is based on the premise that specific lesions block DNA synthesis by *Taq*-polymerase, effectively reducing the amount of available DNA template with a concomitant reduction in the yield of the QPCR reaction. Upon repair, the PCR yield is restored. In this manner we have measured the formation of UV photoproducts, BPDE-induced lesions and their repair.

6.2. Material and Methods

6.2.1. Cell Culture and treatment

Big Blue® rat-2 λLIZ, a rat embryo fibroblast cell line (Stratagene, La Jolla, CA), transgenic for approximately 40 copies of λLIZ insert, was grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% Fetal Bovine Serum (FBS; Gibco), 200 mg/ml geneticin (Sigma, St. Louis, MO) and 10 mg/ml gentamycin for a total volume of 10 ml per 15cm petri dish at 37°C and 7% CO_2.

Cytoxicity was determined by plating 300 cells in a 15 cm petri dish and incubating at 37°C overnight. Cells were washed with PBS and irradiated with a germicidal lamp (254nm, 0.6 J m^{-2}/sec, Sylvania, USA) to the desired fluence, or incubated with selected concentration of BPDE (Sigma) dissolved in DMSO for 30 minutes. Cells were washed and incubated in fresh media for 9 days. The colonies formed were fixed with fix-stain solution (1% methylene blue, 50% methanol) and cell survival in treated dishes was expressed as a percentage of colony formation in untreated control. Suitable UV dose or BPDE concentration was selected to treat plates of R2λLIZ cells (about 80% confluence). Cells were either harvested immediately or allowed to recover for varying lengths of time up to 48 hours.

6.2.2. DNA isolation, restriction, and quantitation

Genomic DNA was isolated using a modification of the RecoverEase™ DNA dialysis method. Briefly, cells were washed with PBS, lysed in cell lysis solution (10 mM Tris-HCl, pH 8.3, 140 mM NaCl, 3 mM KCl, 0.35 M sucrose, 1 mM EDTA, 1% Triton X-100), digested with proteinase K/SDS (2 mg/ml proteinase K, 2% SDS, 100 mM EDTA) and dialyzed against TE for 48-72 hours. The isolated high molecular weight genomic DNA was digested with restriction endonuclease SfiI (New England Biolab) to reduce the viscosity. DNA concentrations were determined by a Hoechst dye-binding fluorescence assay (Labarca and Paigen, 1980) using a QM-1 QuantaMaster Luminescence Spectrometer (Photon Technology International). Absolute DNA concentrations were determined by comparing sample fluorescence values to a standard curve generated for each assay with calf thymus DNA (Sigma). After being diluted to 3

ng/μl with sterile H_2O, the samples were measured again to ensure the accuracy of the DNA concentration. Samples were further diluted to 0.75 ng/μl before use.

6.2.3. Quantitative polymerase chain reaction (QPCR)

A 12.5 kb-target fragment located within the λ insert was amplified from the genomic DNA using the following protocol: DNA templates (8 μl of 0.75ng/μl genomic DNA) were added into 100 μl thin wall PCR tubes along with 32 μl of PCR master mix. The PCR master mix was composed of the following: 1×XL PCR buffer (Perkin-Elmer/Roche, Alameda, CA), 1.3 μM $Mg(OAc)_2$, 0.4 mM dNTPs, and 0.2 μM primers (Forward primer: 5'-CGCCGCCTTGCCCTCGTCT-3', from 28409 to 28427, reverse primer: 5'-AGCTCCGCAAATTCGCCTACAC-3', from 40918 to 40897. Primer positions are taken from wild type λ). The thermal cycling was carried out in a Perkin-Elmer 9600 system. " Hot start" was performed by adding 1.6 units of *rTth* polymerase in 10 μl 1× XLPCR buffer into samples held at 80°C. The samples were amplified as follows: initial denaturation at 94°C for 30s, and then 16 cycles of 94°C for 15s, and 68°C for 10 minutes, followed by 15 cycles of 94°C for 15s, and 68°C for 10 minutes with an increase of 15 seconds extension time per cycle. Samples were then held at 72°C for 10 minutes and stored at 4°C.

6.2.4. Quantitation of the PCR product

The PCR product from each sample was mixed with a standard amount of a 1-kb DNA fragment which acts as a loading control. Ten μl aliquots of the mixture were loaded onto a 0.5% agarose gel and electrophoresed at 60V for 30 minutes. After

electrophoresis, the gel was stained with 0.6% ethidium bromide for 30 minutes and washed with 1× TAE buffer. The gel was digitized with the EagleEye II system (Stratagene, CA, USA) and the band intensity quantified using the ImagePC software package (NIH).

6.3. Results

Measurement of DNA lesions by QPCR is based on the observation that certain lesions can block the progression of thermostable polymerase, e.g. *Taq*, *rTth* polymerase (Cannon et al., 1995; Yakes et al., 1996) in the PCR reaction resulting in a decrease in the yield of the amplification product. Under carefully controlled conditions, the yield of the PCR reaction is linear relative to the amount of undamaged template in the reaction. Assuming a random distribution of DNA lesions, the lesion frequency f per genomic strand can be determined by the Poisson equation: $f = -\ln A_d/A_0$, in which A_d represents the amount of amplification product from the damaged template and A_0 the yield from the control template.

6.3.1. Optimization of λ insert-specific QPCR

Primers located at position 28409 and 40918 in the wild type λ chromosome were used to amplify a 12.5kb DNA fragment. This fragment includes the *cII* gene, but not the LIZ plasmid. To ensure a linear relationship between template DNA and PCR product we performed the following experiments:

1) We determined the maximum number of amplification cycles for which the amount of PCR product generated increased exponentially with increasing cycle number.

Amplification was performed with 0.12 ng/μl of genomic DNA for 24, 27, 30, 32, and 34 cycles and the resulting 12.5 kb λ insert fragment was quantified. The optimal number of cycle was determined to be 31, and a plateau was detected at 34 cycles (Figure 6.3 A). Each point is the average of three independent PCR reactions.

2) In order to establish that amplification is proportional to the amount of intact template in the reaction, amplification of an increasing amount of untreated genomic DNA was performed at 31 cycles which was determined to be in the exponential amplification range. A linear relationship between PCR product and the template concentration was observed between 0 to 0.24 ng/μl of genomic template DNA (Figure 6.3 B).

The study of DNA lesions in the λ insert was therefore performed at 31 amplification cycles with 0.12 ng/μl genomic DNA as the template. Under these conditions, the amount of PCR product is proportional to the amount of intact target sequence.

6.3.2. Cell survival

R2λLIZ cells were treated with 6, 9, 12, 18, and 24 J/m^2 UV (254 nm) to determine a suitable irradiation dose (Figure 6.4A). We selected a range of 3 to 24 J/m^2 to study the formation of UV photoproducts and 9 J/m^2 for determining the DNA repair kinetics. This latter dose results in approximately 70% cell survival. To determine the treatment dose of BPDE for the study of repair we exposed the cells to 0.05, 0.1, 0.5

and1.0 μM BPDE for 30 minutes (Figure 6.4B) and selected 0.5 μM for the repair study. This dose also results in approximately 70% cell survival.

6.3.3. The formation of UV photoproducts after UV exposure

The UV dose needed to produce polymerase blocking damage in genomic DNA was initially determined by irradiating R2λ cells with UV at 9 J/m^2 and 24 J/m^2. A decrease of the yield of the PCR amplification was observed as a function of the UV dose (Figure 6.5A). The standard deviation of the data points is <10%. The dose response of the induction of UV adducts was further studied by irradiating the cells with UV from 3-24 J/m^2. An initial lag at low UV doses followed by a reduction in PCR product yield at high doses was observed (Figure 6.5B).

6.3.4. Repair of UV photoproducts

The repair of UV photoproducts in the transgene was measured after irradiation with 9 J/m^2. Measurement of the amount of PCR product after 24 hours indicated that removal of UV lesions was complete (Figure 6.6A). The kinetics of the repair was assessed in a second experiment by allowing the cells to repair the UV lesions for varying amounts of time. Figure 6.6B shows that the majority of the lesions are repaired within 16 hours. The relative PCR product at each time point was recalculated as the fraction of residual damaged (unamplifiable) DNA, and tested for conformity to a first-order kinetic model. The regression analysis of the remaining DNA lesion and the repair time (Figure 6.6C) revealed a first-order kinetic model (R^2 =0.95). The estimated $t_{1/2}$ for repair is 4.2 hours.

6.3.5. Repair of BPDE-DNA adducts

The frequency of DNA adducts induced in cells lysed immediately after treatment with 0.5 μM BPDE was 2.0 x 10^{-5} adducts/ nucleotide. This is in the reported range of 0.45 –5.2 adducts/ 10^5 nucleotides caused by 0.03 –0.5 μM BPDE (Denissenko et al., 1996). The adduct removal is complete after allowing the cells to repair for 25 hours (Figure 6.7). Regression analysis of the residual DNA damage vs. the repair time demonstrates a first-order repair kinetic model, similar to that of repair after UV samples. The estimated repair $t_{1/2}$ is 5.5 hours.

6.4. Discussion

A major concern regarding the development and use of transgenic mutation assay systems has been whether or not the unusual nature of the transgene reflects the normal mutagenesis process in animals. Information to date suggests that the spectra of mutation induced in the *lacI* transgene and the studied endogenous genes are very similar (Kohler et al., 1991; Provost and Short, 1994). Here we have extended this issue by studying DNA damage induced by UV and BPDE and its subsequent repair in R2λLIZ rat cells using QPCR.

QPCR is a powerful tool for measuring chromosomal damage, as a single UV lesion is sufficient to prevent amplification (Govan et al., 1990). Recently, a QPCR for extra long DNA fragments (10-20 kb) was developed (Cheng et al., 1994; Yakes et al., 1996) which enables the detection of DNA damage and repair progress produced *in vivo* by agents within physiological relevant range.

The formation of DNA lesions after treatment with UV and BPDE and the subsequent repair have been extensively studied (Hunting et al., 1991; Tang et al., 1992; Tung et al., 1996; Denissenko et al., 1998). Two models, a single-exponential (Govan et al., 1990) and a double-exponential model (McCarthy et al., 1996) have been proposed to estimate the formation of UV photoproduct. In our experiment, although the PCR yield decreased with increasing UV doses, a flat curve at low UV doses was observed, possibly due to the limit of the sensitivity or the induction of repair at low doses. As for DNA repair, cyclobutane pyrimidine dimers (CPDs) in the transcribed strand are preferentially repaired in mammalian cells while repair of BPDE adducts shows little gene-specific and strand-specific repair. This difference in repair patterns indicates that the nature of DNA adducts also affects the repair rate. In spite of considerable large standard deviation at some time points, our results indicate that the repair of DNA lesions in the transgene, produced either by UV or BPDE, is rapid and apparently complete. In both cases, the adducts were removed within 25 hours after the treatment.

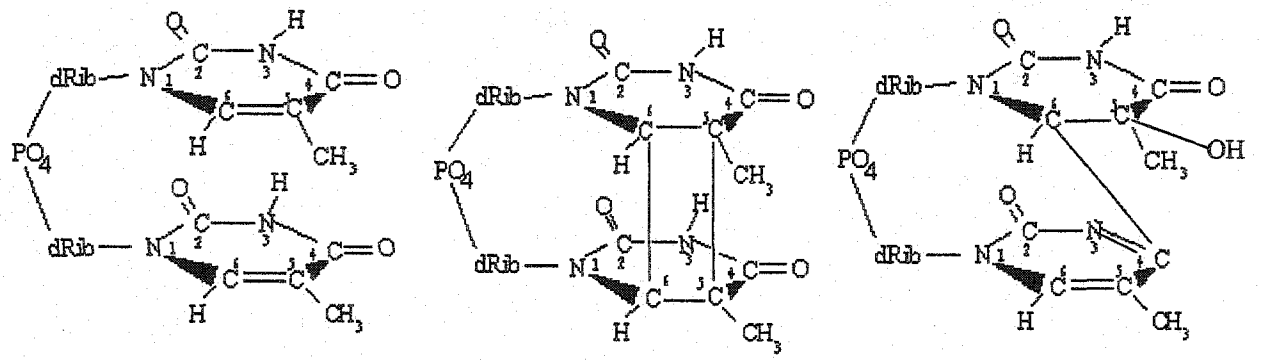
In a recent study of UV response of XP-C fibroblast cells, van Hoffen et al. (1995) reported that both CPDs and 6-4 photoproducts were preferentially repaired in active genes through TCR. This preferential repair was not observed at a high UV dose, in which TCR may be overruled by global repair due to severe inhibition of transcription. The repair rates of a transcribed gene and a nontranscribed gene were reported by McCarthy et al. (1996) using QPCR. The active tPA gene and the inactive hGH gene demonstrated similar repair kinetics in normal fibroblast cells even though they are expected to be repaired via different pathways. We found that UV photoproducts in the

nontranscribed λ insert induced at a low dose were completely repaired within 24 hours. This indicates that global genome repair is not intrinsically slow. In certain regions it can be as efficient as TCR, and this may depend on the chromatin organization and accessibility to DNA repair enzymes.

Chromatin structure is suspected to play an important role in modulating DNA repair. On the basis of ultrastructural analysis, genomic domains appear to include stretches of supercoiled DNA which in some cases have been observed to extend from a central scaffold. Bohr et al. (1986) proposed that such DNA loop domains were anchored on the nuclear matrix and that genes located in the middle of these loops may be more accessible to DNA repair enzymes. Active genes apparently packaged in altered nucleosome structures were in these less condensed genomic regions, which may be associated with their preferential repair to inactive genes. Moreover, Shanower and Kantor (1997) found that damage-free regions were created around the active genes as a result of TCR. These regions (~50kb) were much larger than the active genes, but correspond well with the estimated size of DNA loop domains (50~63kb). In the case of the λ insert, the lengthy 40-copies of λLIZ head-to-tail tandem repeats are attached to the chromosome core by flanking mouse sequences as seen during meiotic prophase, forming a 1.8 Mb loop domain (Heng et al., 1994), thus creating a unique nontranscribed region with similar structure of active genes. Assuming the λ insert forms such a special region which is more accessible to repair enzymes, the fast repair rate of UV photoproducts and BPDE-adducts may be explained. Finally, the fast repair of DNA adducts in the λ insert may be due to the low initial adduct level. DNA excision repair is constitutive in

character and the overall efficiency is inversely correlated with genotoxic load. The removal of BPDE adducts in our study is faster than found previously by Chen et al. (1992) who found a $t_{1/2}$ of 10.2 hours. However, our study used a lower concentration of BPDE, resulting in a lower level of adducts. This reduced level of adducts may not challenge the repair mechanisms as completely as in the study of Chen et al. (1992), allowing for the faster and more complete repair. Another approach regarding the role of nucleotide excision repair (NER) in mutagenesis in transgenic mice was reported by Frijhoff et al. (1998). $XPA^{-/-}$ *lacZ* transgenic mice, deficient in NER, have higher mutation frequency and adduct levels than $XPA^{+/+}$ *lacZ* mice. This also indicates that DNA adducts in the transgenes can be efficiently repaired by NER.

In summary, DNA damage induced by UV and BPDE and the subsequent repair in the λ insert can be detected by QPCR. The presence of 40 copies of target sequence in the R2λLIZ cell significantly reduces the amount of template required in the long-PCR reactions, and results in an improved signal to noise ratio. Our results indicate that the DNA adducts induced by these two agents in the λ construct in the R2λLIZ cells can be efficiently repaired, possibly by global nucleotide excision repair. This finding has potential implications for the understanding of mutational spectra recovered from the *lacI* transgenic rodent in mutagenesis studies.



Adjacent Thymine Residues Thymine Dimer 6-4 Photoproduct

Figure 6.1. Structures of adjacent thymines in DNA (left), cyclobutane thymine dimer (center) and 6-4 photoproduct (right); dRib=2'-deoxyribose.

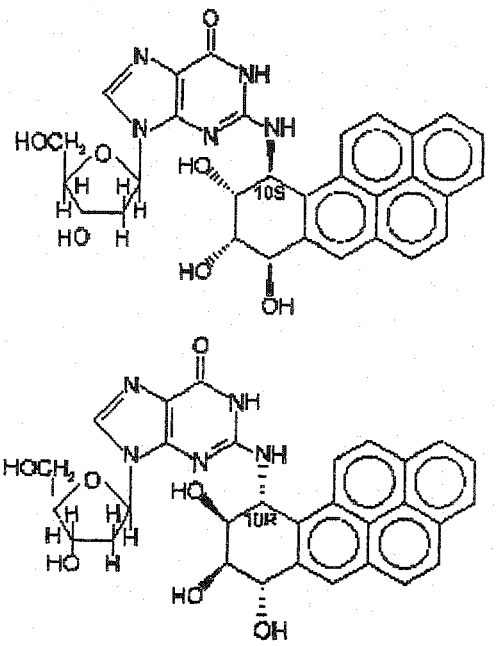
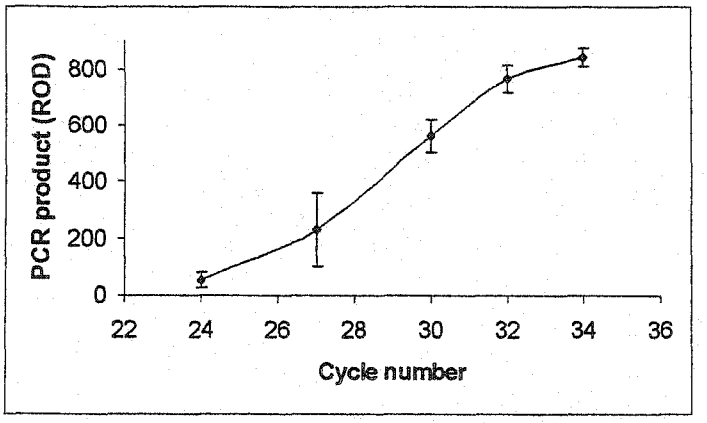


Figure 6.2. Structures of DNA adducts formed between BPDE and guanine

A



B

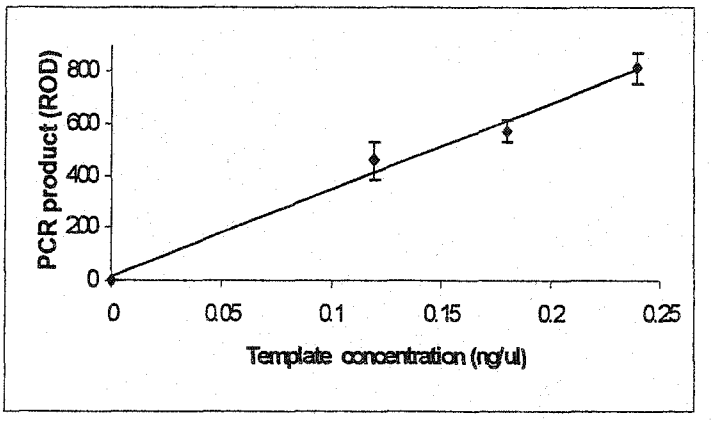
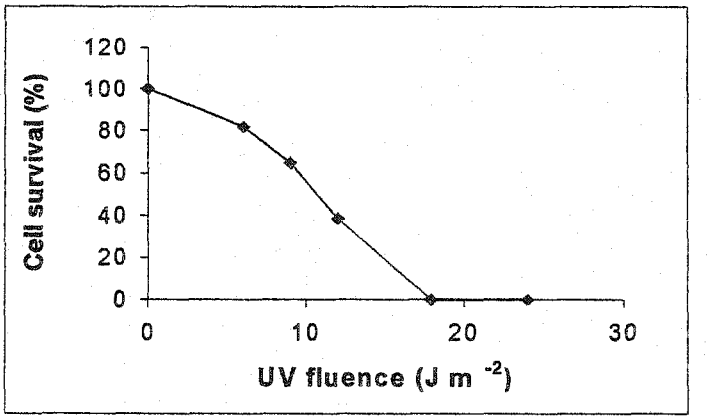


Figure 6.3. **A**. Determination of cycle number for exponential amplification of the 12.5 kb target fragment. Samples were removed at the completion of 24, 27, 30, 32 and 34 cycles respectively. **B**. Dependence of PCR on the template concentration. Linear increase of the PCR product was observed when template concentration is between 0-0.25 ng/μl. The amount of PCR product is represented by the band density in the agarose gel given in relative optical density (ROD) and expressed as mean ± SD from three independent PCR reactions.

A



B

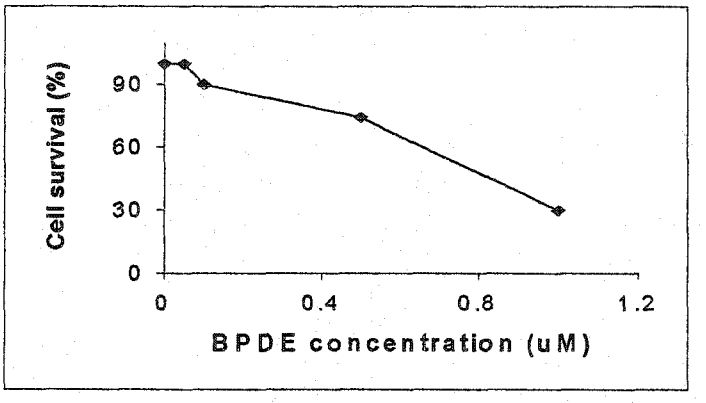
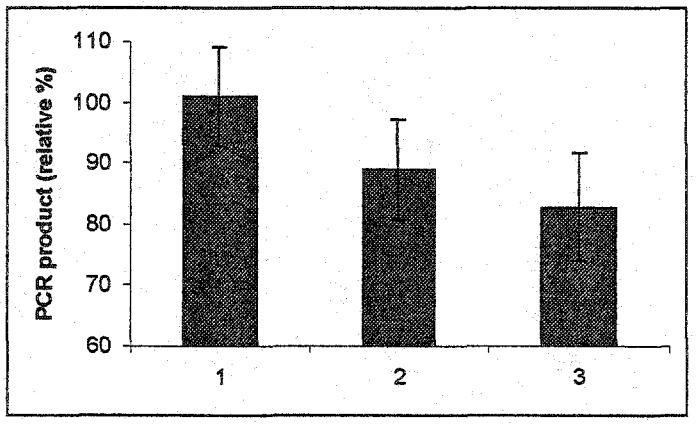


Figure 6.4. **A**. Survival of R2λLIZ cells after UV irradiation. **B**. Survival of R2λLIZ cells after BPDE treatment. Cell survival was measured by counting colonies 9 days after UV or BPDE exposure. Each point represents the mean of colonies from three plates.

A



B

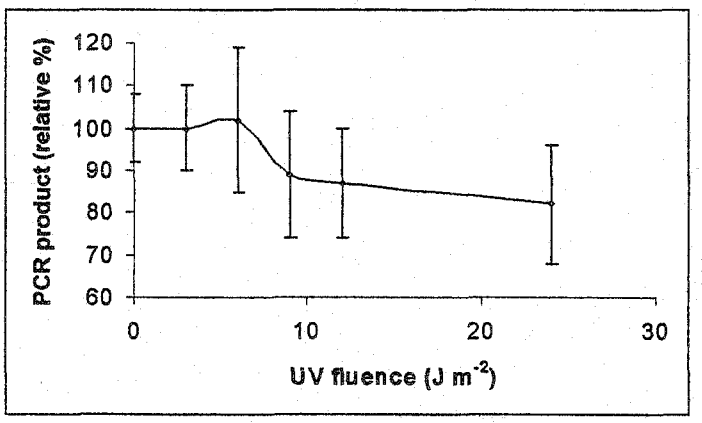


Figure 6.5. The effect of UV irradiation of cellular DNA on amplification of the 12.5 kb λ target fragment. The data is relative to the PCR product from an untreated control sample, normalized to 100%, and expressed as mean ± SD from three individual PCR reactions. **A**. Decrease of PCR yield as a function of increasing UV dose from 9 J m^{-2} to 24 J m^{-2}. 1-3 are samples treated with different doses of UV. 1: untreated control; 2: 9 J m^{-2}; 3: 24 J m^{-2}. The SD is ~8.1%. **B**. Induction of adducts with 3-24 J m^{-2} UV irradiation.

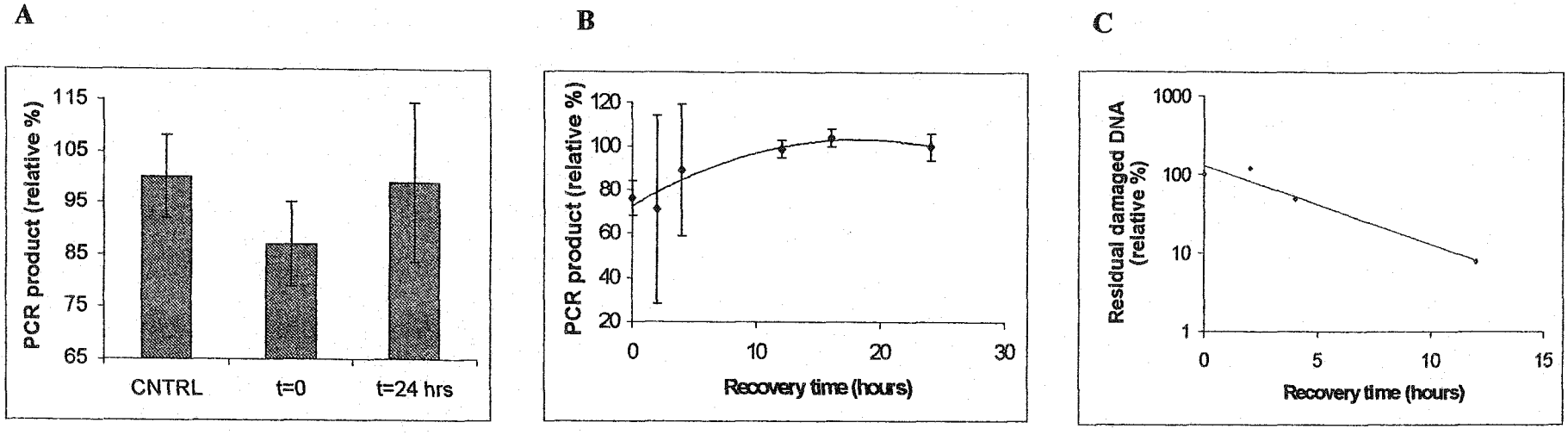
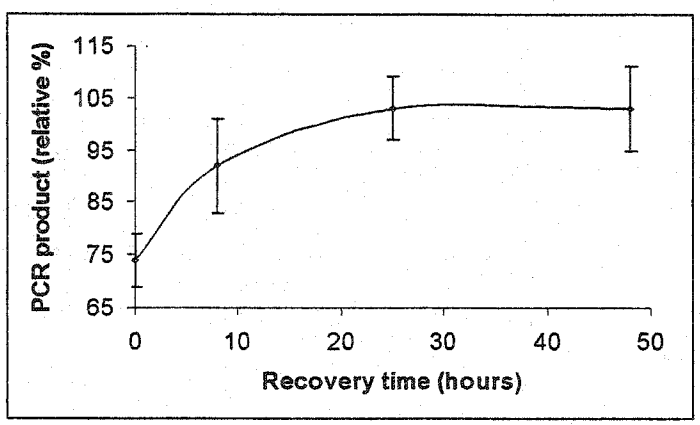


Figure 6.6. Repair of UV photoproducts in the 12.5 kb λ target fragment in the R2λLIZ cells. PCR product accumulation was measured as a function of incubation time following 9 J m^{-2} of 254 nm UV irradiation. **A**. The repair trend of UV adducts during post-incubation. **B**. Repair kinetic of UV photoproduct. All the data in A and B are relative to the PCR product from an untreated control sample, normalized to 100%, and expressed as mean ± SD from three individual PCR reactions. **C**. Regression analysis of the repair kinetics. The relative PCR product at each time point was recalculated as the fraction of residual damaged (unamplifiable) DNA.

A



B

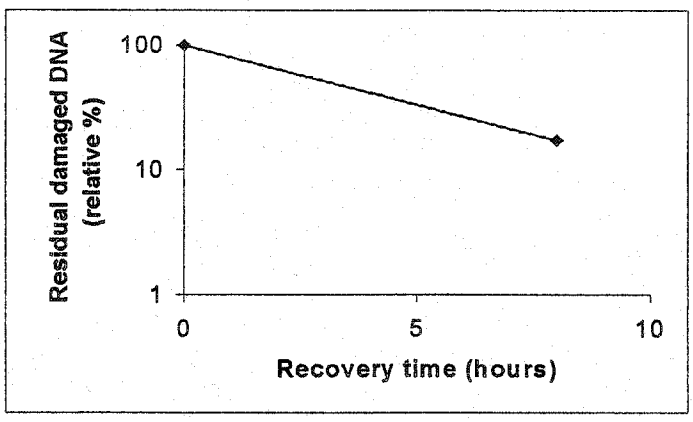


Figure 6.7. Repair kinetics of BPDE adducts in the 12.5 kb λ target fragment in the R2λLIZ cells. **A**. R2λLIZ cells were treated with 0.5 μM BPDE for 30 minutes and incubated for the indicated time for repair. The data is relative to the PCR product from an untreated control sample, normalized to 100%, and expressed as mean ± SD from three individual PCR reactions. **B**. Regression analysis of BPDE-DNA adduct repair. The relative PCR product at each time point was recalculated as the fraction of residual damaged (unamplifiable) DNA.

Chapter 7. Mutagenicity of CLA in Mismatch Repair Deficient and Proficient Bacteria Cells

Abstract
The effect of conjugated linoleic acid on the formation of mutations in mismatch repair proficient (NR9102) and deficient (*mutS* and *mutL*) *Escherichia coli* strains was investigated using the rifampicin mutagenesis assay. At the concentrations tested (10 mM and 25 mM), CLA inhibited the growth of all the test strains. In the mismatch repair proficient cell line, CLA treatment caused a dose-dependent increase of rifampicin-resistant mutants, while having no apparent effect in the mismatch repair deficient cell lines. It appears that CLA may interfere with the mismatch repair pathway although further investigation is necessary.

7.1. Introduction

Antimutagens can be classified into desmutagens and bio-antimutagens based on their antimutagenic properties (Kuroda and Inoue, 1988). Desmutagens refer to those which cause chemical and biochemical modifications of mutagens before they can produce DNA damage, while bio-antimutagens modulate cellular processes of mutation fixation such as DNA repair and replication (Kada et al., 1986a; Kada et al., 1986b). Bio-antimutagenic activities can be achieved by: (1) increase of fidelity of DNA replication; (2) stimulation of error-free repair of DNA damage; and (3) inhibition of error-prone repair systems (Simic et al., 1998). It is also possible for an agent to appear as a bio-animutagen if it results in the preferential loss of damaged cells from a population.

Conjugated linoleic acid (CLA) was first identified as an antimutagen from ground beef when it was observed to inhibit chemically induced mutagenesis catalyzed by an S9 liver extract from untreated rats but not from rats treated with phenobarbital or

Aroclor 1254 (Pariza et al., 1979). This property has been proposed to relate to its inhibition of the activation of (pro-)mutagens by affecting cytochrome P450 enzymes or prostaglandin H synthase, although direct evidence has not yet been obtained (Pariza et al., 1983; Liew et al., 1995; Josyula et al., 1998). Weak mutagen-trapping properties of CLA have been observed (including electrophile scavenging and anti-oxidant properties) of CLA have been observed, but this is thought unlikely to account for CLA's anitmutagenic effect (Liew et al., 1995). When the antimutagenic effect of CLA was examined *in vivo* in the *lacI* transgenic rats, we observed that CLA changed the mutational spectra in the prostate in a pattern reminiscent of the enhancement of mismatch repair (Yang et al., submitted). It therefore seemed appropriate to explore the possible interference of CLA with mismatch repair.

We thus chose to investigate this question in *Escherichia coli* where wild type and various repair deficient isogenic strains are available to detect bio-antimutagens and elucidate their mechanisms (Simic et al., 1998). Specifically, we wished to study the influence of CLA on *E. coli* strains that were proficient or deficient in mismatch repair.

7.2. Materials and methods

7.2.1. Bacterial strains

All the bacterial strains (Table 7.1) were generous gifts from Dr. Roel M. Schaaper of the National Institute of Environmental Health Sciences. NR9102 has been described by Schaaper and Dunn (1991). The mismatch repair deficient strains were

NR3996 (*mutS*101), NR9562 (*mut*L::Tn*5*) and NR9322 (*mut*S::Tn*5*), among which NR9562 and NR9322 are derivatives of NR9102.

7.2.2. Chemicals

Rifampicin (approximately 95% HPLC pure) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (Oakville, Ontario). CLA (>94% pure; the composition of CLA is: ~41% *cis*-9,*trans*-11 CLA, ~44% *trans*-10,*cis*-12 CLA, ~10% *cis*-10,12 CLA, ~5% *cis*-9,12 linoleate) was obtained from Nu-Chek-Prep (Elysian, MN).

7.2.3. Mutagenesis assay

LB broth and glucose minimal medium plates containing Vogel-Bonner salts (MM plates) were prepared as described by Schaaper et al. (1986). Rifampicin selective plates are MM plates containing 100 μg/ml rifampicin (MM-Rif plates). CLA was dissolved in DMSO at a concentration of 1 M. The mutagenesis assay was performed as described by Miller (1992). Briefly, an overnight cell culture of each test strain was incubated with CLA at the indicated concentrations at 37°C with agitation for 30 minutes. An appropriate aliquot (100 μl of 10^{-5} – 10^{-6} dilution) of the cell culture of each strain was plated on MM plates and incubated at 37°C overnight. The colonies formed were counted to determine the effect of CLA on the survival of each test strain. New overnight cultures were generated by diluting each CLA-treated sample 1:20 in LB medium and incubating at 37°C with agitation. Aliquots of appropriate dilutions of the overnight cultures were spread on MM plates (non-selective, 100 μl of 10^{-5} – 10^{-6} dilution) and MM-Rif plates (selective, 100 μl of 10^{0} – 10^{-1} dilution) to determine the total cell counts

and mutants, respectively. Mutant frequencies were calculated by dividing the average number of mutants per culture by the average number of total cells per culture.

7.3. Results and discussion

The role of DNA repair in maintaining genetic integrity has been documented numerous times. A high conservation of several DNA repair genes in mammals, lower eukaryotes and bacteria is reported in an editorial on DNA repair and mutagenesis (Friedberg et al., 1995). Moreover, there is direct evidence that defective mismatch repair and nucleotide excision repair contribute to particular types of cancer (Kraemer et al., 1994; Wei et al., 1995; Peltomaki, 2001). In the current study, we used a bacterial system to examine whether the potential chemopreventive agent CLA affects the mismatch repair pathway, since our *in vivo* data showed that the alteration of *lacI* mutational spectra in the prostate by CLA matches the function of a mismatch repair gene MSH2 (Yang et al., submitted).

We first determined the toxicity of CLA on the test strains. Cell viability was determined after growth in different concentrations of CLA. All of the tested cell lines were inhibited by CLA in a dose-dependent manner (Figure 7.1).

The mutagenicity of CLA in both mismatch-repair proficient and deficient (*mutL* and *mutS*) strains was determined using the rifampicin resistant assay. Rifampicin specifically inhibits bacterial RNA polymerase, the enzyme responsible for DNA transcription, by forming a stable drug-enzyme complex. Mutations leading to a change in the structure of the beta subunit of RNA polymerase would result in bacterial resistance to rifampicin and can be identified in rifampicin-containing selective plates.

Two concentrations of CLA were tested (10 and 25 mM) and the data are presented in Table 7.2. The spontaneous frequency of rifampicin-resistant (Rif^r) mutants in the wild type strain, NR9102, was 18.2×10^{-9}, which is consistent with the reported spontaneous mutant frequency of NR9102 (Schaaper, 1988; Schaaper, 1993). A linear dose-dependent increase of mutant frequency was observed with increasing CLA concentrations. Incubating with 10 and 25 mM CLA resulted in a mutant frequency of 35.4×10^{-9} and 67.7×10^{-9}, respectively.

The spontaneous mutant frequencies in the mismatch-repair deficient *mutL* and *mutS* strains were 50- to 150- fold higher than that of the wild type. However, in contrast to the wild type strain, increasing CLA concentration did not induce extra mutants in either the *mutL* or *mutS* strains. The absence of an effect of CLA on those strains may be explained in two ways. Firstly, the increase in mutant frequency in the wild type cells may reflect an impairment of mismatch repair by CLA. If this is correct, then it follows that strains lacking mismatch repair capacity will not demonstrate this enhanced mutant frequency in the presence of CLA. Alternatively, the spontaneous background in the mismatch repair deficient strains may hide the modest effect of CLA. Indeed, if CLA increases the formation of mutants in the presence of an active mismatch repair system, it would be an effect opposite to that observed in the prostate of the rats, in which it decreases the mutational frequencies in the animals tissue, in particular the frequencies of G:C→A:T transitions and –1 frameshifts. The change in the Rif^r mutants has not been characterized. However, long-chain fatty acids have been found to interact differently with mammalian DNA polymerases and prokaryotic DNA polymerases. Linoleic acid,

the isomer of CLA, can bind to mammalian DNA polymerases and subsequently interfere with the binding of a template-primer DNA to the polymerase (Mizushina et al., 2000). Linoleic acid was found to inhibit the activities of mammalian DNA polymerase α and β *in vitro*, and less potently, plant DNA polymerases, while hardly influencing the activities of prokaryotic DNA polymerases (Mizushina et al., 1996; Mizushina et al., 1998). This observation raises the possibility that the activities of CLA in the mismatch repair pathways may not be consistent between bacteria and animals.

In conclusion, we have shown that CLA increased the formation of Rif^r mutants in a mismatch repair proficient *E. coli* strain, but not in mismatch repair deficient strains. The degree to which these observations relate to a direct effect of CLA on mismatch repair pathway needs to be further clarified.

Acknowledgements

I would like to thank Dr. Roel M. Schaaper of the National Institute of Environmental Health Sciences, for providing the four *E. coli* strains. I also appreciate the assistance of Virginia Haslett in this experiment.

Table 7.1. Bacterial strains used in this study.

Strain	Relevant marker
NR9102	ara, thi, Dprolac, F'prolac(F'128-27)
NR9562	ara, thi, Dprolac, F'prolac(F'128-27), mutL::Tn5
NR9322	ara, thi, Dprolac, F'prolac(F'128-27), mutS::Tn5
NR3996	ara, thi, Dprolac, F'prolac, mutS101

Table 7.2. Mutant frequencies (per 10^9 cells) of CLA in mismatch repair proficient and deficient *E. coli* strains.

CLA concentration (mM)	NR9102	NR9562	NR9322	NR3996
0	18.2	2700	2450	930
10	35.4	2290	1900	977
25	67.7	2760	2560	854

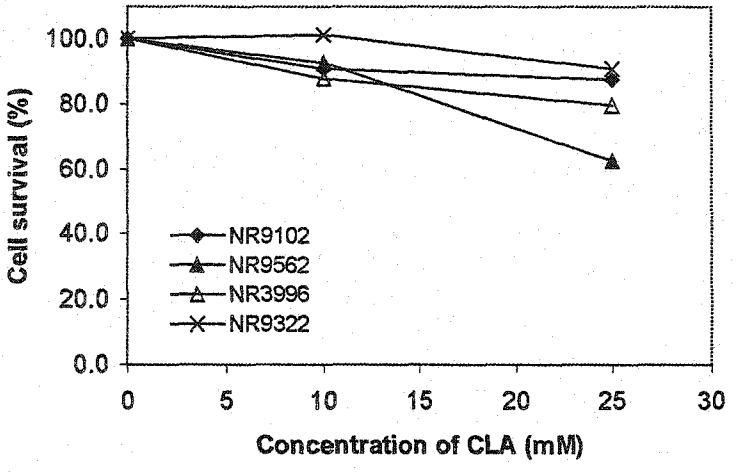


Figure 7.1. Effect of CLA on the survival of mismatch repair proficient and deficient cell strains. A dose-dependent inhibition of cell survival was observed in all the strains tested.

Chapter 8. General Discussion

The chemical induction of mutation in animal organs and tissues is a process that generally involves metabolic activation of parent compounds in the liver, distribution of activated metabolites, uptake and often further local metabolism by target cells, DNA adduct formation, and finally DNA repair and replication processes that either eliminate or fix the lesion as a mutation. For a variety of reasons, mutagenicity of a chemical in a transgenic animal assay has been proposed as a reasonable estimation of its carcinogenicity. Conversely, it is thought that decreasing the rate of mutation accumulation corresponds to a reduction in cancer risk (Liviero and von Borstel, 1996). This model provides the essential intellectual framework for chemoprevention studies. This dissertation is concerned with determining the distribution and specificity of mutations in Big Blue® transgenic rats after exposure to PhIP and how conjugated linoleic acid (CLA) modulates PhIP-induced mutagenicity in a tissue-, sex-, and dose-related manner.

8.1. Mutagenicity of PhIP

Various endpoints have been used to predict the carcinogenic risk to mutagens and carcinogens (Hasegawa and Ito, 1992; Poulsen and Loft, 1995; Schulte, 1995; Mitchelmore and Chipman, 1998). A common method for heterocyclic amines (HCAs) is the measurement of HCA-DNA adducts (Turteltaub et al., 1997; Lightfoot et al., 2000). However, from the data collected it appears that the HCA-DNA adduct level does not provide a reliable estimate of cancer risk (Nagao et al., 2001). It therefore becomes

important to develop a better biomarker for the evaluation of cancer risk related to HCA exposure. In this study, the appearance of mutations, a process that incorporates both the formation of DNA adducts and their repair, as well as cell proliferation, was chosen as the endpoint to evaluate the risk to tumor-induction by PhIP.

The induction of tumors by PhIP treatment occurs in the general order: colon (male) = mammary gland (female) > prostate (Shirai et al., 1995; Shirai et al., 1999). Tumors in other tissues are rarely detected (Hasegawa et al., 1994). Consequently, it is these three tissues that are suspected mutation target tissues for PhIP. Indeed, elevated mutation frequencies (MFs) have been observed in the rodent colon (Chapter 2 and 3), prostate (Chapter 4), and mammary gland upon dietary exposure to 100 ppm PhIP, being $40 \pm 6 \times 10^{-5}$, $15 \pm 3.2 \times 10^{-5}$, and $11 \pm 2.8 \times 10^{-5}$ (unpublished data), respectively. As an example of a non-target tissue of PhIP, a relatively small induction of mutation was observed in the kidney of male ($7.7 \pm 0.3 \times 10^{-5}$) and female rats ($4.7 \pm 1.0 \times 10^{-5}$, Chapter 5), whereas no evidence of mutation induction was observed in either the skin (3.9×10^{-5}, unpublished data) or liver (4.4×10^{-5}, unpublished data). These tissue-specific mutational responses differ from the tissue-specific formation of PhIP-DNA adducts, in which similar or even higher levels were observed in the kidney and prostate than in the colon (Takayama et al., 1989; Friesen et al., 1996; Purewal et al., 2000). It thus appears that the PhIP-induced MF rather than DNA adduct levels better reflects the susceptibility of various tissues to PhIP-induced carcinogenicity. We have, however, observed an exception in the colon of female rats and in the cecum. PhIP does not induce tumors in the colon of female rats nor in the cecum of either sex, whereas a significant increase of

MF after exposure to PhIP was seen in both tissues. As shown in Chapter 2 and 3, exposure to 200 ppm PhIP for 61 days or to 100 ppm PhIP for 47 days resulted in a MF of $36.9 \pm 3.7 \times 10^{-5}$ in the colon of female rats and $20.1 \pm 2.3 \times 10^{-5}$ in the cecum, respectively.

Besides the tissue-specific mutational response, the dose- and sex-responses of Big Blue® rats to PhIP were also examined. Two doses of PhIP were tested (total PhIP intake was 0.071 g and 0.181 g, respectively) and tissue-specific dose-response curves were obtained. PhIP-induced mutations were found to accumulate linearly in the distal part of the colon (DC) and cecum, but exponentially in the prostate. The existence of sex differences in PhIP-induced mutations was examined in the cecum, colon, and kidney. Only in the kidney was a sex difference observed. PhIP induced a 3-fold increase of MF in male rats and a 0.6-fold increase of MF in female rats. We did not find a difference in the induced MF and MS in the cecum and colon between male and female animals, a finding in concurrence with Okonogi et al. (1997a). Masumura et al. (1999) also failed to detect any significant difference in PhIP-induced MF in the colon of male and female mice, using the *gpt* Δ transgenic mouse. These findings indicate that the critical steps in male-specific colon tumor development in rats take place after initiation. Our data, taken together with the carcinogenesis data from others, tend to suggest that male rats are more susceptible to PhIP-induced carcinogenesis and mutagenesis. More information such as a sex difference in adduct level and mutagenicity of PhIP in other non-target tissues is needed before we can understand the basis for this conclusion.

Mutation spectra (MS) were analyzed in order to better understand the mutational mechanisms involved. Significant alterations in the MS after PhIP treatment were seen in the cecum, colon (divided into proximal and distal sections, PC and DC), and prostate as compared with the background MS. In the kidney, due to the small number of independent mutants acquired from the control animals, a statistical comparison was not practical. The PhIP-induced MS from the colon, cecum, prostate and kidney share several characteristics: (1) The spectra consist mainly of point mutations, with G:C→T:A transversions and –1 frameshifts predominating although their proportion varies among tissues; (2) More than 60% of the induced mutations occurred at G:C base pairs, consistent with the observation that PhIP-DNA adducts are primarily formed at G:C base pairs; (3) A number of positions are mutational hotspots in more than one tissue, including nucleotide (nt) position 90-92, nt 790, and nt 877, each of which have been identified as hotspots in other PhIP studies (Okonogi et al., 1997a; Okonogi et al., 1997b; Okochi et al., 1999). In addition, the sequence context of substitutions at guanine residues showed a preference of G, C>T>A as the 5' flanking base, similar to the observed context preference of substitutions resulting from dG-C8-PhIP in *in vitro* study (Shibutani et al., 1999); and (4) The mutational fingerprints of PhIP, G:C deletions at 5'-GGGA-3' sequences, were recovered in all these tissues in a range of 3-10%. The observed sequence specificity is consistent with mutations induced *in vitro* by the PhIP-DNA adduct, dG-C8-PhIP, and the PhIP-induced spectra from other *in vivo* studies (Okonogi et al., 1997a; Okonogi et al., 1997b; Okochi et al., 1999).

Despite these similarities, statistical comparisons of the PhIP-induced MS from different tissues by Monte Carlo analysis indicate that most of these spectra differ significantly from each other, with the exception of the spectra from colon and cecum. Compared with the PhIP-induced MS from the colon and cecum, the PhIP-induced spectrum in the prostate has a significantly higher proportion of –1 frameshifts, mostly involving single G:C base pairs. This heightened fraction of –1 frameshifts was also noted in the prostate background mutation spectrum. In the kidney, -A:T base frameshifts are more common following PhIP treatment than in other tissues.

8.2. Modulation of mutation by CLA

The mitigating effect of CLA against PhIP (200 ppm) exposure was initially investigated at a concentration of 0.5%. A reduction of PhIP-induced mutations was observed in the distal colon. In PC, CLA did not alter the frequency of PhIP-induced mutations. However, CLA unexpectedly increased the PhIP-induced mutation frequency in the cecum. A subsequent experiment using 1% CLA confirmed that CLA inhibited the PhIP-induced MF DC, but in this experiment CLA had no effect on mutation levels in the cecum. Inhibition of PhIP-induced mutation by CLA appears to be dose-dependent since the PhIP-induced MF was reduced by 14% and 25% in DC when CLA was given at 0.5% and 1%, respectively. The biological significance of the suppression was confirmed by the absence of PhIP-induced ACF in DC with CLA addition. However, the discrepancy between the effects of two doses of CLA on PhIP-induced mutations in the cecum remains to be clarified.

CLA was also found to inhibit mutation induction in the prostate in which the mutation induction was reduced by 38%. CLA showed its greatest effect in the kidney of female rats where the PhIP-induced MF was brought back to the level of control animals, while in the male rats no effect of CLA on PhIP-induced mutagenesis was detected. The same female specific protection was seen with TCDD against AFB_1 mutagenesis in the liver (Thornton, in preparation).

The effect of CLA on PhIP-induced spectra was determined by comparisons of the MS recovered from PhIP and PhIP + CLA treatment. With the exception of the prostate, CLA treatment does not significantly alter the types of mutations recovered in the tissues examined, when analyzed by the Monte Carlo method recommended by Adams and Skopek (1987). In the prostate, the frequencies of G:C→A:T transitions and −1 frameshifts were selectively decreased by the addition of CLA. Interestingly, this reduction in the frequencies of these two classes of mutations by CLA is opposite to the effect of the loss of function of the mismatch repair (MMR) gene MSH2 on spontaneous and PhIP-induced mutagenesis (Zhang et al., in press) as determined in an MSH2 knock-out *lacI* transgenic mouse model. The similar effect of CLA and MMR suggests that the antimutagenic effect of CLA in the prostate is consistent with an enhancement of MMR. Analysis of the context sequence of substitutions at G:C base pairs in DC indicates that CLA preferentially inhibited mutations at 5'-G<u>G</u>-3' (<u>G</u> is the mutated base). This sequence-specific inhibition was not observed in the cecum. The sequence-related inhibitory effect further points out the possible involvement of a DNA repair pathway in the modulation effect of CLA, since the repair of DNA adducts is sequence-dependent.

The possibility of an effect on MMR was examined in mismatch repair proficient and deficient *E. coli* cell lines (Chapter 7). CLA was found to increase the formation of mutants in repair-proficient, but not in repair-deficient cells. To further understand the modifying mechanism of CLA, the antimutagenic effect of 0.5% CLA was compared with that of DTT (Chapter 2). The addition of DTT to the diet decreased the PhIP-induced MF in DC but had no effect on PC and cecum, suggesting that CLA modifies mutagenesis via pathways different from DTT. The chemopreventive mechanism of DTT has been attributed to the induction of an array of phase II enzymes, particularly glutathione transferase, which would increase the detoxification of activated metabolites (Clapper et al., 1994; Begleiter et al., 1997). Therefore, the antimutagenic effect of CLA might not be associated with the induction of phase II enzymes, and indeed, CLA has been shown not to induce phase II enzymes (Martin et al., 2000).

8.3. Using the transgenic rodent model as a screening tool for antimutagens

The Big Blue® *lacI* transgenic assay is a classified mutation-based assay because the endpoint of this assay is the direct measurement of mutation. In the past several years, mutation-based transgenic animal models have been extended to include *lacZ*, *cII*, lambda/*spi* and *E. coli gpt* gene as inserted reporter genes (Lynch et al., 1996; Masumura et al., 1999; Stuart et al., 2000b). The development of mutation-based transgenic models provides a unique opportunity to study mutations *in vivo*. These models have been extensively applied to determining the mutagenicity of different chemicals. This approach has provided *in vivo* mutation data in a wide range of highly relevant tissues

(Walker et al., 1996; Bol et al., 2000). Common environmental carcinogens such as aflatoxin B_1 and B(*a*)P have been found to be strongly mutagenic, especially in their carcinogenic target tissues (Dycaico et al., 1996; Shane et al., 2000). Therefore, induced mutational data acquired from transgenic assays is predictive for the carcinogenicity of the component of interest. However, due to the rather complicated relationship between mutation induction and cancer occurrence, a direct extrapolation of mutation induction to cancer is not always appropriate. In general, exposure to a mutagenic carcinogen results in an increased MF of one or more types of mutations in its tumor target tissues (Sills et al., 2001; Nishikawa et al., 2001), but induction of these changes is not always limited to target tissues (Cruz-Munoz et al., 2000). Additional factors, including induced cell proliferation, determine whether an increased MF will result in tumor formation (de Boer et al., 1996)

In this study, a transgenic mutagenesis assay was used for the identification and evaluation of chemopreventive agents and their efficacies. Within the current model involving mutation as the "initiating" event in cancer, the use of these transgenic animal models may have practical and scientific merit. The application of *in vitro* assays, e.g. the Ames *Salmonella* test, for identification of antimutagens is attenuated by the fact that bacteria lack many metabolic enzyme pathways present in mammalian cells and can only detect certain antimutagenic mechanisms such as inactivation of mutagens by direct binding (Waters et al., 1996; Schwab et al., 2000). Compounds that influence the activation or detoxification of mutagens through affecting phase I or phase II enzymes can not be detected. At the same time, evidence shows that a chemical can act as a

carcinogen or anticarcinogen depending on cell or tissue type, or as a function of exposure dose (van Borstel and Higgins, 1998). For example, in female F344 rats, caffeine inhibited the induction of mammary tumors by PhIP, while enhancing its carcinogenicity in the colon, possibly due to the tissue specific induction of P450 enzymes (Hagiwara et al., 1999). In this sense, transgenic mutagenesis assays represent far more practical models than *in vitro* tests for identifying chemopreventive agents.

At least two strategies can be employed that use transgenic animal models to the investigation of antimutagens. One approach is to examine the effects of the potential agents on spontaneous mutation. This approach suffers from the problem that the effect must be substantial in order to be detected and that it is probably limited to the detection of antioxidants, as oxidative damage represents the largest subset of preventable mutations that are recovered as background events (Poulsen et al., 1998; Jackson and Loeb, 2001). Potential antioxidants can be further confirmed if the frequencies of G:C→T:A and G:C→C:G transversions, mutations induced by oxidative DNA damage (Jackson and Loeb, 2001), are specifically inhibited. The second approach involves the screening of potential protective agents in studies of chemically induced mutation, and allows the identification of compounds that affect the metabolism of mutagens, enhance DNA repair, either directly or by delaying DNA replication, or inhibit cell proliferation.

8.4. A possible follow-up on this project

The experiments reported in this thesis demonstrate the potential value of Big Blue® transgenic mutagenesis assay in identifying chemopreventive compounds and

elucidating their mechanism(s) of action. In order to increase the value of this system, more biological endpoints should be examined. Endpoints could include DNA adduct levels, cell proliferation, gene expression profiling, and comparative protein profiling. Such an integrated approach will provide a powerful new tool to the study of chemoprevention.

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