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Efficacy of 9-cis retinoic acid (9-cis-RA) in preventing methylnitrosourea (MNU)-induced mammary cancers.

Mark Bradley Cope
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EFFICACY OF 9-CIS RETINOIC ACID (9-CIS-RA) IN PREVENTING METHYLNITROSOUREA (MNU)-INDUCED MAMMARY CANCERS

by

MARK BRADLEY COPE

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

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2001

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Program Nutrition Sciences

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Title Efficacy of 9-cis Retinoic Acid (9-cis-RA) In Preventing Methylnitrosourea (MNU)-Induced Mammary Cancers

Chemopreventive agents inhibit the process of carcinogenesis. 9-cis Retinoic Acid (9-cis-RA) is known to be an effective inhibitor of cancer formation in the mammary gland. The biochemical mechanism(s) involved, however, are not understood. At the beginning of this effort, it was unclear how 9-cis-RA, at various dose levels or in combination with other chemopreventive agents, affected cancer incidence in the MNU-induced mammary cancer model.

In an initial study, conducted with animals that received a range of 9-cis-RA dose levels, a dose-dependent response was found within the groups. Another study demonstrated that, to achieve a strong chemopreventive effect, 9-cis-RA had to be administered continuously.

To investigate the effects of 9-cis-RA combined with N-(4-hydroxyphenyl)-retinamide (4-HPR), younger animals were administered agents in the diet, starting at 53 days of age. The combination caused significant reductions in cancer incidence and number per animal. Older animals (administered agents in the diet at 93 days of age) showed an even greater effect when treated with the combination of 4-HPR and 9-cis-RA.

Another study was designed to determine if the combination of vitamin D3 with 9-cis-RA would inhibit mammary carcinogenesis in young rats. The two groups of animals fed vitamin D3 alone had no reductions in mammary cancer compared to controls. When combined with 9-cis-RA, however, both dose levels of vitamin D3 inhibited mammary cancer to a greater extent than 9-cis-RA alone.

A final study was conducted in an attempt to understand the mechanism(s) in which 9-cis-RA may decrease mammary cancers. The diets of animals were supplemented with 9-cis-RA for either 3 days or 4 weeks, and various parameters were evaluated. Mammary epithelial cells were isolated from the animals, and the total RNA was extracted. mRNA expression for the retinoid nuclear receptor, retinoic acid receptor β (RARβ), was measured in the epithelial cells; no differences in expression were found among the groups. Morphologic evaluation of the mammary glands showed no increased differentiation in rats treated with 9-cis-RA.

In conclusion, these studies showed that 9-cis-RA, at various dose levels, alone and in combination with other chemopreventive agents strongly inhibited MNU-induced mammary cancer. Additional studies are needed to determine the mechanism(s) involved in this effect.

DEDICATION

Dedicated to my grandparents, parents, brothers, sister, and loving wife who have all played valuable roles in allowing me to be where I am today.

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From day one, Dr. Clinton Grubbs has been here with his tremendous knowledge and continuous kindness. I would not be where I am today without his dedication to his work. He invested many hours of his time in me and his guidance and friendship, I will always cherish. Thank you, Dr. Grubbs.

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TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

LIST OF TABLES viii

LIST OF FIGURES x

LIST OF ABBREVIATIONS xiii

INTRODUCTION 1

REVIEW OF LITERATURE 3

- Carcinogenesis 3
- MNU-Induced Mammary Cancer Model 5
- Chemoprevention 8
- Retinoids 8
- Retinoid Receptors in Cancer 12
- 9-cis-RA 13
- N-(4-Hydroxyphenyl)retinamide 15
- Vitamin D3 17

HYPOTHESIS AND SPECIFIC AIMS 19

- Hypothesis 19
- Specific Aims 19

MATERIALS AND METHODS 21

- General Animal Care and Treatment 21
- Necropsy Tissue Collection 22
  - Tumors and major organs 22
  - Blood samples 22
  - Mammary gland wholemounts 23

TABLE OF CONTENTS (Continued)

Page

Experimental Studies 24
Experiment 1 24
Experiment 2 24
Experiment 3 26
Experiment 4 26
Experiment 5 27
Experiment 6 29
Isolation of mammary epithelial cells 30
RNA extraction and Northern blot analysis 31
Statistical Considerations 33
Statistical power 33
Statistical analysis 33

RESULTS 35

Experiment 1 35
Experiment 2 39
Experiment 3 42
Experiment 4 46
Experiment 5 49
Experiment 6 53

DISCUSSION 104

LIST OF REFERENCES 117

APPENDIX

A INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE DOCUMENTATION 134

B UNIVERSAL NUMBERING SYSTEM FOR THE INDENTIFICATION OF RODENTS 137

LIST OF TABLES

Table		Page
1	Experiment 1 Protocol for Determining the Effect of Various Doses of 9-cis-RA on MNU-Induced Mammary Cancers Prior to Initiation and Promotion in Older Rats	25
2	Experiment 2 Protocol for Determining the Effect of Limited and Continuous Treatment With 9-cis-RA on MNU-Induced Mammary Cancers in Young Rats	25
3	Experiment 3 Protocol for Determining the Effect of 9-cis-RA Alone and Combined With 4-HPR on MNU-Induced Mammary Cancers in Young Rats	27
4	Experiment 4 Protocol for Determining the Effect of 9-cis-RA Alone and Combined With 4-HPR on MNU-Induced Mammary Cancers in Older Rats	28
5	Experiment 5 Protocol for Determining the Effect of 9-cis-RA Alone and Combined With Vitamin D3 on MNU-Induced Mammary Cancers in Young Rats	29
6	Experiment 6 Protocol for Determining the Effects of 9-cis-RA on Mammary Gland Morphology and Levels of RARβ mRNA in Isolated Mammary Epithelial Cells	30
7	Effect of 9-cis-RA at 60 and 150 mg/kg diet on Body, Liver, and Uterus Weights	36
8	Effect of 9-cis-RA at Various Dose Levels on MNU-Induced Mammary Cancers in Older Sprague-Dawley Rats	37
9	Effect of 9-cis-RA on Organ Weights in Young Sprague-Dawley Rats After 20 Weeks of Treatment	40
10	Effect of Limited and Continuous Treatment With 9-cis-RA on MNU-Induced Mammary Cancers in Young Rats	41
11	Effect of 9-cis-RA Alone and Combined With 4-HPR on Organ Weights in Young Sprague-Dawley Rats	43
12	Effect of 9-cis-RA Alone and Combined With 4-HPR on the Percent Incidence and Number of MNU-Induced Mammary Cancers in Young Sprague-Dawley Rats	45

LIST OF TABLES (Continued)

Table — Page

13 Effect of 9-cis-RA Alone and Combined With 4-HPR on Organ Weights in Older Sprague-Dawley Rats 47

14 Effect of 9-cis-RA Alone and Combined With 4-HPR on the Percent Incidence and Number of MNU-Induced Mammary Cancers in Older Sprague-Dawley Rats .. 48

15 Effect of 9-cis-RA Alone and Combined With Vitamin D3 on Organ Weights in Young Sprague-Dawley Rats 51

16 Effect of 9-cis-RA Alone and Combined With Vitamin D3 on the Percent Incidence and Number of MNU-Induced Mammary Cancers in Young Sprague-Dawley Rats 52

17 Effect of 3 Days of Treatment With 9-cis-RA on Body and Liver Weights of Sprague-Dawley Rats 54

18 Effect of 4 Weeks of Treatment With 9-cis-RA on Organ Weights of Sprague-Dawley Rats 55

19 Average Levels of Retinyl Palmitate in Livers of 50-Day-Old Female Sprague-Dawley Rats After a 3-Day Feeding of 9-cis-RA. 57

20 Average Levels of Retinyl Palmitate in Livers of 75-Day-Old Female Sprague-Dawley Rats After a 4-Week Feeding of 9-cis-RA 58

21 Average Expression Levels of RARβ mRNA From Mammary Epithelial Cells of 50-Day-Old Female Sprague-Dawley Rats After a 3-Day Feeding of 9-cis-RA 59

22 Average Expression Levels of RARβ mRNA From Mammary Epithelial Cells of 75-Day-Old Female Sprague-Dawley Rats After a 4-Week Feeding of 9-cis-RA 60

LIST OF FIGURES

Figure — Page

1 Visible MNU-induced mammary cancer on Sprague-Dawley rat and photomicrographs of normal mammary gland, an MNU-induced mammary adenocarcinoma, and a fibroadenoma 61

2 Average body weight (in grams) of rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 60 mg/kg diet (Group 3), 75 mg/kg diet (Group 4), and 150 mg/kg diet (Group 5) 63

3 Percentage of mammary cancer incidence in rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 60 mg/kg diet (Group 3), 75 mg/kg diet (Group 4), and 150 mg/kg diet (Group 5) 65

4 Average number of mammary cancers per rat receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 60 mg/kg diet (Group 3), 75 mg/kg diet (Group 4), and 150 mg/kg diet (Group 5) 67

5 Average body weight (in grams) of rats receiving Teklad diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet for 10 weeks (Group 2), and 60 mg/kg diet for 20 weeks (Group 3) 69

6 Percentage of mammary cancer incidence in rats receiving Teklad diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet for 10 weeks (Group 2), and 60 mg/kg diet for 20 weeks (Group 3) 71

7 Average number of mammary cancers per rat receiving Teklad diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet for 10 weeks (Group 2), and 60 mg/kg diet for 20 weeks (Group 3) 73

8 Average body weight (in grams) of rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), 4-HPR: 586 mg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and 4-HPR: 586 mg/kg diet (Group 4) 75

9 Average number of mammary cancers per rat receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), 4-HPR: 586 mg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and 4-HPR: 586 mg/kg diet (Group 4) 77

LIST OF FIGURES (Continued)

Figure Page

10 Percentage of mammary cancer incidence in rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), 4-HPR: 586 mg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and 4-HPR: 586 mg/kg diet (Group 4) 79

11 Average body weight (in grams) of rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 4-HPR: 196 mg/kg diet (Group 3), and 9-cis-RA: 30 mg/kg diet and 4-HPR: 196 mg/kg diet (Group 4) 81

12 Percentage of mammary cancer incidence in rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 4-HPR: 196 mg/kg diet (Group 3), and 9-cis-RA: 30 mg/kg diet and 4-HPR: 196 mg/kg diet (Group 4) 83

13 Average number of mammary cancers per rat receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 4-HPR: 196 mg/kg diet (Group 3): and 9-cis-RA, 30 mg/kg diet and 4-HPR: 196 mg/kg diet (Group 4) 85

14 Average body weight (in grams) of rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 3.3 μg/kg diet (Group 4), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 3.3 μg/kg diet (Group 6)..... 87

15 Average body weight (in grams) of rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 10 μg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 10 μg/kg diet (Group 5)..... 89

16 Percentage of mammary cancer incidence in rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 3.3 μg/kg diet (Group 4), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 3.3 μg/kg diet (Group 6) 91

17 Percentage of mammary cancer incidence in rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 10 μg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 10 μg/kg diet (Group 5) 93

18 Average number of mammary cancers per rat receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 3.3 μg/kg diet (Group 4), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 3.3 μg/kg diet (Group 6) 95

19 Average number of mammary cancers per rat receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 10 μg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 10 μg/kg diet (Group 5) 97

LIST OF FIGURES (Continued)

Figure Page

20 Average body weight (in grams) of rats receiving Teklad (4%) diet ad libitum (Group 4), 9-cis-RA: 60 mg/kg diet (Group 5), and 120 mg/kg diet (Group 6) 99

21 Levels of retinyl palmitate in the livers after 3 days or 4 weeks of feeding 9-cis-RA.. 101

22 Autoradiographs showing retinoic acid receptor β mRNA expression levels in mammary epithelial cells from rats fed 9-cis retinoic acid for 3 days or 4 weeks.... 102

LIST OF ABBREVIATIONS

4-HPR	N-(4-hydroxyphenyl)retinamide
9-cis-RA	9-cis retinoic Acid
AF	activation function
atRA	all-trans retinoic Acid
bcl	B-cell leukemia
BT	breast tumor
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
DMBA	7,12-dimethylbenz(a)anthracene
HL	human leukemia
MCF	mammary cancer follicle
MNU	N-methyl-N-nitrosourea, methylnitrosourea
MOPS	morpholino-propane sulphonic acid
^{32}P-dATP	^{32}phosphorus- deoxyadenosine triphosphate
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP	retinol binding protein
RXR	retinoid x receptor
RXRE	retinoid x response element
SSPE	saline sodium phosphate ethylenediamine tetracetic acid

TR	thyroid receptor
VDR	vitamin D receptor

INTRODUCTION

To investigate potential chemopreventive agents for their effectiveness in vivo, rodent models that resemble the human carcinogenesis process are commonly used. Our laboratory, for many years, has used the methylnitrosourea (MNU)-induced mammary cancer model in the Sprague-Dawley rat.

9-cis retinoic acid (9-cis-RA) is a naturally occurring derivative of vitamin A. Members of this class of compounds affect the growth and differentiation of epithelial cells as they progress from normal cells to malignant cells (Lotan, 1980). Toxic side effects occur, however, if retinoids are given at high doses for extended lengths of time (Kamm, 1982). 9-cis-RA has shown effectiveness in the MNU-induced rat model (Anzano, Byers, et al., 1994; Anzano et al., 1996). Nevertheless, it was not effective therapeutically when given to humans with advanced cancers (Rizvi et al., 1998). Two classes of nuclear hormone receptors, retinoic acid receptors (RARs; Benbrook, Lernhardt, & Pfahl, 1988; Brand et al., 1988; Giguere, Ong, Segui, & Evans, 1987; Giguere, 1990; Krust, Kastner, Petkovich, Zelent, & Chambon, 1989; Petkovich, Brand, Krust, & Chambon, 1987) and retinoid x receptors (RXRs; Hamada et al., 1989; Leid, Kastner, Lyons, et al., 1992; Mangelsdorf et al., 1992; Mangelsdorf, Ong, Dyck, & Evans, 1990; Mangelsdorf, Umesono, & Evans, 1994; Yu et al., 1991), mediate retinoic acid-dependent transcription. Since 9-cis-RA binds to both receptor families (Kurie et al., 1996), it has the potential for a variety of biological activities. In general, the anti-

carcinogenic activities of retinoids appear to be mediated through their binding to the regulatory proteins that can induce or suppress gene activity.

Studies have indicated that vitamin A deficiency may correlate with abnormal growth patterns (Underwood, 1984; West, 1991). Animal studies evaluating vitamin A deficiency show increased cancer incidence for various cancer models (Cohen, Wittenberg, & Bryan, 1976; Rogers, Herndon, & Newberne, 1973). The mechanism for how retinoids play a role in carcinogenesis has not been found. Understanding this mechanism could lead to the development of other chemopreventive agents that are more effective and/or less toxic. Further, combinations of chemopreventive agents may be more effective and less toxic than when they are given separately. The studies presented contribute to the understanding of 9-cis-RA as a chemopreventive agent in mammary carcinogenesis, to the assessment of combinations for chemoprevention of mammary carcinogenesis, and to the evaluation of possible biochemical mechanisms of 9-cis-RA in the mammary epithelial cell.

REVIEW OF LITERATURE

In the United States, the second most common type of cancer in women is breast cancer. About 182,800 women will be diagnosed with the disease this year, and more than 40,000 women will die from this disease. After having a 4% increase per year in the 1980s, incidence rates leveled off in the 1990s. Mostly due to improved early detection and treatment, mortality rates declined significantly between 1992-1996, with the greatest decreases in young women. In most cases, there is no clear understanding about the etiology of breast cancer, but there are several risks factors. Breast cancer incidence increases with age, in women with family history of the disease, in women with a long menstrual history, in women who have never had children or had their first child after age 30, and in women who consume two or more drinks of alcohol daily (American Cancer Society, 2000). Epidemiological studies show that there is a correlation between increased breast cancer incidence and decreased dietary intake of certain nutrients such as vitamin A (Graham, 1984; Graham et al., 1982; Longnecker, Newcomb, Mittendorf, Greenberg, & Willett, 1997).

Carcinogenesis

The process of carcinogenesis, in general, can be divided into the "initiation stage," which is a permanent and irreversible change in a cell; the "promotion stage," which involves conversion of the initiated cell into a tumor; and the "progression stage," in which the tumors become malignant neoplasms (Pitot & Dragan, 1991).

Carcinogenesis is a slow process that may cover many decades of a person's life before detection of the cancer (Wattenberg, 1997). Regulation of cell growth, differentiation, and chromosome stability are all involved with the eventual development of neoplasms (Fingert, Campisi, & Pardee, 1993). Both exogenous and endogenous factors may be involved in the development of a fully malignant neoplasm (Weinstein, 1988). The complex interactions of these factors cause genetic and biochemical changes in the cell during the three stages of carcinogenesis.

In order for a cell to be initiated, it has to be exposed to an active carcinogen that induces an irreversible genetic alteration in the cell. This change in the cellular genome becomes permanent when the cell undergoes cell division without repairing the damaged DNA (Pitot & Dragan, 1991). Unrepaired DNA can have mutations due to transversions, transitions, and small deletions, as well as combinations of these defects (Pitot, 1993). Initiation is a common event that often occurs spontaneously. Chemicals can induce this process, and experimental animal models using these chemicals are readily developed (Pitot).

Following initiation, the cell may enter promotion, which is a stage characterized by the reversible expansion of initiated cells (Pitot & Dragan, 1991). Promoting agents cause alterations in gene expression, stimulate increased rate of cell division, and inhibit programmed cell death or apoptosis in initiated cells (Pitot, 1993).

Characteristics of the final stage of carcinogenesis, progression, are malignant growth of cells and instability of chromosomes (Pitot, 1993). Malignant cells release proteases that allow for the invasion of distant tissues. When cancer cells spread to other tissues, the tumors that develop are irreversible malignant neoplasms (Pitot & Dragan,

1991). In this stage of carcinogenesis, proto-oncogenes are activated, and tumor suppressor genes lose their function. Because there are permanent genetic alterations at this stage, progression is irreversible.

MNU-Induced Mammary Cancer Model

For decades, researchers have attempted to design animal models that are similar to the human development of certain diseases such as breast cancer. To investigate breast cancer and the possible means of preventing and treating the disease, it is imperative to have an animal model that has characteristics similar to those seen in humans. An appropriate animal model for breast cancer would have the following characteristics: (a) the tumor should be easily and reliably induced, (b) the organ site should be specific, (c) the tumors should originate from ducts, (d) the tumor histopathology should resemble the human tumor, (e) most of the tumors should be hormone dependent, and (f) the process should allow examination of the initiation and promotion stages (Welsch, 1985).

There have been several attempts to develop appropriate animal models for breast cancer. One such model, involving administration of 2-acetylaminofluorene to rats that yielded high cancer incidence but was not organ specific, had limited use (Wilson, DeEds, & Cox, 1941). Another model developed by Huggins, Grand, and Brillantes (1961) used 7,12-dimethylbenzanthracene (DMBA), which had to be further metabolized to become an active carcinogen. With only one gavage administration of DMBA (Huggins et al.), this procedure produced hormone-responsive tumors (Huggins, 1965) of ductal origin (Middleton, 1965; Rigden, Brigham, Nelson, & Hershey, 1964). The major disadvantages of this model were that the tumors did not display local invasion or

metastasis to distant sites and there were a large number of benign tumors relative to carcinomas (McCormick, Adamowski, Arsen, & Moon, 1981).

Another breast cancer model involved use of MNU, which is an alkylating agent that requires no metabolic activation, to induce mammary cancer in Sprague-Dawley rats. Bots and Willighagen (1975) developed the first MNU model for mammary cancer. Female Lewis rats (75-day-old) were intravenously injected with MNU (25 mg/kg body weight) on a monthly schedule (for 3 months), and 65% of the animals developed mammary cancer at 6 months after dosing. Following three monthly intravenous injections of MNU to 50-day-old animals, there were incidences of 73% and 89% of mammary cancers in female Sprague-Dawley and Fischer 344 rats, respectively (Gullino, Pettigrew, & Grantham, 1975). Important characteristics of these cancers are that they metastasize and are hormonally similar to breast cancer in women (Gullino et al.).

The age of an animal at the time of initiation of a carcinogen determines the extent of cancer development. In the mammary gland, there are terminal end buds, terminal ducts, and alveolar buds. Time-related susceptibility of the rat mammary gland to chemically induced carcinogenesis has been determined (Russo & Russo, 1980a, 1980b; Russo, Tait, & Russo, 1983; Russo, Wilgus, & Russo, 1979). Rats aged 30-55 days, after treatment with DMBA, had the highest incidence of mammary cancers in the terminal end buds (Russo et al., 1979). The aging rat has decreased terminal end buds and increased numbers of terminal ducts and alveolar buds. The susceptibility of the mammary gland to MNU-induced carcinogenesis was dependent on the age of the rat at the time of dosing (Grubbs, Peckham, & Cato, 1983). There was 100% incidence of mammary cancer in animals dosed between the ages of 35 and 57 days.

MNU-induced mammary cancers are responsive to hormone manipulations (Arafah, Finegan, Roe, Manni, & Pearson, 1982; Rose & Noonan, 1981). When the hypothalamus was removed from rats with established MNU-induced mammary cancers, there was an eventual reduction in the number of tumors (Rose & Noonan). Ovariectomy, accomplished for rats prior to MNU dosing, inhibited the formation and growth of tumors (Arafah, Gullino, Manni, & Pearson, 1980; Grubbs, Peckham, & McDonough, 1983). By treating rats with 17 β-estradiol and progesterone at pharmacological doses, there were decreased numbers of tumors after MNU treatment (Grubbs, Peckham, & McDonough, 1983).

The reproductive status of rats can influence the carcinogenic effects of MNU (Grubbs, Juliana, Hill, & Whitaker, 1986). Rats that became pregnant after MNU administration had suppressed mammary carcinogenesis. Pregnancy with lactation in rats resulted in a suppressed incidence of mammary cancers. Parous rats receiving prolactin after MNU injections had a reduced rate of mammary carcinoma development (Grubbs et al., 1986).

The MNU model appears to mimic human breast cancer better than the DMBA model. MNU, which is an alkylating agent that does not require metabolic activation, has a short half-life in the plasma of dosed animals (Thompson & Meeker, 1983). Because of the chemical characterisitics of MNU, this model can be used to examine the initiation and promotion phases of carcinogenesis. The high incidence and number of mammary cancers, the hormone dependence of the tumors that develop, and the invasiveness of the cancers make the MNU model an appropriate model for human breast cancer.

Chemoprevention

Sporn, Dunlop, Newton, and Smith (1976) coined the term "chemoprevention," and today an extensive effort is ongoing to find compounds that can inhibit or prevent the process of carcinogenesis. Chemopreventive agents consist of three categories that are based on the stage of carcinogenesis at which the compounds act. First, direct inhibitors prevent metabolic conversion of noncarcinogenic precursors to their carcinogenic forms. Second, blocking agents act to prevent carcinogens from causing damage to target tissue by removing the active form of the carcinogen before it can react with DNA. Examples of blocking agents include phenols, indoles, and flavones. Most direct acting and blocking agents are effective only if administered before or with the cancer-causing agent. Third, suppressing agents act at the cellular level to prevent promotion and progression in the carcinogenic process. Because they can be effective after exposure to carcinogens, suppressing agents such as retinoids have been studied intensively.

Retinoids

Retinoids include vitamin A and its products that occur naturally in many types of foods (meat and plant) and analogs that are specially designed and made synthetically. The natural retinoids are involved in vision (Saari, 1994), immunity (Ross & Hammerling, 1994), cell proliferation (Dion, Blalock, & Gifford, 1977; Lotan & Dennert, 1979), and cell differentiation (Hashimoto et al., 1996; Peck, Elias, & Wetzel, 1977). They also may play a role in apoptosis (Lotan, 1995a). There are two classes of nuclear receptors for retinoids, the RARs (Benbrook et al., 1988; Brand et al., 1988; Giguere et al., 1987, 1990; Krust et al., 1989; Petkovich et al., 1987) and RXRs (Hamada et al.,

1989; Leid, Kastner, Lyons, et al., 1992: Mangelsdorf et al., 1990, 1992, 1994; Yu et al., 1991). Through the promotion or silencing of genes as mediated by the nuclear receptors, retinoids may exert their anticarcinogenic effects by modulating cell growth, differentiation, and/or programmed cell death.

Over the last decade, many studies have investigated the chemopreventive efficacy of retinoids. There is evidence in humans that vitamin A deficiency has a strong correlation with cancer development (Peto, Doll, Buckley, & Sporn, 1981; Wald, Idle, Boreham, & Bailey, 1980), and there are animal experiments that show increased tumor incidence with decreased vitamin A intake (Moon, Mehta, & Rao, 1994). A common hypothesis is that physiological levels of retinoids can protect an organism against the development of some premalignant and malignant lesions. At pharmacological levels, retinoids have been effective in the prevention of mammary cancer in experimental carcinogenic models (Moon et al., 1994). Human clinical trials have indicated that some retinoids will potentially be effective chemopreventive agents against breast and ovarian cancer (Lippman, Heyman, Kurie, Benner, & Hong, 1995).

An active form of vitamin A, retinol, supports visual and reproductive functions; in contrast, retinoic acid promotes growth and cellular differentiation. Two enzymes are responsible for the esterification and absorption of retinol. Retinol is absorbed by facilitated diffusion at physiologic concentrations and by passive diffusion at pharmacological levels (Blomhoff, Green, Green, Berg, & Norum, 1991). Lecithin-retinol acyltransferase and acetyl coenzyme A-retinol acyltransferase facilitate retinoid absorption (Ong, 1993). The presence of dietary fat is required for the hydrolysis and absorption of retinoids.

In the upper small intestinal lumen, long chain retinyl esters are hydrolyzed by pancreatic lipase and pancreatic carboxyl ester lipase to retinol. Through a carrier-mediated process facilitated by cellular retinol binding protein-II (CRBP-II), retinol is taken into the intestinal mucosal cells. The retinol is then re-esterified with free fatty acids to form retinyl esters (Ong, Kakkad, & MacDonald, 1987). Eventually, chylomicrons incorporate the retinyl esters and transport them to vascular circulation. The hepatocytes of the liver take up retinyl esters as part of the chylomicron remnants (Mahley & Hussain, 1991; Redgrave, 1983).

The liver is the primary site of retinol storage and release. Parenchymal cells are responsible for the uptake of chylomicron remnants, rehydrolysis of retinyl esters to retinol, and the synthesis and secretion of retinol binding protein (RBP; Blaner et al., 1985; Hendriks, Verhoofstad, Brouwer, de Leeuw, & Knook, 1985; Yamada et al., 1987). Retinyl esters are stored in the stellate cells of the liver (Blaner et al., 1985; Blomhoff et al., 1985). RBP forms a complex with retinol, and the complex is transported from the liver into plasma. Transthyretin, formerly called prealbumin (Kopelman, Cogan, Mokady, & Shinitzky, 1976; Van Jaarsveld et al., 1973), is complexed with RBP-retinol and may decrease the elimination of retinol from plasma. Epithelial cells in target tissues take up this complex (Olson, 1990). The retinol bound to RBP is relatively nontoxic, while unbound retinol and retinyl esters are toxic, disrupting cell membranes (Hinds, West, & Knight, 1997).

Target tissues can receive retinol through specific cell surface membrane receptors; however, these processes are not clearly understood. Cellular retinol binding protein-I, CRBP-I, appears to be involved with retinoid transport and metabolism within

the target cells (Boerman & Napoli, 1991). Once in a target cell, retinol can undergo oxidation to retinaldehyde and then retinoic acid. Via the cellular retinoic acid binding proteins, CRABP-I and CRABP-II, retinoic acid can be transported to cytoplasmic or nuclear receptors.

In order for retinoids to be effective suppressors of carcinogenesis, there must be specific mechanisms that allow these compounds to modulate cell differentiation, proliferation, and/or apoptosis. The two classes of nuclear retinoid receptors, which are members of the steroid hormone receptor superfamily, are involved in these cell processes (Mangelsdorf et al., 1994). Both classes, RARs and RXRs, are composed of three subtypes (α, β, and γ). The RARs can bind all-trans retinoic acid (atRA) and 9-cis-RA, but RXRs can only bind 9-cis-RA. RARs form heterodimers with RXRs, bind to specific DNA sequences (retinoic acid response elements, RARE, or retinoid x response elements, RXRE) in promoter regions of genes, and, when activated by a ligand, enhance the transcription of target genes (Mangelsdorf et al., 1994). Because the subtypes are expressed in different adult tissues, each one is thought to regulate distinct genes (Chambon, 1996). One study showed that the regulation of specific genes was abrogated in cells that had receptor genes knocked out (Boylan et al., 1995).

Cancer development requires changes in genes involved with the control of growth and differentiation. Retinoids, as mentioned earlier, regulate expression of genes for growth factors, signal transducers, proto-oncogenes, transcription factors, and cell surface and extracellular matrix adhesion molecules (Lotan, 1995b). Due to the various effects shown by retinoids, it is important to understand which receptors are present in the tissue in which cancer develops. For breast cancer, studies have been accomplished

with cell cultures and with some animal models. There have only been a few clinical trials of retinoids as preventive agents for human breast cancer.

Retinoid Receptors in Cancer

Within the RARs and RXRs, there are six distinct domains (A-F) that contain functional units of the transcription factor (Krust et al., 1986; Mangelsdorf et al., 1994). Regions A and B contain the ligand-independent transcription activation function, AF-1. The DNA-binding domain is located in Region C. A hinge region that is involved with nuclear translocation and corepressor binding is found in Region D. Region E is the domain that allows dimerization, ligand binding, and ligand dependent transcriptional activation function, AF-2. No function has been discovered for Region F. Unique A regions are the cause of the diversity in protein sequences between the receptors. Generally, the isoforms of the receptors have identical protein sequences in the B-F regions (Sommer, Chen, Treuting, Smith, & Swisshelm, 1999).

The nuclear retinoid receptors mediate the effects of the retinoids on gene expression, thereby altering the growth and differentiation of normal as well as tumor cells (DeLuca, 1991; Lotan et al., 1995). The highly pleiotropic effects of retinoids can be explained by the complexity of the retinoid signaling pathways and their convergence with other signaling pathways by means of heterodimeric interactions between nuclear retinoid receptors and other members of the steroid/thyroid receptor superfamily (Leid, Kastner, & Chambon, 1992).

Many studies investigated the relationship of receptor levels and the process of carcinogenesis. RARβ, which is primarily expressed in epithelial cells, exhibited induced

expression in response to retinoic acid, which was mediated by an RARE within its promoter (de The, Vivanco-Ruiz, Tiollais, Stunnenberg, & Dejean, 1990). Under-expression of the RARβ gene has been demonstrated in several types of human epithelial cell cancers (Gebert, Moghal, Frangioni, Sugarbaker, & Neel, 1991; Hu, Crowe, Rheinwald, Chambon, & Gudas, 1991; Roman, Clarke, Hall, Alexander, & Sutherland, 1992; Seewaldt, Johnson, Parker, Collins, & Swisshelm, 1995; Swisshelm et al., 1994; van der Leede, van den Brink, & van der Saag, 1993; Xu et al., 1994; Zhang, Liu, Lee, & Pfahl, 1994). X. S. Li et al. (1995) found that RARβ functions as a negative regulator of growth in breast epithelial cells, and Liu et al. (1996) showed that RARβ can mediate retinoid action in breast cancer cells by promoting apoptosis.

9-cis-RA

9-cis-RA was identified as an agonist for RARs and as the physiological ligand for the RXRs (Heyman et al., 1992; Levin et al., 1992). A naturally occurring isomer of atRA, 9-cis-RA is a bifunctional ligand that binds and transactivates both retinoid receptor families, the RARs and RXRs (Chambon, 1994; Heyman et al.). It has the potential for a variety of biological activities. However, only very low levels of 9-cis-RA are found in foods; human consumption is approximately 10-100 μg/day. 9-cis-RA is rapidly metabolized in the body; it is not stored in the liver and does not accumulate over time (Blaner & Olson, 1994). CRABP, which is an RA carrier protein that does not transduce retinoid signals, has an affinity for 9-cis-RA that is substantially less than atRA (Davies & Lippman, 1996; Redfern, Lovat, Malcolm, & Pearson, 1994). With less carrier protein binding, 9-cis-RA may have reduced vulnerability for oxidative

degradation, which could lead to its unique activity profile (Napoli, Boerman, Chai, Zhai, & Fiorella, 1995).

Mangelsdorf et al. (1992) found that 9-cis-RA is a signaling molecule that mediates its action through the RXRs. Each of the RXRs is activated by 9-cis-RA more potently and efficiently than atRA. In human tissues RXRα has a high affinity for 9-cis-RA. RXRα can function as a homodimer that binds specific DNA sequences (Heyman et al., 1992), and it serves as a common heterodimer with vitamin D receptor (VDR), thyroid receptor (TR), and the other RXRs and RARs (Kliewer, Umesono, Mangelsdorf, & Evans, 1992). The role of 9-cis-RA in gene transcription related to RXRα signaling is not fully understood.

In vitro and in vivo studies show that 9-cis-RA can inhibit carcinogenesis. 9-cis-RA induced differentiation in human leukemia (HL-60) cells (Breitman, Selonick, & Collins, 1980). When treated with 9-cis-RA mammary cancer follicle (MCF-7) cells had inhibited growth and downregulation of the estrogen receptor (Rubin et al., 1994). Myeloblastic leukemia cells had increased apoptosis and downregulation of B-cell leukemia (bcl-2) expression when exposed to 9-cis-RA (Nagy et al., 1995). 9-cis-RA was an effective chemopreventive agent in the rat model for MNU-induced mammary cancer when administered in the diet starting at 1 week after the carcinogen (Anzano, Byers, et al., 1994, Anzano et al., 1996), but it was not effective therapeutically in humans who had advanced cancers (Rizvi et al., 1998). Toxic effects were noted at therapeutic dose levels (Armstrong, Ashenfelter, Eckhoff, Levin, & Shapiro, 1994; Kamm, Ashenfelter, & Ehmann, 1984; Kelloff et al., 1996; Nau, Chahoud, Dencker,

Lammer, & Scott, 1994). The maximum tolerated oral dose in humans was 80 mg/m^2 per day (Rizvi et al.).

N-(4-Hydroxyphenyl)retinamide

A synthetic retinoid, N-(4-hydroxyphenyl)retinamide (4-HPR), has been investigated as a chemopreventive agent in many in vitro studies. Steele, Kelloff, Wilkinson, and Arnold (1990) showed that 4-HPR suppressed the transformation of rat tracheal epithelial cells induced by benzo[a]pyrene. PC3 prostate carcinoma cells treated with 4-HPR had a reduction in the proliferation rate, and this inhibition was associated with the suppression of c-myc gene expression (Igawa, Tanabe, Chodak, & Rukstalis, 1994). 4-HPR induced apoptosis in a number of cancer models, including head and neck (Oridate, Lotan, Xu, Hong, & Lotan, 1996; Sun, Kurie, et al., 1999), ovarian (Sabichi, Hendricks, Bober, & Birrer, 1998; Supino et al., 1996), prostate (Roberson et al., 1997; Sun, Yue, & Lotan, 1999), and breast (Pellegrini et al., 1995; Sheikh et al., 1994, 1995; Swisshelm et al., 1994). However, 4-HPR was not effective at inducing differentiation in HL-60 cells (Delia et al., 1993) or neuroblastoma cells (Ponzoni et al., 1995).

The mechanism(s) involved with the inhibitory effects of 4-HPR in vivo are not clearly understood. It does not bind substantially to the nuclear receptors (Sheikh et al., 1995). One hypothesis is that it induces apoptosis (Sun, Li, Yue, Lippman, Hong, & Lotan, 1999).

Animal studies evaluating the effectiveness of 4-HPR as a chemopreventive agent have been accomplished in a number of models. 4-HPR reduced the percent incidence and multiplicity of cancers in C3H mice (Welsch, DeHoog, & Moon, 1983). Moon et al.

(1979, 1989) and Grubbs, Eto, Juliana, Hardin, and Whitaker (1990) showed that 4-HPR is effective at inhibiting mammary cancer in Sprague-Dawley rats injected with MNU.

4-HPR slightly reduced breast cancer occurrence in premenopausal women in a clinical trial (Veronesi et al., 1999). In this trial, 4-HPR displayed minimal toxicity (Costa et al., 1995). Common side effects included cutaneous toxicity and night blindness that are due to reduced plasma retinal, but these effects were reversed by giving patients a 3-day drug "holiday" (Formelli, Barua, & Olson, 1996; Kelloff et al., 1994). Breast tissue from women in a clinical trial had 4-HPR concentrated in the epithelial cells of the breast (Mehta, Moon, Hawthorne, Formelli, & Costa, 1991).

In a study done by Canfield (1995), the MNU-induced mammary cancer model was used to observe the effects of 4-HPR on mammary cancer initiation. The study was designed to investigate the influence of 4-HPR on enhancement of tumor formation if administered prior to carcinogen. It was known that 4-HPR was highly effective in inhibiting tumor formation in rats when administered at the same time as the carcinogen. It needed to be shown whether prior 4-HPR supplements would have the same effect. Grubbs et al. (1990) reported a 93% increase in the number of tumors in animals who were given 4-HPR 60 days prior to carcinogen administration. This was an enhancement of the initiation phase of carcinogenesis. Continued administration of 4-HPR did produce a significant reduction in cancers.

Molecular biomarker measurements did not show definitive quantitative products to indicate how 4-HPR was enhancing initiation (Canfield, 1995). The precise mechanisms for how 4-HPR increases early tumorigenesis are still not known, but, nonetheless, this retinoid is currently being evaluated in clinical trials for prevention of

various cancers. 4-HPR can be used as a chemopreventive agent to inhibit breast cancer only if it is administered at the appropriate time; otherwise, it may actually cause a significant increase in the initiation of carcinogenesis.

Vitamin D3

Results of the National Health and Nutrition Examination Survey (I) epidemiologic follow-up study from 1971-1975 to 1992 indicated that dietary vitamin D intake might reduce the risk of breast cancer (John, Schwartz, Dreon, & Koo, 1999). 1,25 dihydroxyvitamin D3 [1,25-$(OH)_2$ D3], which is the biologically active form of vitamin D, has possible chemopreventive effects. Vitamin D3 showed inhibitory effects on melanoma cell proliferation (K. W. Colston, Colston, & Feldman, 1981). Other reports have identified vitamin D3 as a potent negative growth regulator on breast cancer cell lines (Chouvet, Berger, & Coombes, 1986; K. Colston, Berger, & Coombes, 1989; J. Eisman et al., 1989). Vitamin D3 had significant inhibitory effects in studies for leukemias, melanoma, colon cancer, osteosarcoma, and breast cancer (Bouillon, Okamura, & Norman, 1995; J. A. Eisman, Barkla, & Tutton, 1987; Tsuchiya, Morishita, Tomita, Ueda, & Tanaka, 1993; Zhou et al., 1990).

1α (OH) D3, which is a synthetic analog of vitamin D3 that is rapidly converted to 1,25-$(OH)_2$ D3 in vivo, suppressed MNU-induced rat mammary tumors when the compound was administered intraperitoneally three times a week at a dose of 0.1 μg per rat (K. W. Colston, Chander, Mackay, & Coombes, 1992). Higher doses of 1α (OH) D3 (0.25 μg per day), however, caused hypercalcemia to develop even while feeding the rats low levels of calcium (K. W. Colston et al., 1992).

More than 80% of breast tumors contain receptors for vitamin D3 (Berger, Wilson, & McClelland, 1987). The VDRs, which interact with 1,25-$(OH)_2$ D3, belong to the steroid/thyroid/retinoic acid superfamily of nuclear receptors and modulate expression of genes that cause biological responses attributed to vitamin D3 (Studzinski, McLane, & Uskokovic, 1993). The effects of vitamin D3 on cell lines expressing VDR include induction of differentiation markers, cell cycle arrest, and/or activation of apoptosis (Elstner et al., 1995, 1997; Shabahang et al., 1994; Tsuchiya et al., 1993; Welsh, Simboli-Campbell, Narvaez, & Tenniswood, 1995).

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

9-cis-RA, in the MNU-induced mammary cancer model, will be effective in preventing mammary cancer at various dose levels, with limited or continuous treatment, alone or in combination with 4-HPR and vitamin D3, and it will cause changes in the morphology of the mammary glands and alter levels of RARβ mRNA in isolated mammary epithelial cells.

Specific Aims

The following specific aims were established to test the hypothesis:

1. The effect of increasing dose levels of 9-cis-RA on the average number of MNU-induced mammary adenocarcinomas per rat in older rats will be determined.

2. The effect of limited and continuous treatment of 9-cis-RA on the average number of MNU-induced mammary adenocarcinomas per rat in young rats will be determined.

3. The effects of 9-cis-RA and 4-HPR, alone or combined, on the average number of MNU-induced mammary adenocarcinomas per rat in young rats will be determined.

4. The effects of 9-cis-RA and 4-HPR, alone or combined, on the average number of MNU-induced mammary adenocarcinomas per rat in older rats will be determined.

5. The effects of 9-cis-RA and vitamin D3, alone or combined, on the average number of MNU-induced mammary adenocarcinomas per rat in young rats will be determined.

6. The effect of acute (3 days) and chronic (4 weeks) feeding of high doses of 9-cis-RA on the gross and histological morphology of the rat mammary gland will be determined.

7. The effect of acute (3 days) and chronic (4 weeks) feeding of high doses of 9-cis-RA on the mRNA expression level of RARβ will be determined.

MATERIALS AND METHODS

General Animal Care and Treatment

The guidelines from the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham were strictly followed for all animal studies (Appendix A). Female Sprague-Dawley rats were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Rats were housed in polycarbonate cages (five per cage) throughout the studies. Animals were fed Teklad 4% rodent diet and given tap water ad libitum. The rooms, where the animals were housed, had 12 hr of artificial light each day and were kept at a temperature of 72 ±2 °F.

Prior to dietary supplementation of chemopreventive agents, rats were randomly placed in the experimental or control groups. Each group had mean body weights within ±3 g. Holes or notches were punched in the ears to give each animal a unique identify-cation (Appendix B).

9-cis-RA was obtained from Southern Research Institute International and was mixed with ethanol: trioctanoin (12 g and 19 g/kg of diet, respectively) prior to incorp-oration into the diet. 4-HPR was obtained from Chemical Industry Laboratory and was mixed with the same vehicle prior to adding to the diet. Tenox-20 and d,l-alpha-tocopherol (0.05 ml of each/kg of diet) were also added as antioxidants to the 9-cis-RA and 4-HPR mixes. Photospectroscopy and high performance liguid chromatography verified purity and stability of the dietary agents.

The carcinogen, MNU (Ash Stevens Inc., Detroit, MI), was prepared by dissolving it in physiological saline. The pH of this solution was adjusted to 5.0 with 3% acetic acid. This preparation was done immediately prior to administration. Rats anesthetized by ether received MNU (50 mg/kg body weight) by a single intravenous injection.

All animals were checked daily for signs of toxicity. Body weights were measured weekly, and animals receiving the carcinogen were palpated twice weekly. Palpable tumors were recorded for location and date of appearance. Throughout the studies some animals were checked for stage of the estrus cycle by vaginal smears.

Necropsy Tissue Collection

Tumors and major organs. During necropsy, all tumors and major organs were examined. Organ weights were recorded, and tumors were removed for pathological evaluation (Young & Hallowes, 1973). The mammary tumors were fixed in neutral-buffered formalin and sectioned for hematoxylin and eosin staining. Each tumor was classified as either a cancer or benign tumor. The mammary cancers were adeno-carcinomas; the benign tumors included fibromas, adenomas, and fibroadenomas.

Blood samples. During necropsy, blood samples were taken in stoppered centrifuge tubes. The samples were then centrifuged at 4 °C for 15 min at 2,800 rpm. Serum (supernatant) was removed from the tubes, placed in new tubes, and stored.

Mammary gland whole mounts. The mammary gland was dissected away from the skin by using iris scissors and forceps beginning at the fourth abdominal teat. The mammary gland was then placed on a microscope slide and spread as thin as possible using forceps. Slides were placed in formalin until staining.

Solutions were prepared for staining the slides. A 1:3 ratio of glacial acetic acid: 100% ethanol was prepared. Solutions of 35%, 50%, 70%, and 95% ethanol were prepared by diluting 100% ethanol with distilled water. Alum carmine stain was prepared as a combination of 1 g carmine, 2.5 g potassium sulfate, and 500 ml distilled water. The mixture was boiled for 20 min. After cooling in a water bath, the final volume was adjusted to 500 ml by adding distilled water. The stain was filtered, and thymol (50-100 mg) was added as a preservative.

Prior to staining, slides were placed in (1:3) glacial acetic acid:ethanol solution for 1 hr. Each slide was then placed in acetone for 2 hr, 70% ethanol for 15 min, and distilled water for 5 min. Slides were stained overnight in alum carmine and then washed in 35%, 50%, 70%, 95%, and 100% ethanol for 20 to 30 min increments each. Mammary glands were placed in toluene until the gland was clear. Mammary glands were removed from slides, trimmed, and mounted on new slides using cover slips and Permount. By observing the size and number of terminal end buds, alveolar buds, alveoli, and terminal end ducts, each mammary gland could be qualitatively evaluated for degree of proliferation and differentiation.

Experimental Studies

Experiment 1. This study was designed to evaluate the effectiveness of 9-cis-RA at various dose levels in older rats. As presented in the literature review, 9-cis-RA had been shown to be an effective inhibitor of mammary cancer in the MNU-induced rat model of young rats. The present study was accomplished to determine whether increasing doses of 9-cis-RA would have preventive effects or if the retinoid would lose its effectiveness and become toxic in older animals.

Female Sprague-Dawley rats were obtained at 28 days of age and placed on Teklad (4%) diet upon arrival. The animals were fed ad libitum. At 93 days of age, 9-cis-RA was initiated in the diets of the treated groups, and the control group (Group 1) was fed a vehicle diet as indicated in Table 1. Diets were analyzed for the appropriate amounts of 9-cis-RA at initial feeding and at the end of the study.

At 100 days of age, MNU was administered to rats by intravenous injection (50 mg/kg of body weight) via the jugular vein. All animals were palpated for mammary tumors twice a week, and the location and time of appearance of all tumors found were recorded. The study was terminated at 180 days after MNU administration.

Experiment 2. This study was designed to investigate the influence of limited and continued treatment with 9-cis-RA on the MNU-induced mammary cancer model in young rats (Table 2). Group 1 received vehicle diet throughout the study. Group 2 received 9-cis-RA (60 mg/kg diet), beginning at 53 days of age, for 10 weeks. After 10 weeks, Group 2 received vehicle diet for the remainder of the study. Group 3 received 9-

Table 1

Experiment 1 Protocol for Determining the Effect of Various Doses of 9-cis-RA on MNU-Induced Mammary Cancers Prior to Initiation and Promotion in Older Rats

Group	Number of rats	Carcinogen[a]	Treatment[b]
1	40	MNU	Teklad vehicle
2	40	MNU	9-cis-RA, 30 mg/kg diet
3	40	MNU	9-cis-RA, 60 mg/kg diet
4	40	MNU	9-cis-RA, 75 mg/kg diet
5	40	MNU	9-cis-RA, 150 mg/kg diet

Note. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.
[a] MNU (50 mg/kg body weight) was administered by intravenous injection to female Sprague-Dawley rats at 100 days of age. [b] Beginning at 93 days of age, retinoid treatment was initiated.

Table 2

Experiment 2 Protocol for Determining the Effect of Limited and Continuous Treatment With 9-cis-RA on MNU-Induced Mammary Cancers in Young Rats

Group	Number of rats	Carcinogen[a]	Treatment[b]
1	20	MNU	Vehicle
2	20	MNU	9-cis-RA, 60 mg/kg diet (10 Weeks)
3	20	MNU	9-cis-RA, 60 mg/kg diet (20 Weeks)

Note. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.
[a] MNU (50 mg/kg body weight) was administered by intravenous injection to female Sprague-Dawley rats at 50 days of age. [b] Beginning at 53 days of age, 9-cis-RA treatment was initiated.

cis-RA (60 mg/kg diet), beginning at 53 days of age, for 20 weeks. Diets were analyzed for the appropriate concentration of 9-cis-RA during the study.

At 50 days of age, the rats were injected with MNU (50 mg/kg body weight) via the jugular vein. Body weights were recorded weekly, and the animals were palpated twice a week to monitor tumor development. When tumors were found, the location and date were recorded. The study was terminated 20 weeks after MNU administration.

Experiment 3. This study was designed to look at the effects of 9-cis-RA alone and combined with 4-HPR on MNU-induced mammary carcinogenesis in young animals. The control group (Group 1) was fed a vehicle diet throughout the study. Group 2 was fed 9-cis-RA at a dose of 60 mg/kg of diet, and Group 3 was fed 4-HPR at a dose of 586 mg/kg of diet. Group 4 was fed the 9-cis-RA and 4-HPR in combination (Table 3). All treated rats were placed on the supplemented diets at 53 days of age. Throughout the study, diets were analyzed for the appropriate amounts of agents.

MNU was administered at 50 days of age by intravenous injection (50 mg/kg of body weight). Animals were palpated twice a week, and all palpable tumors were recorded for location and time of appearance. The study was terminated at 150 days after MNU administration.

Experiment 4. This study was designed to evaluate the effects of 9-cis-RA alone and combined with 4-HPR on MNU-induced mammary carcinogenesis in older animals. The control group (Group 1) was fed a vehicle diet throughout the study. Group 2 was fed 9-cis-RA at a dose of 30 mg/kg of diet, and Group 3 was fed 4-HPR at a dose of 196

Table 3

Experiment 3 Protocol for Determining the Effect of 9-cis-RA Alone and Combined With 4-HPR on MNU-Induced Mammary Cancers in Young Rats

Group	Number of rats	Carcinogen[a]	Treatment[b]
1	25	MNU	Vehicle
2	25	MNU	9-cis-RA, 60 mg/kg diet
3	25	MNU	4-HPR, 586 mg/kg diet
4	25	MNU	9-cis-RA, 60 mg/kg diet + 4-HPR, 586 mg/kg diet

Note. 9-cis-RA = 9-cis retinoic acid; 4-HPR = 4-hydroxyphenylretinamide; MNU = N-methyl-N-nitrosourea.
[a] MNU (50 mg/kg body weight) was administered by intravenous injection to female Sprague-Dawley rats at 50 days of age. [b] Beginning at 53 days of age, retinoid treatment was initiated.

treated rats went on the supplemented diets at 93 days of age. Dietary analysis was performed to evaluate for the appropriate concentrations of agents.

MNU was administered at 100 days of age by intravenous injection (50 mg/kg of body weight). Animals were palpated twice a week, and all palpable tumors were recorded for location and time of appearance. The study was terminated at 180 days after MNU administration.

Experiment 5. This study was designed to investigate the effects of 9-cis-RA and vitamin D3, alone and combined, on the MNU-induced mammary cancer model in young animals (Table 5). Group 1 was fed a vehicle diet throughout the study. The chemo-

Table 4

Experiment 4 Protocol for Determining the Effect of 9-cis-RA Alone and Combined With 4-HPR on MNU-Induced Mammary Cancers in Older Rats

Group	Number of rats	Carcinogen[a]	Treatment[b]
1	40	MNU	Vehicle
2	40	MNU	9-cis-RA, 30 mg/kg diet
3	40	MNU	4-HPR, 196 mg/kg diet
4	40	MNU	9-cis-RA, 30 mg/kg diet + 4-HPR, 196 mg/kg diet

Note. 9-cis-RA = 9-cis retinoic acid; 4-HPR = 4-hydroxyphenylretinamide; MNU = N-methyl-N-nitrosourea.
[a] MNU (50 mg/kg body weight) was administered by intravenous injection to female Sprague-Dawley rats at 100 days of age. [b] Beginning at 93 days of age, retinoid treatment was initiated.

preventive groups were Group 2, 9-cis-RA (60 mg/kg diet); Group 3, vitamin D3 (10 μg/kg diet); Group 4, vitamin D3 (3.3 μg/kg diet); Group 5, 9-cis-RA (60 mg/kg diet) and vitamin D3 (10 μg/kg diet); and Group 6, 9-cis-RA (60 mg/kg diet) and vitamin D3 (3.3 μg/kg diet). The chemopreventive agents were incorporated into the diets beginning when the rats were 53 days of age. Analysis of the diets was performed to insure that adequate amounts of the agents were incorporated.

At 50 days of age, the rats were injected with MNU (50 mg/kg body weight) via the jugular vein. Body weights were recorded weekly, and the animals were palpated twice a week to monitor tumor development. When tumors were found, the location and

Table 5

Experiment 5 Protocol for Determining the Effect of 9-cis-RA Alone and Combined With Vitamin D3 on MNU-Induced Mammary Cancers in Young Rats

Group	Number of rats	Carcinogen[a]	Treatment[b]
1	25	MNU	Vehicle
2	25	MNU	9-cis-RA, 60 mg/kg diet
3	25	MNU	Vitamin D3, 10 μg/kg diet
4	25	MNU	Vitamin D3, 3.3 μg/kg diet
5	25	MNU	9-cis-RA, 60 mg/kg diet + Vitamin D3, 10 μg/kg diet
6	25	MNU	9-cis-RA, 60 mg/kg diet + Vitamin D3, 3.3 μg/kg diet

Note. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.
[a] MNU (50 mg/kg body weight) was administered by intravenous injection to female Sprague-Dawley rats at 50 days of age. [b] Beginning at 53 days of age, chemopreventive treatment was initiated.

date were recorded in order to monitor the latency time and multiplicity of mammary cancers throughout the study. The study was terminated 150 days after MNU injections.

Experiment 6. This study was accomplished to evaluate the effects of 9-cis-RA on the morphology of mammary glands and to determine levels of RARβ mRNA in isolated epithelial cells from mammary glands (Table 6). For 3 days before being sacrificed, Group 1 received Teklad diet only, Group 2 received 9-cis-RA (60 mg/kg of diet), and Group 3 received 9-cis-RA (120 mg/kg of diet). For 4 weeks before being sacrificed, Group 4 received Teklad diet only, Group 5 received 9-cis-RA (60 mg/kg of diet), and

Table 6

Experiment 6 Protocol for Determining the Effects of 9-cis-RA on Mammary Gland Morphology and Levels of RARβ mRNA in Isolated Mammary Epithelial Cells

Group	Number of rats	Treatment
1	10	Teklad diet only (3 days)
2	10	9-cis-RA, 60 mg/kg diet (3 days)
3	10	9-cis-RA, 120 mg/kg diet (3 days)
4	10	Teklad diet only (4 weeks)
5	10	9-cis-RA, 60 mg/kg diet (4 weeks)
6	10	9-cis-RA, 120 mg/kg diet (4 weeks)

Note. 9-cis-RA = 9-cis retinoic acid. 9-cis-RA was initiated beginning at 47 days of age.

Group 6 received 9-cis-RA (120 mg/kg of diet). The 9-cis-RA was incorporated into the diets when the rats were 47 days of age. Diets were analyzed for appropriate amounts of 9-cis-RA at initial feeding and at the end of the study. The animals were checked daily for signs of toxicity, and each animal was weighed weekly.

Isolation of mammary epithelial cells. The incubation solution (50 ml) was prepared by combining 5 ml of medium 199 10X concentration (Life Technologies Gibco BRL), 4 ml of 2.8% sodium bicarbonate (Sigma), 1 ml of penicillin-streptomycin 5,000 units per ml mixture (Life Technologies, Gibco BRL), and 40 ml of sterile water. Ten milliliters of the incubation solution were added to a 50 ml Erlenmeyer flask containing 35 mg of Collagenase Type II (Life Technologies Gibco BRL). Approximately 1 g of

normal mammary gland from the fat pad lying below the abdominal-inguinal teats was removed from rats and immediately cut into several 1-cm pieces. The pieces were placed in the flask, and the flask was sealed with Parafilm. The flask was then placed in a Dubnoff metabolic shaking incubator for 1-1.5 hr at 37 °C with shaking of 160 oscillations/min.

After incubation was complete, the medium and tissue fragments were transferred to a conical bottom, glass centrifuge tube and centrifuged (400 x g) for 20 min. The floating tissue and medium were discarded using an aspirator. The remaining cells were resuspended in 0.9% sodium chloride (Sigma) by inverting the tubes and centrifuged (400 x g) for 10 min. The aspiration of supernatant, resuspension of cells in 0.9% sodium chloride, and centrifugation were repeated twice. After the final wash, the cells were transferred to 15-ml polypropylene tubes for RNA extraction.

RNA extraction and Northern blot analysis. The cells were analyzed for retinoid receptor expression by use of a Northern blot assay. Two to 2.5 ml of Trizol reagent (Life Technologies, Gibco BRL) was added to cells, and the cells were immediately ground with a Polytron homogenizer. The homogenized samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then 0.2 ml chloroform per 1 ml of Trizol used for the initial homogenization was added, and the tubes were shaken vigorously for 15 s and were left to stand at room temperature for 2-3 min.

The homogenates were centrifuged (2,000 x g) for 30 min at 4 °C. After centrifugation, the colorless upper aqueous phase (which contains RNA) was transferred

to a fresh tube. Isopropanol (0.5 ml per ml of Trizol used for the initial homogenization) was added to the aqueous phase. Samples were mixed and stored for 5-10 min at room temperature and then centrifuged (2,000 x g) for 30 min at 4 °C. The RNA precipitated as a white pellet in the bottom of the tube.

The supernatant was decanted, and the pellet was washed by adding 1 ml of 75% ethanol per ml of Trizol used for the initial homogenization. The mixture was vortexed and centrifuged (2,000 x g) for 30 min at 4 °C. The supernatant was decanted, and the pellet was dried for 15 min at room temperature. The pellet was dissolved in 50 to 150 µl of formamide and stored at -80 °C. The RNA concentration and purity were estimated based on absorbance at 260 and 280 nm. Samples of RNA (15 µg per lane) were heated at 55 °C for 15 min in formaldehyde-morpholino-propane sulphonic acid (MOPS) buffer, after which time the samples were loaded onto a 1.2% agarose-formaldehyde gel and electrophoresed at 5 V per cm.

After electrophoresis, gels were washed and transferred overnight onto a positively charged nylon membrane (Boehringer-Mannheim) using a Transblotter apparatus (Schleicher and Schuell) and 10X saline sodium phosphate ethylenediamine tetracetic acid (SSPE) buffer. After transfer, the membrane was washed for 5 min using 2X SSPE buffer, and RNA was crosslinked to the membrane using a crosslinking apparatus (Fisher Scientific). The membrane was stained with methylene blue solution to visualize 18S and 28S bands of RNA and stored at 4 °C in an airtight bag until it was hybridized with labeled probe.

Mouse RARβ cDNA probes were labeled with ^{32}phosphorous-deoxyadenosine triphosphate (^{32}P-dATP) using the random-primed labeling method, and the labeled

probes were hybridized overnight at 62 °C with the nylon membrane containing transferred RNA. The hybridization was accomplished using a high-efficiency hybridization buffer (Molecular Research Center). The membranes were washed three times at room temperature with 1X prehybridization/wash solution and followed by three washes at 65 °C with the same solution. The membranes were exposed to autoradiography film for 16-24 hr. Bands corresponding to RARβ and 18S RNA were scanned and quantitated using Adobe Photoshop (Adobe Systems). RARβ expression is presented as the ratio of the intensity of RARβ mRNA bands to 18S RNA bands.

Statistical Considerations

Statistical power. The average numbers of adenocarcinomas among groups were compared for Experiments 1-5. To determine statistical power, the average number of adenocarcinomas, which was the outcome variable, was used. In the younger animal model, either 20 or 25 animals per group were studied to achieve the required power, and 40 animals were used in the older animal model. Experiment 6 was a preliminary study to determine if mammary epithelial cells could be isolated and if RNA could be extracted from the cells; therefore, statistical power was not achieved in comparing the ratios of RNA expression.

Statistical analysis. The Armitage (Armitage, 1966) test was used for analysis of the number of cancers per rat. The Logrank test (Peto, 1988) was used to analyze the percentage incidence of mammary cancers between treated groups and controls. Body weights, organ weights, and RARβ expression were compared using unpaired t test and

Wilcoxon rank-sum test. In order to evaluate the effect of individual and combination treatments in Experiments 3, 4, and 5, a regression model using Poisson distribution was used. The intervention of the combined agents can be evaluated using this method.

In additon, longitudinal analysis was used to compare the number of cancers per rat at two time points in Experiment 2. The total numbers of cancers per rat for 70 and 140 days after MNU were calculated for each rat. The longitudinal analysis using Poisson distribution was used to compare the number of cancers per rat across the two time points.

RESULTS

In Experiments 1-5, MNU was used to induce mammary tumors in female Sprague-Dawley rats. Figure 1 illustrates a rat with a visible MNU-induced mammary cancer and histological characteristics of normal mammary gland tissue, an adenocarcinoma, and a fibroadenoma.

Experiment 1

This study was conducted to determine the effects of 9-cis-RA at various dose levels on the initiation and promotional phases of carcinogenesis in the MNU-induced mammary cancer model in older animals. Figure 2 shows the average body weights for each group throughout the study. Average body weights were elevated (t test, $p < 0.001$) in Group 4 (75 mg/kg diet) compared to Group 1 (vehicle diet). None of the other groups had differences in body weights compared to the controls. For approximately 10 days following MNU administration, body weights for all groups were reduced. Liver and uteri weights were measured in Groups 1, 3, and 5 (Table 7). Group 5 animals had a significant increase in liver weights compared to the controls (t test, $p < 0.001$), and Groups 3 and 5 had significant decreases in uteri weights compared to Group 1 (t test, $p < 0.001$).

Table 8 summarizes the overall effects from increasing dose levels of 9-cis-RA on the percentage incidence and average number per rat of adenocarcinomas. Seventy percent of the rats in Group 1 developed adenocarcinomas, with an average of 1.58

Table 7

Effect of 9-cis-RA at 60 and 150 mg/kg diet on Body, Liver, and Uterus Weights

Group	Treatment[a]	Final body weights	Liver[b,c]	Uterus[b,c]
1	Vehicle	261	3.6 ± 0.1	0.30 ± 0.01
3	9-cis-RA (60 mg/kg diet)	262	3.7 ± 0.2	0.19 ± 0.02[d]
5	9-cis-RA (150 mg/kg diet)	258	4.2 ± 0.1[d]	0.10 ± 0.04[d]

Note. 9-cis-RA = 9-cis retinoic acid. Study terminated when rats were 280 days of age.
[a] Diet supplementation with 9-cis-RA was initiated when the rats were 93 days of age. [b] Mean ± SEM. [c] Expressed as g/100 g body weight. [d] Significantly different from vehicle group ($p < 0.001$, t test).

Table 8

Effect of 9-cis-RA at Various Dose Levels on MNU-Induced Mammary Cancers in Older Sprague-Dawley Rats

Group	Number of rats	Carcinogen[a]	Treatment[b]	Adenocarcinomas Percentage incidence	Adenocarcinomas Average number/rat
1	40	MNU	Vehicle	70	1.58
2	40	MNU	9-cis-RA, 30 mg/kg diet	35[c]	0.43[d] (73%)
3	40	MNU	9-cis-RA, 60 mg/kg diet	15[c]	0.18[d] (87%)
4	40	MNU	9-cis-RA, 75 mg/kg diet	3[c]	0.03[d] (98%)
5	40	MNU	9-cis-RA, 150 mg/kg diet	0[c]	0.00[d] (100%)

Note. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea. Study was terminated 180 days after MNU administration.
[a] MNU (50 mg/kg body weight) was administered by intravenous injection to female Sprague-Dawley rats at 100 days of age.
[b] Beginning at 93 days of age, 9-cis-RA treatment was initiated. [c] $p < 0.01$, Logrank test. [d] $p < 0.01$, Armitage test.

per rat. Treating the animals with 9-cis-RA at 30, 60, 75, and 150 mg/kg diet caused the number of mammary cancers per rat to decrease by 73, 87, 98, and 100%, respectively. The control animals had 15% incidence of benign tumors, and Groups 2 and 3 had 13 and 15% incidence of benign tumors, respectively (data not shown). Groups 4 and 5 had no benign tumors.

Figure 3 indicates the percentage incidence of adenocarcinomas for each group of animals administered MNU. Groups 2, 3, and 4 did not develop cancer until 45, 85, and 90 days after MNU administration, respectively. Group 5 never developed mammary cancer. Logrank test probability values for the percentage incidence of mammary cancers in the 9-cis-RA treated groups compared to the control group were as follows: 0.01 (Group 2), 0.001 (Group 3), 0.001 (Group 4), and 0.001 (Group 5). All groups had a significant decrease in percentage incidence of mammary cancers.

The average number of mammary cancers for each group is shown in Figure 4, with Groups 2, 3, 4, and 5 having 0.43, 0.18, 0.03, and 0.00 cancers per rat, respectively, at the end of the study. The Armitage test was performed to evaluate the significance of 9-cis-RA on the average number of cancers per rat between groups. Probability values for the 9-cis-RA treated groups compared to Group 1 were as follows: 0.01 (Group 2), 0.001 (Group 3), 0.001 (Group 4), and 0.001 (Group 5). All groups had a significant reduction in the average number of mammary cancers.

Overall, increasing dietary levels of 9-cis-RA resulted in a decrease in tumor multiplicity and extended the latency time of cancer development in older Sprague-Dawley rats.

Experiment 2

This study was designed to determine the effect of 9-cis-RA on the promotional phase of carcinogenesis in young rats and to determine whether continuous supplementation of 9-cis-RA is required for its inhibitory effects to be present. After administering MNU to 50-day-old rats, 9-cis-RA was added to the diet 3 days later for either 10 or 20 weeks to determine the effectiveness of 9-cis-RA for a limited treatment time (10 weeks) and a prolonged treatment time (20 weeks). The body weights of each group throughout the study are shown in Figure 5; none of the groups had reduced weight gain. Estrus cycles were not affected by the treatment. Table 9 shows liver, uterus, and ovarian weights for Groups 1 and 3. Group 3 had slight increases in liver (t test, $p < 0.001$) and uterus weights (not significant) and a significant decrease in ovarian weight (t test, $p < 0.01$).

Control animals had 95% incidence of mammary cancers with 9.95 mammary cancers per rat (Table 10). Figure 6 shows the percentage incidence of mammary cancers for each group throughout the study. By the end of the study both groups of animals fed 9-cis-RA (Groups 2 and 3) had a 100% incidence of mammary cancers. The 9-cis-RA treated animals did not develop cancers until 40 days after MNU administration, while the control animals had 30% incidence after 40 days. 9-cis-RA extended the latency period of cancer formation.

Figure 7 depicts the average number of mammary cancers per rat throughout the study. At the end of the study, Groups 2 and 3 had an average of 6.95 (a 30% reduction compared to Group 1) and 4.30 (a 57% reduction compared to Group 1) mammary cancers per rat, respectively. Five weeks after removing 9-cis-RA from the diet (105

Table 9

Effect of 9-cis-RA on Organ Weights in Young Sprague-Dawley Rats After 20 Weeks of Treatment

Group	Treatment[a]	Liver[b,c]	Uterus[b,c]	Ovary[b,c]
1	Teklad (4%) diet	3.2 ± 0.0	0.18 ± 0.02	0.045 ± 0.001
3	9-cis-RA (60 mg/kg diet) - 20 weeks	3.8 ± 0.1[d]	0.19 ± 0.01	0.038 ± 0.002[d]

Note. 9-cis-RA = 9-cis retinoic acid. Study terminated when rats were 180 days of age.
[a] Diet supplementation with 9-cis-RA was initiated when the rats were 53 days of age. [b] Mean ± SEM. [c] Expressed as g/100 g body weight. [d] Significantly different from vehicle group ($p < 0.01$, t test).

Table 10

Effect of Limited and Continuous Treatment With 9-cis-RA on MNU-Induced Mammary Cancers in Young Rats

Group	Number of rats	Carcinogen[a]	Treatment[b]	Adenocarcinomas[c] Percentage incidence	Adenocarcinomas[c] Average number/rat
1	25	MNU	Teklad (4%) diet	95	9.95
2	25	MNU	9-cis-RA, 60 mg/kg diet--10 weeks	100	6.95
3	24	MNU	9-cis-RA, 60 mg/kg diet--20 weeks	100	4.30[d]

Note. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

[a] MNU (50 mg/kg body weight) was administered to female Sprague-Dawley rats at 50 days of age. [b] Beginning at 53 days of age, 9-cis-RA was administered in the diet. [c] Incidence and number of adenocarcinomas at the end of the study (140 days after MNU). [d] $p < 0.01$, Armitage test.

days after MNU), Group 2 had an increase in tumor multiplicity compared to Group 3 (3.45 and 2.60, respectively), and this trend continued throughout the remainder of the study (6.95 and 4.30 at the end of the study, respectively).

By employing a change-point test, two time points (70 and 140 days after MNU) showing significant changes in the number of cancers during the study were determined. At 70 days after dosing with MNU, Group 2 had 9-cis-RA removed from the diet. Poisson distribution evaluating the two time points showed that there was a significant difference across the three groups. Groups 2 and 3 were significantly different from Group 1 at 70 days after MNU ($p < 0.001$), but these two groups were not different from one another ($p = 0.1053$). However, at 140 days after MNU, Group 2 had a significant increase in mammary cancers compared to Group 3 ($p < 0.001$), and there was no difference between Groups 1 and 2 ($p = 0.2935$). At the end of the study, Group 3 had a significant reduction in mammary cancers compared to Group 1 ($p < 0.001$).

In summary, continued treatment with 9-cis-RA (Group 3) was required to cause a significant reduction in multiplicity of mammary cancers. Limited treatment with 9-cis-RA did not result in a significant reduction of mammary cancers.

Experiment 3

This study was designed to look at the effects of 9-cis-RA and 4-HPR, alone and in combination, on the prevention of the promotional phase of carcinogenesis in the MNU-induced mammary cancer model in young rats. For all of the groups at the end of the study, body weights were not affected by the agents, but animals fed 4-HPR alone did have significant decreased body weight gain for most of the study (Figure 8). Table 11

Table 11

Effect of 9-cis-RA Alone and Combined With 4-HPR on Organ Weights in Young Sprague-Dawley Rats

Group	Treatment[a]	Liver[b]	Uterus[b]	Ovary[b]
1	Vehicle	3.1 ± 0.1	0.22 ± 0.02	0.042 ± 0.003
2	9-cis-RA (60 mg/kg diet)	3.6 ± 0.1[c]	0.18 ± 0.01	0.043 ± 0.004
3	4-HPR (586 mg/kg diet)	3.6 ± 0.1[c]	0.25 ± 0.02	0.035 ± 0.003[c]
4	9-cis-RA (60 mg/kg diet) + 4-HPR (586 mg/kg diet)	3.5 ± 0.1[c]	0.18 ± 0.02	0.040 ± 0.003

Note. 9-cis-RA = 9-cis retinoic acid; 4-HPR = 4-hydroxyphenylretinamide. Study was terminated when rats were 200 days of age. [a] Diet supplementation with the chemopreventive agents initiated when the rats were 53 days of age. [b] Mean ± SEM and expressed as g/100 g body weight. [c] Significantly different from Group 1 ($p < 0.05$, Wilcoxon rank-sum test).

shows liver, uterus, and ovarian weights for each group. 9-cis-RA and 4-HPR alone and combined caused increases in liver weights compared to controls (t test, $p < 0.05$). 4-HPR caused a significant decrease in ovary weights compared to controls (t test, $p < 0.05$).

The average number of adenocarcinomas and benign tumors as well as percentage incidence of each is summarized in Table 12. Control animals developed an average of 6.68 cancers per rat. 9-cis-RA or 4-HPR, alone, caused 23 and 16% decreases in the number of cancers per rat, respectively. Figure 9 reveals the effects of 9-cis-RA and 4-HPR, alone and in combination, on the multiplicity of mammary cancer development, with the combination group having only 3.42 cancers per rat (a 49% reduction compared to the control group). The Armitage test revealed that at the end of the study the combination group had a significant decrease in mammary cancers per rat ($p < 0.01$).

Percentage incidence of mammary cancers for each group is depicted in Figure 10. Groups 1, 2, 3, and 4 had 100, 96, 96, and 75% incidence of mammary cancers, respectively. At 75 days after MNU administration, all of the treated groups had approximately 50% incidence, and the control group had 80% incidence. The latency time for cancer development was extended in all of the treated groups. A Logrank test compared percentage incidence of mammary cancers between groups at the end of the study. The analysis showed that the combination group had a significant reduction in percentage incidence of cancers ($p < 0.01$).

To determine if there was a significant interaction between 9-cis-RA and 4-HPR, a regression model using Poisson distribution was performed. The results from this

Table 12

Effect of 9-cis-RA Alone and Combined With 4-HPR on the Percent Incidence and Number of MNU-Induced Mammary Cancers in Young Sprague-Dawley Rats

				Adenocarcinomas[c]		Benign mammary tumors[c]	
Group	Number of rats	Carcinogen[a]	Treatment[b]	Percentage incidence	Average number/rat	Percentage incidence	Average number/rat
1	25	MNU	Vehicle	100	6.68	4	0.04
2	25	MNU	9-cis-RA, 60 mg/kg diet	96	5.12	8	0.08
3	25	MNU	4-HPR, 586 mg/kg diet	96	5.56	4	0.04
4	24	MNU	9-cis-RA, 60 mg/kg diet + 4-HPR, 586 mg/kg diet	75[d]	3.42[e]	8	0.08

Note. 9-cis-RA = 9-cis retinoic acid; 4-HPR = 4-hydroxyphenylretinamide; MNU = N-methyl-N-nitrosourea.
[a] MNU (50 mg/kg body weight) was administered to female Sprague-Dawley rats at 50 days of age. [b] Beginning at 53 days of age, the retinoids were administered in the diet. [c] Incidence and number of adenocarcinomas and benign tumors at the end of study (150 days after MNU). [d] $p < 0.01$, Logrank test. [e] $p < 0.01$, Armitage test.

analysis revealed that there was not a significant interaction between 9-cis-RA and 4-HPR ($p = 0.2211$). During the study, the two agents were as effective alone as they were combined. At the end of the study, the combination of the agents did have a significant effect on the final number of cancers per rat, but there was no significant interaction between the two agents.

Experiment 4

This study was performed in order to evaluate the effectiveness of 9-cis-RA and 4-HPR, alone and in combination, on the MNU-induced mammary cancer model in older rats. By feeding the animals the chemopreventive agents prior to administration of MNU, this experiment evaluated the effects of these agents on the initiation and promotional phases of carcinogenesis.

Body weights varied among groups, with the group receiving the combination having a higher average body weight than the other groups (Figure 11). Table 13 summarizes the organ weights that were recorded at necropsy (data for all groups were recorded, except for Group 2). Liver weights were slightly increased in the animals treated with 4-HPR, alone and combined with 9-cis-RA. Uterus weights were decreased in the combination group, but ovarian weights were not different among groups.

The percentage incidence and average number of adenocarcinomas and benign tumors per rat are shown in Table 14. Figure 12 indicates the percentage incidence of mammary cancers among the groups. Groups 1, 2, 3, and 4 had incidences of 57, 35, 43, and 5%, respectively. Groups 1 and 3 developed palpable tumors at 30 days after MNU. Groups 2 and 4 showed tumor induction at 40 and 155 days after MNU, respectively.

Table 13

Effect of 9-cis-RA Alone and Combined With 4-HPR on Organ Weights in Older Sprague-Dawley Rats

Group	Treatment[a]	Liver[b]	Uterus[b]	Ovary[b]
1	Vehicle	3.1 ± 0.1	0.26 ± 0.03	0.037 ± 0.001
3	4-HPR (196 mg/kg diet)	3.4 ± 0.1[c]	0.24 ± 0.03	0.035 ± 0.003
4	9-cis-RA (30 mg/kg diet) + 4-HPR (196 mg/kg diet)	3.5 ± 0.1[c]	0.20 ± 0.02[c]	0.038 ± 0.003

Note. 9-cis-RA = 9-cis retinoic acid; 4-HPR = 4-hydroxyphenylretinamide. Study was terminated when rats were 280 days of age. [a] Diet supplementation with the chemopreventive agents was initiated when the rats were 93 days of age. [b] Mean ± SEM and expressed as g/100 g body weight. [c] Significantly different from Group 1 ($p < 0.01$, Wilcoxon rank-sum test).

Table 14

Effect of 9-cis-RA Alone and Combined With 4-HPR on the Percent Incidence and Number of MNU-Induced Mammary Cancers in Older Sprague-Dawley Rats

Group	Number of rats	Carcinogen[a]	Treatment[b]	Adenocarcinomas[c] Percentage incidence	Adenocarcinomas[c] Average number/rat	Benign mammary tumors[c] Percentage incidence	Benign mammary tumors[c] Average number/rat
1	40	MNU	Vehicle	57	1.12	13	0.13
2	40	MNU	9-cis-RA, 30 mg/kg diet	35[d]	0.43[e]	13	0.13
3	40	MNU	4-HPR, 196 mg/kg diet	43[d]	0.60[e]	10	0.10
4	39	MNU	9-cis-RA, 30 mg/kg diet + 4-HPR, 196 mg/kg diet	5[d]	0.05[e]	3[d]	0.03[e]

Note. 9-cis-RA = 9-cis retinoic acid; 4-HPR = 4-hydroxyphenylretinamide; MNU = N-methyl-N-nitrosourea.
[a] MNU (50 mg/kg body weight) was administered by intravenous injection to female Sprague-Dawley rats at 100 days of age.
[b] Beginning at 93 days of age, the retinoids were administered in the diet. [c] Incidence and number of adenocarcinomas and benign tumors at the end of study (180 days after MNU). [d] $p < 0.01$, Logrank test. [e] $p < 0.01$, Armitage test.

When combined, the agents caused an extended latency period. The Logrank test showed that, at the end of the study, the groups on chemopreventive agents had significant reductions in percentage mammary cancer incidence ($p < 0.01$).

Figure 13 depicts the average number of adenocarcinomas per rat in each group throughout the study. Group 2 had a 62% decrease in the average number of mammary cancers per rat compared to Group 1, and Group 3 had a 46% decrease. A 96% decrease was found in Group 4, which developed only 0.05 mammary cancers per rat. Cancer multiplicity was significantly decreased in the treated groups. The Armitage test revealed that the three treated groups had significant reductions in mammary cancers per rat ($p < 0.01$) compared to the control group at the end of the study.

Using a regression model with Poisson distribution, the interaction between 9-cis-RA and 4-HPR was evaluated during the study. Results showed that the agents did have a significant interaction ($p = 0.042$). The effect of 9-cis-RA on the number of cancers per rat differs according to the level of 4-HPR. Likewise, effects of 4-HPR are different when 9-cis-RA is combined with it. The average numbers of cancers per rat were 1.12, 0.43, 0.60, and 0.05 for Groups 1, 2, 3, and 4, respectively. These averages indicate that 9-cis-RA and 4-HPR alone significantly reduced mammary cancers; the combination of these agents resulted in a significant reduction in the multiplicity of mammary cancers.

Experiment 5

This study was accomplished to investigate the effects of 9-cis-RA and vitamin D3, alone and in combination, on the promotional phase of carcinogenesis in young rats. Body weights did not differ among groups throughout the study (Figures 14 and 15).

Table 15 summarizes the organ weights from each group. Liver weights were significantly increased in Groups 2 and 5. Uterus weights were significantly decreased in Group 2. Ovarian weights did not differ in any group compared to the controls.

Table 16 depicts the effects of 9-cis-RA and vitamin D3, alone and combined, on percentage incidence and average number per rat of adenocarcinomas and benign tumors. Percentage incidence among the groups is presented in Figures 16 and 17. At the end of the study, Groups 1, 3, and 4 had a 100% incidence, and Groups 2, 5, and 6 had 96, 83, and 96% incidences, respectively. A Logrank test showed that, at the end of the study, Group 5 had a significant reduction in percentage incidence compared to Group 1 ($p < 0.01$). Sixty days after MNU, Groups 2 and 3 had 25 and 40% incidences of mammary cancer. The combination group (Group 5) had only a 5% incidence of mammary cancer, while the control group had 65% cancer incidence. These results for percentage incidence at 60 days after MNU treatment indicate that the combination of 9-cis-RA and vitamin D3 increased the latency period of mammary cancer development in young rats.

Figures 18 and 19 indicate the effects of these agents on the average number of cancers per rat throughout the study. The control group had 6.68 cancers per rat; the 9-cis-RA treated animals (Group 2) had 5.16 cancers per rat (a 23% reduction compared to Group 1). Animals treated with vitamin D3 (Groups 3 and 4) had 7.84 and 7.72 cancers per rat, respectively (17 and 16% enhancement compared to Group 1, respectively). The combination of 9-cis-RA and vitamin D3 (Groups 5 and 6) caused 44 and 37% reductions in the number of cancers per rat, respectively. The Armitage test revealed that the reductions in the number of cancers per rat in the combination groups at the end of the study were significant compared to the controls ($p < 0.01$). Vitamin D3, alone, enhanced

Table 15

Effect of 9-cis-RA Alone and Combined With Vitamin D3 on Organ Weights in Young Sprague-Dawley Rats

Group	Treatment[a]	Liver[b,c]	Uterus[b,c]	Ovary[b,c]
1	Vehicle	3.1 ± 0.1	0.22 ± 0.02	0.042 ± 0.003
2	9-cis-RA (60 mg/kg diet)	3.6 ± 0.1[d]	0.18 ± 0.01[d]	0.043 ± 0.004
3	Vitamin D3 (10 µg/kg diet)	3.1 ± 0.1	0.21 ± 0.01	0.039 ± 0.003
5	9-cis-RA (60 mg/kg diet) + Vitamin D3 (10 µg/kg diet)	3.7 ± 0.0[d]	0.19 ± 0.01	0.038 ± 0.003

Note. 9-cis-RA = 9-cis retinoic acid. Study was terminated when rats were 200 days of age.
[a] Diet supplementation with the chemopreventive agents was initiated when the rats were 53 days of age. [b] Expressed as g/100 g body weight. [c] Mean ± SEM. [d] Unpaired t test ($p < 0.05$).

Table 16

Effect of 9-cis-RA Alone and Combined With Vitamin D3 on the Percent Incidence and Number of MNU-Induced Mammary Cancers in Young Sprague-Dawley Rats

			Adenocarcinomas[c]		Benign Mammary Tumors[c]	
Group[a]	Number of rats	Treatment[b]	Percentage incidence	Average number/rat	Percentage incidence	Average number/rat
1	25	Vehicle	100	6.68	4	0.04
2	25	9-cis-RA (60 mg/kg of diet)	96	5.16	8	0.08
3	25	Vitamin D3 (10 µg/kg of diet)	100	7.84	8	0.08
4	25	Vitamin D3 (3.3 µg/kg of diet)	100	7.72	16	0.16
5	25	9-cis-RA (60 mg/kg of diet) + Vitamin D3 (10 µg/kg of diet)	83[d]	3.71[d]	4	0.04
6	25	9-cis-RA (60 mg/kg of diet) + Vitamin D3 (3.3 µg/kg of diet)	96	4.21[d]	8	0.08

Note. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.
[a] Each animal was given MNU (50 mg/kg BW) at 50 days of age by intravenous injection. [b] Female Sprague-Dawley rats were administered the chemopreventive agents in the diet beginning at 53 days of age. [c] Study was terminated at 150 days after MNU.
[d] Statistically different from Group 1 (Incidence, Logrank, $p < 0.01$; multiplicity, Armitage, $p < 0.01$).

mammary cancer multiplicity, but 9-cis-RA, alone, reduced the number of cancers. Combining the agents enhanced the inhibition of carcinogenesis greater than 9-cis-RA alone.

A regression model with Poisson distribution was applied to determine if the 9-cis-RA and vitamin D3 had a significant interaction during the study. Results showed that neither dose level of vitamin D3 was effective at inhibiting mammary cancers but that 9-cis-RA was an effective inhibitor ($p < 0.001$). The interaction of vitamin D3 and 9-cis-RA was significant in preventing mammary cancers for both dose levels of vitamin D3 ($p = 0.0127$, 3.3 μg/kg diet group; $p = 0.0046$, 10 μg/kg diet group). The average numbers of cancers per rat were 6.68, 5.16, 7.84, 7.72, 3.71, and 4.21 in Groups 1, 2, 3, 4, 5, and 6, respectively. Prevention with 9-cis-RA alone was effective, but combining vitamin D3 with 9-cis-RA enhanced the effect significantly.

Experiment 6

This study was conducted to determine the effects of 9-cis-RA on mammary gland morphology and to analyze the RARβ mRNA expression level in isolated mammary epithelial cells. After receiving a diet supplemented with 9-cis-RA for 3 days, the rats in Groups 2 and 3 did not show any reduced body weight gain compared to the control group (Group 1), and liver weights were not different between Groups 1, 2, and 3 (Table 17). As shown in Figure 20, animals fed 9-cis-RA for 4 weeks (Groups 5 and 6) did not differ in body weight from the control group (Group 4). Groups 5 and 6 had significant increases in liver weight. Group 6 had a slight decrease in uterus and ovarian weights compared to Group 4 (Table 18). Levels of retinyl palmitate in the liver were

Table 17

Effect of 3 Days of Treatment With 9-cis-RA on Body and Liver Weights of Sprague-Dawley Rats

Group	Treatment[a]	Body weights[b]	Liver[b,c]
1	Teklad (4%) diet	156 ± 2	4.9 ± 0.1
2	9-cis-RA (60 mg/kg diet)	161 ± 2	4.9 ± 0.1
3	9-cis-RA (120 mg/kg diet)	163 ± 3	4.9 ± 0.1

Note. 9-cis-RA = 9-cis retinoic acid. Study terminated after 3 days of feeding.
[a] Diet supplementation with 9-cis-RA was initiated when the rats were 47 days of age. [b] Mean ± SEM. [c] Expressed as g/100 g body weight.

Table 18

Effect of 4 Weeks of Treatment With 9-cis-RA on Organ Weights of Sprague-Dawley Rats

Group	Treatment[a]	Body weights[b]	Liver[b,c]	Uterus[b,c]	Ovary[b,c]
4	Teklad (4%) diet	212 ± 4	3.9 ± 0.1	0.19 ± 0.01	0.067 ± 0.004
5	9-cis-RA (60 mg/kg diet)	208 ± 5	4.4 ± 0.1[d]	0.19 ± 0.01	0.068 ± 0.004
6	9-cis-RA (120 mg/kg diet)	206 ± 4	4.4 ± 0.1[d]	0.15 ± 0.01	0.062 ± 0.003

Note. 9-cis-RA = 9-cis retinoic acid. Study terminated after 4 weeks of feeding.
[a] Diet supplementation with 9-cis-RA was initiated when the rats were 47 days of age. [b] Mean ± SEM. [c] Expressed as g/100 g body weight. [d] Significantly different from Group 4 (t test, $p < 0.001$).

measured for all groups (Tables 19 and 20). Groups 5 and 6 had significant decreases in retinyl palmitate compared to Group 4 (Figure 21).

Autoradiographs showing levels of RARβ mRNA in the isolated mammary epithelial cells from the rats fed 9-cis-RA for 3 days and 4 weeks are shown in Figure 22. Tables 21 and 22 summarize the RARβ expression levels from each group. Groups 2 and 3 had slightly reduced levels of RARβ mRNA compared to Group 1. Groups 5 and 6 had a slight reduction in RARβ mRNA expression compared to Group 4. Treating rats with 9-cis-RA, for 3 days or 4 weeks, did not cause a significant change in RARβ mRNA expression levels.

Table 19

Average Levels of Retinyl Palmitate in Livers of 50-Day-Old Female Sprague-Dawley Rats After a 3-Day Feeding of 9-cis-RA

Group	Treatment[a]	Number of rats	Retinyl palmitate μg/g liver[b]
1	Teklad diet only	10	319.01± 11.61
2	9-cis-RA, 60 mg/kg diet	10	309.63 ± 4.21
3	9-cis-RA, 120 mg/kg diet	10	301.94 ± 11.93

Note. 9-cis-RA = 9-cis retinoic acid.
[a] Diet supplementation with 9-cis-RA when rats were 47 days of age. [b] Mean ± SEM.

Table 20

Average Levels of Retinyl Palmitate in Livers of 75-Day-Old Female Sprague-Dawley Rats After a 4-Week Feeding of 9-cis-RA

Group	Treatment[a]	Number of rats	Retinyl palmitate µg/g liver[b]
4	Teklad diet only	10	567.64 ± 20.63
5	9-cis-RA, 60 mg/kg diet	10	416.63 ± 11.67[c]
6	9-cis-RA, 120 mg/kg diet	10	346.94 ± 10.42[c,d]

Note. 9-cis-RA = 9-cis retinoic acid.

[a] Diet supplementation with 9-cis-RA when rats were 47 days of age. [b] Mean ± SEM. [c] Significantly different from Group 4 (t test, $p < 0.0001$). [d] Significantly different from Group 3 (t test, $p < 0.001$).

Table 21

Average Expression Levels of RARβ mRNA From Mammary Epithelial Cells of 50-Day-Old Female Sprague-Dawley Rats After a 3-Day Feeding of 9-cis-RA

Group	Treatment[a]	Number of rats	RARβ mRNA ratio (RARβ/18S)
1	Teklad diet only	5	1.54 ± 0.01
2	9-cis-RA, 60 mg/kg diet	5	1.49 ± 0.03
3	9-cis-RA, 120 mg/kg diet	5	1.44 ± 0.04

Note. 9-cis-RA = 9-cis retinoic acid.
[a] Diet supplementation with 9-cis-RA when rats were 47 days of age. [b] Mean ± SEM.

Table 22

Average Expression Levels of RARβ mRNA From Mammary Epithelial Cells of 75-Day-Old Female Sprague-Dawley Rats After a 4-Week Feeding of 9-cis-RA

Group	Treatment[a]	Number of rats	RARβ mRNA ratio (RARβ/18S)
4	Teklad diet only	5	1.58 ± 0.04
5	9-cis-RA, 60 mg/kg diet	5	1.45 ± 0.04
6	9-cis-RA, 120 mg/kg diet	5	1.48 ± 0.03

Note. 9-cis-RA = 9-cis retinoic acid.
[a] Diet supplementation with 9-cis-RA when rats were 47 days of age. [b] Mean ± SEM.

Figure 1. **Visible methylnitrosourea (MNU)-induced mammary cancer on Sprague-Dawley rat (A) and photomicrographs of normal mammary gland (B), an MNU-induced mammary adenocarcinoma (C), and a fibroadenoma (D).**

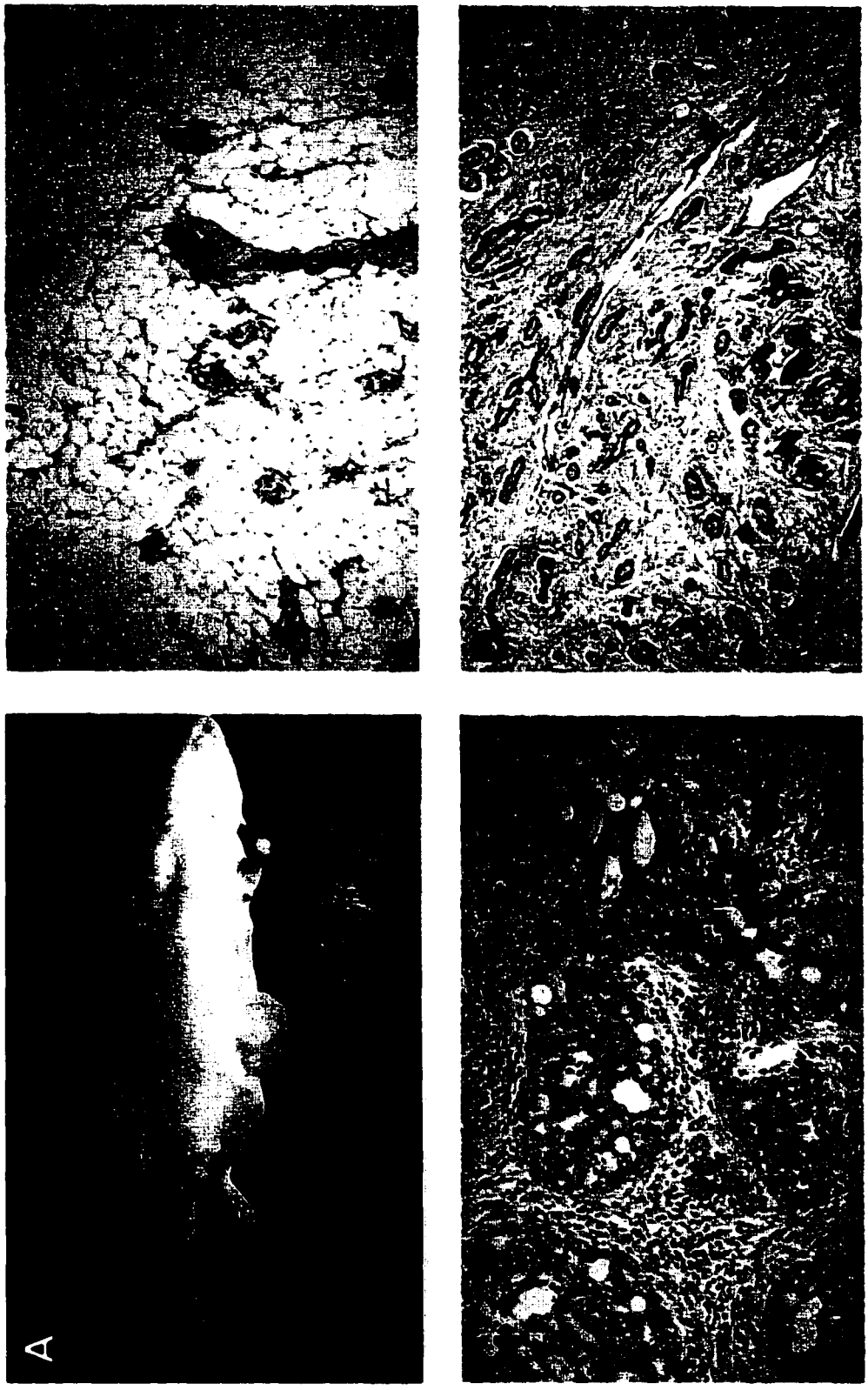


Figure 2. Average body weight (in grams) of rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 60 mg/kg diet (Group 3), 75 mg/kg diet (Group 4), and 150 mg/kg diet (Group 5). Treatment with 9-cis-RA was initiated at 93 days of age. MNU (50 mg/kg body weight) was administered at 100 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

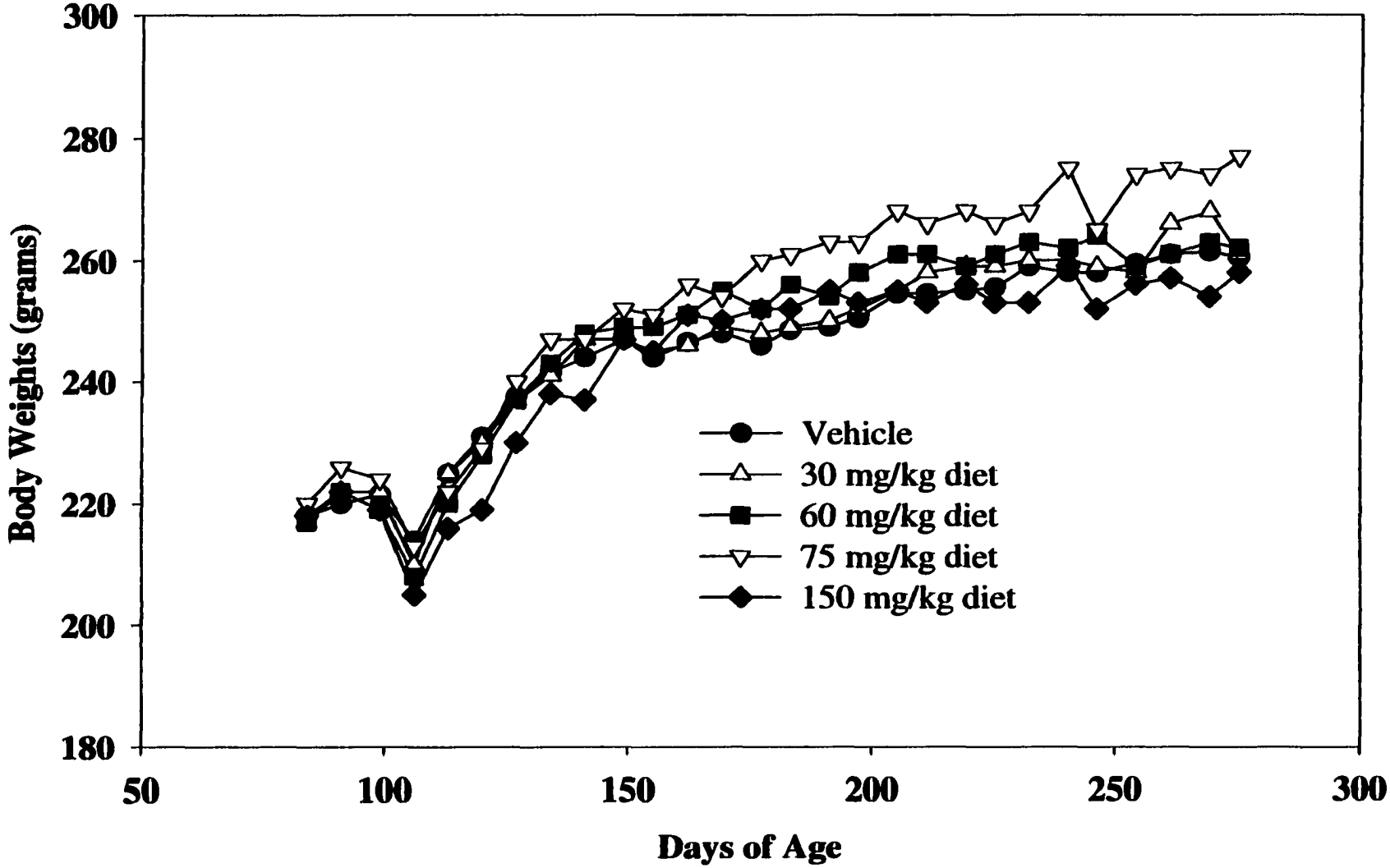


Figure 3. Percentage of mammary cancer incidence in rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 60 mg/kg diet (Group 3), 75 mg/kg diet (Group 4), and 150 mg/kg diet (Group 5). Treatment with 9-cis-RA was initiated at 93 days of age. MNU (50 mg/kg body weight) was administered at 100 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

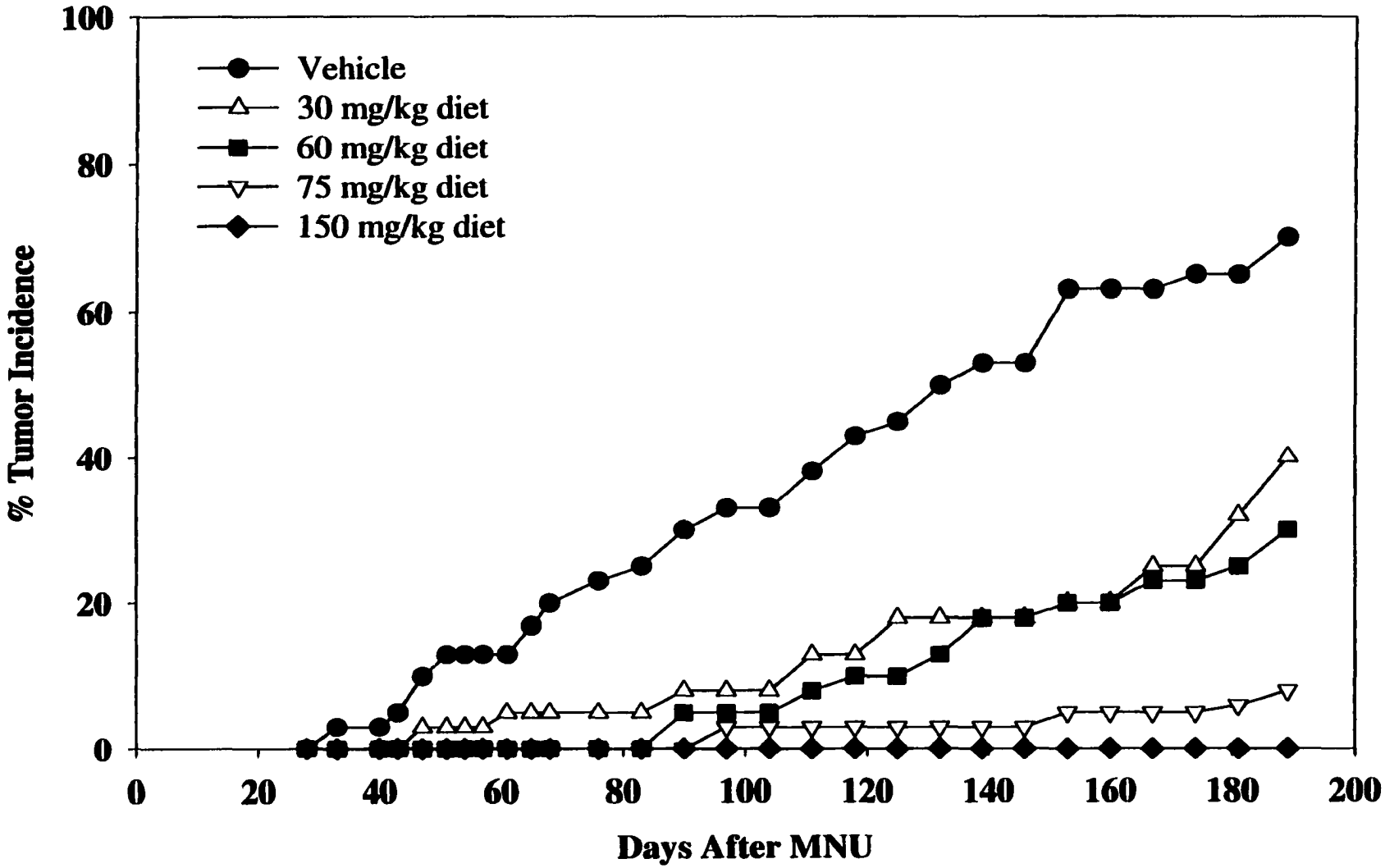


Figure 4. Average number of mammary cancers per rat receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 60 mg/kg diet (Group 3), 75 mg/kg diet (Group 4), and 150 mg/kg diet (Group 5). Treatment with 9-cis-RA was initiated at 93 days of age. MNU (50 mg/kg body weight) was administered at 100 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

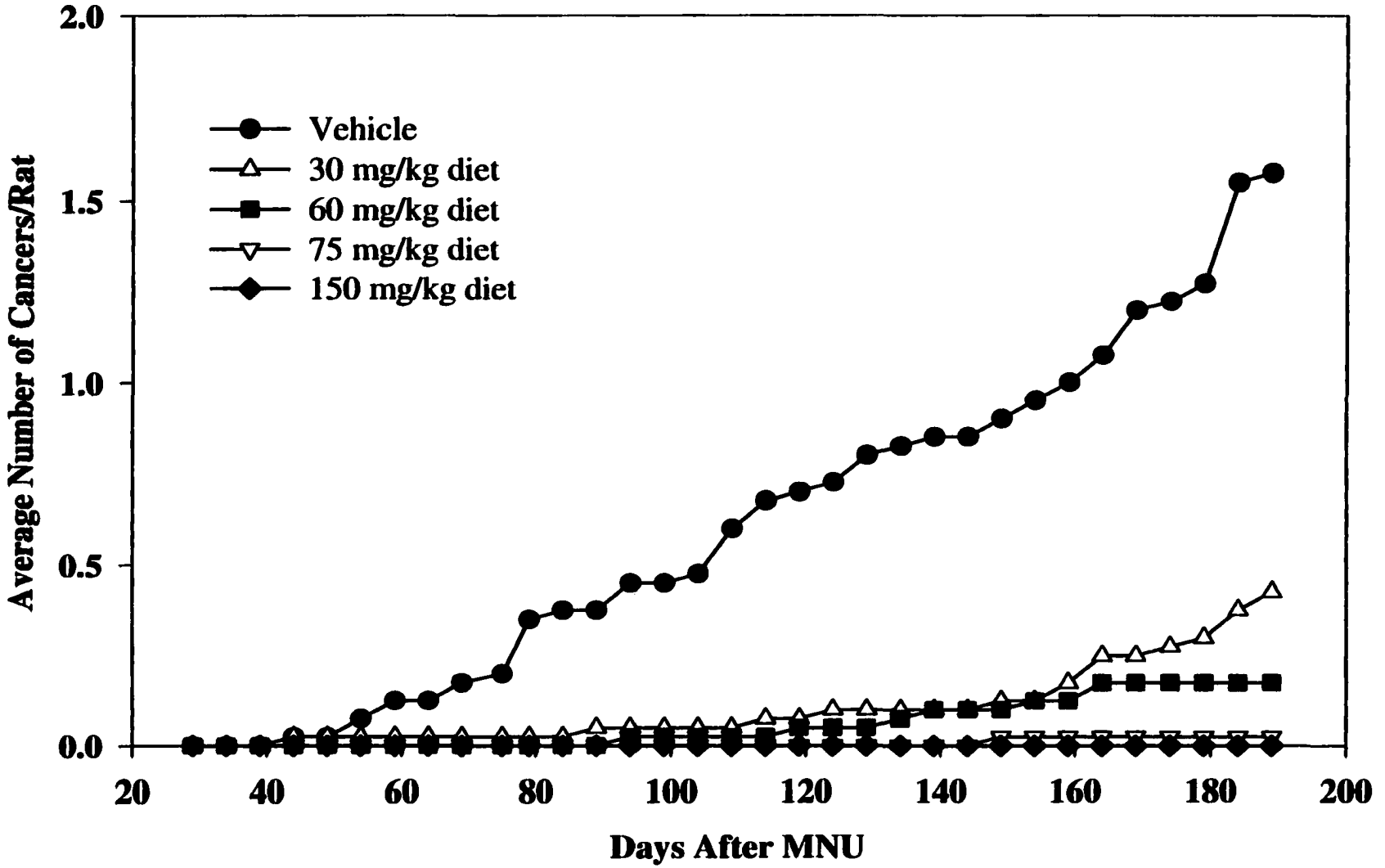


Figure 5. Average body weight (in grams) of rats receiving Teklad diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet for 10 weeks (Group 2), and 60 mg/kg diet for 20 weeks (Group 3). Treatment with 9-cis-RA was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

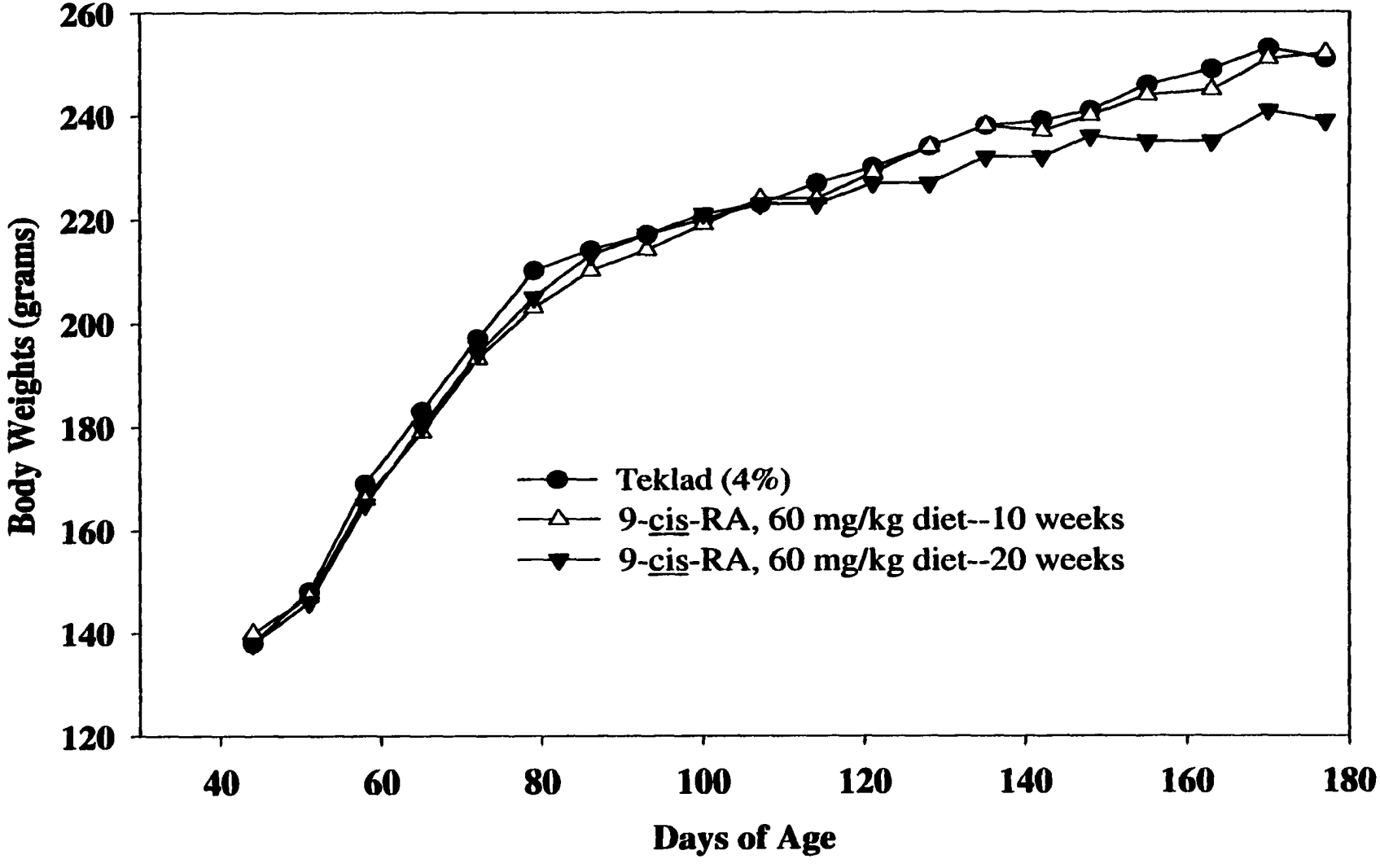


Figure 6. Percentage of mammary cancer incidence in rats receiving Teklad diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet for 10 weeks (Group 2), and 60 mg/kg diet for 20 weeks (Group 3). Treatment with 9-cis-RA was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

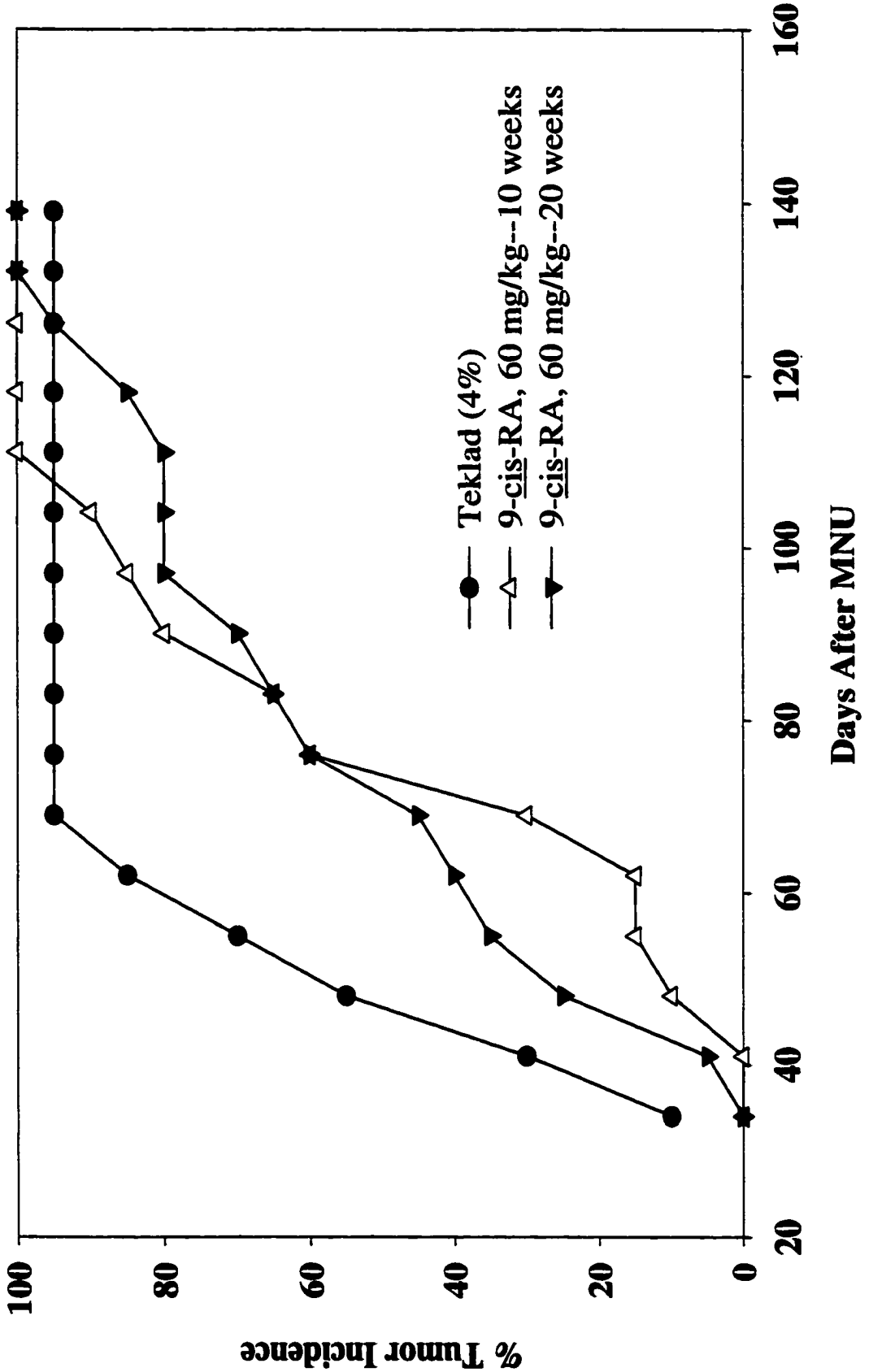


Figure 7. Average number of mammary cancers per rat receiving Teklad diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet for 10 weeks (Group 2), and 60 mg/kg diet for 20 weeks (Group 3). Treatment with 9-cis-RA was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

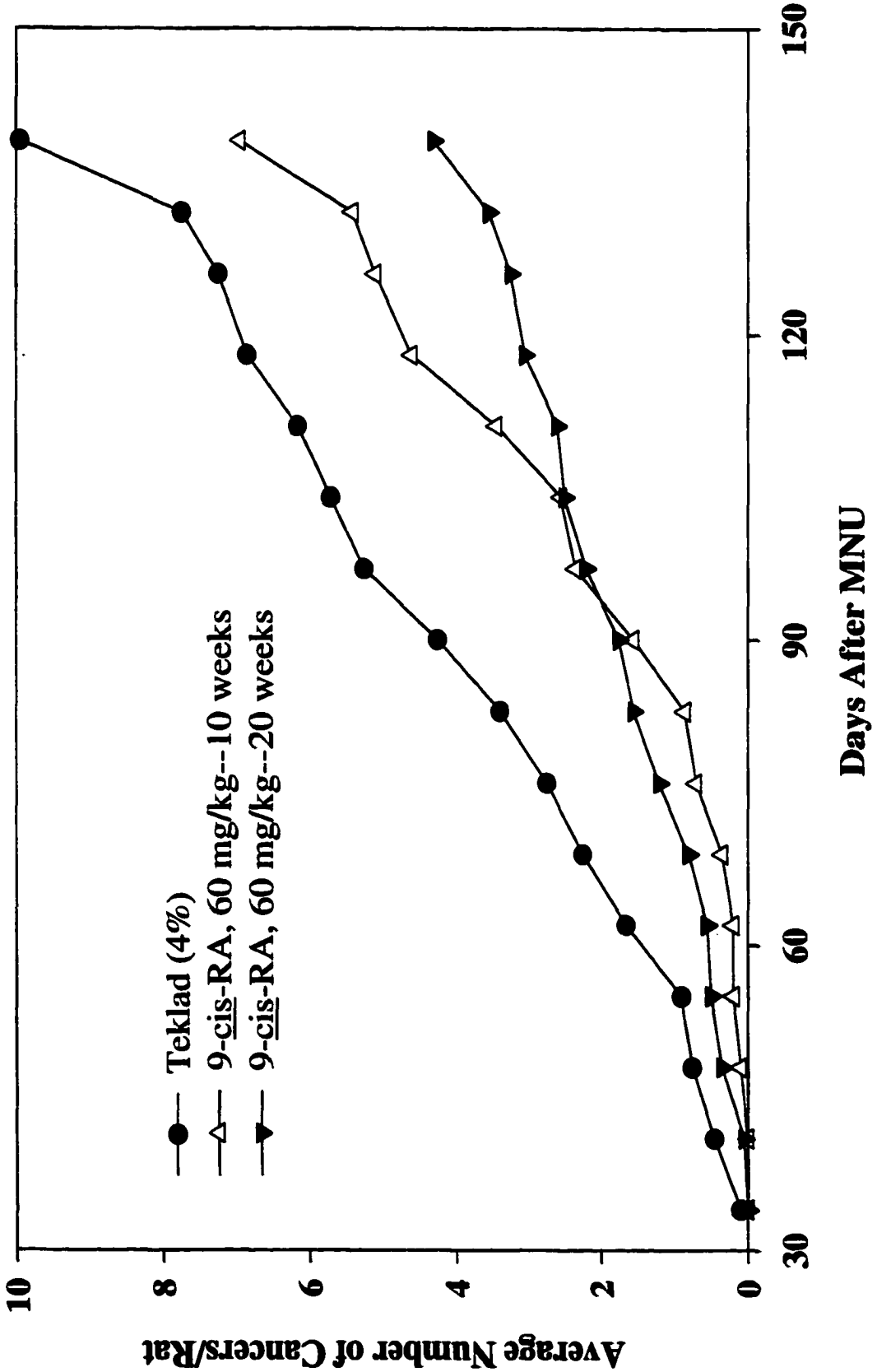


Figure 8. Average body weight (in grams) of rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), 4-HPR: 586 mg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and 4-HPR: 586 mg/kg diet (Group 4). Treatment with chemopreventive agents was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; 4-HPR= 4-hydroxyphenylretinamide; MNU = N-methyl-N-nitrosourea.

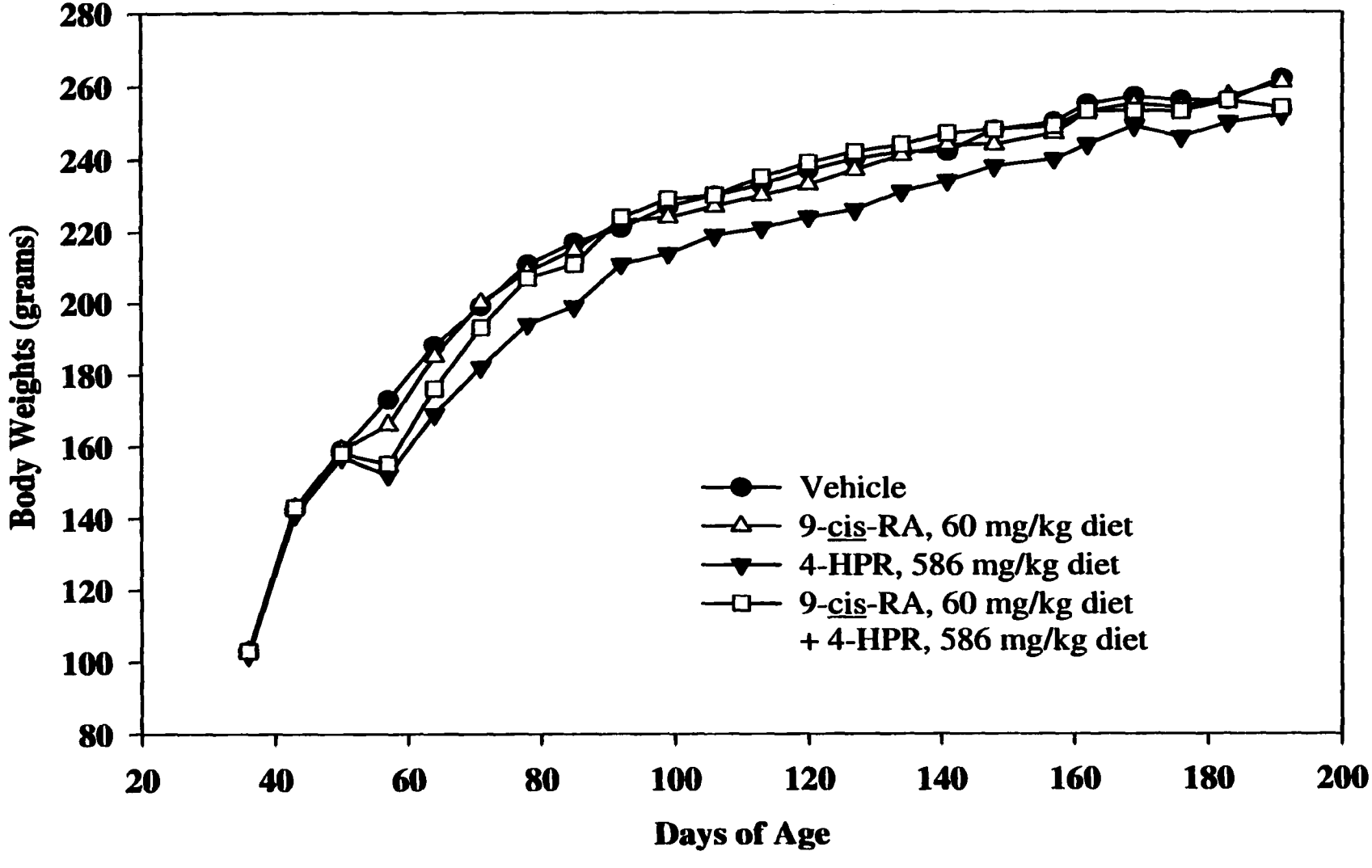


Figure 9. Average number of mammary cancers per rat receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), 4-HPR: 586 mg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and 4-HPR: 586 mg/kg diet (Group 4). Treatment with chemopreventive agents was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; 4-HPR= 4-hydroxyphenylretinamide; MNU = N-methyl-N-nitrosourea.

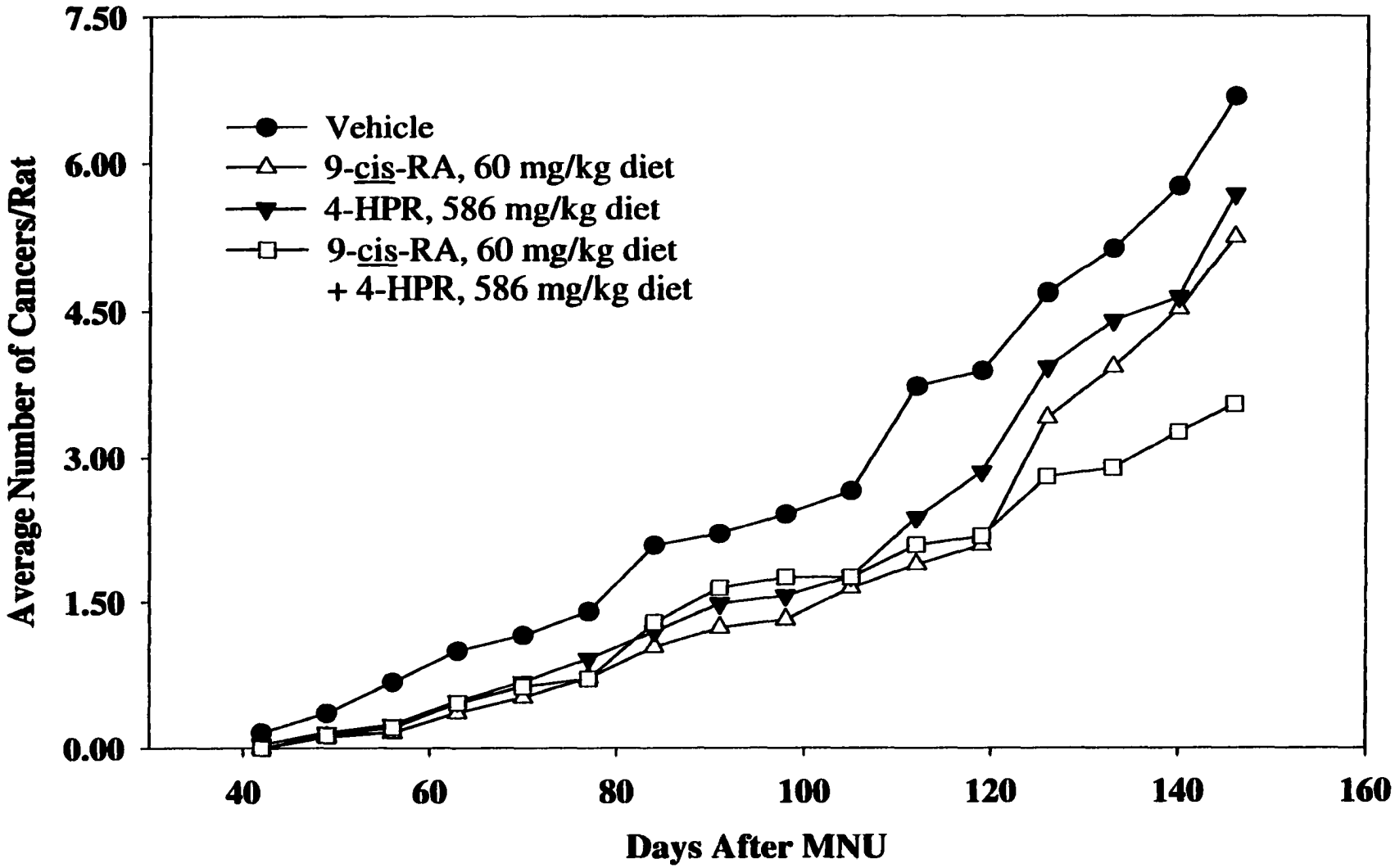


Figure 10. Percentage of mammary cancer incidence in rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), 4-HPR: 586 mg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and 4-HPR: 586 mg/kg diet (Group 4). Treatment with chemopreventive agents was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic Acid; 4-HPR= 4-hydroxyphenylretinamide; MNU = N-methyl-N-nitrosourea.

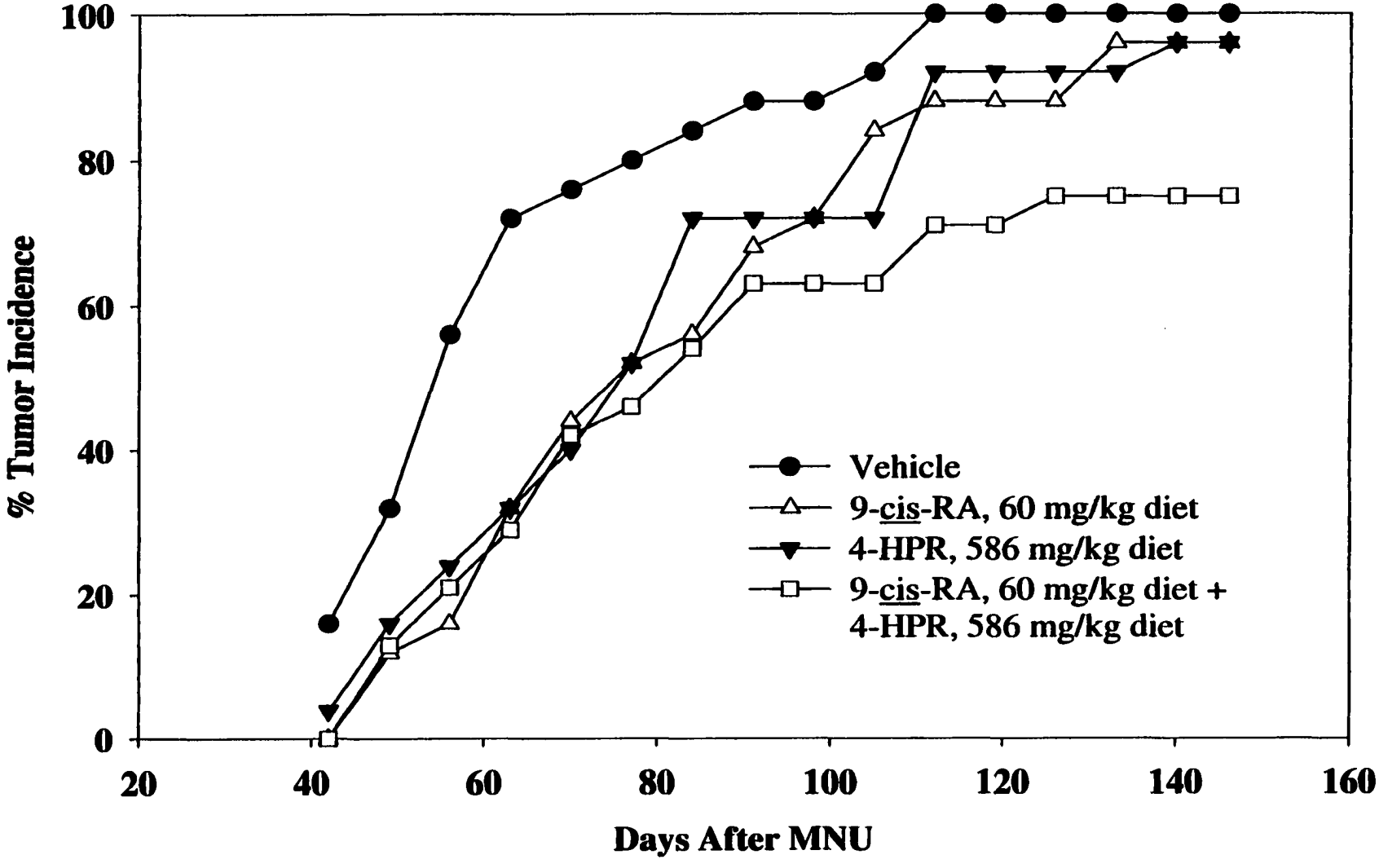


Figure 11. Average body weight (in grams) of rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 4-HPR: 196 mg/kg diet (Group 3), and 9-cis-RA: 30 mg/kg diet and 4-HPR: 196 mg/kg diet (Group 4). Treatment with chemo-preventive agents was initiated at 93 days of age. MNU (50 mg/kg body weight) was administered at 100 days of age. 9-cis-RA = 9-cis retinoic acid; 4-HPR= 4-hydroxyphenylretinamide; MNU = N-methyl-N-nitrosourea.

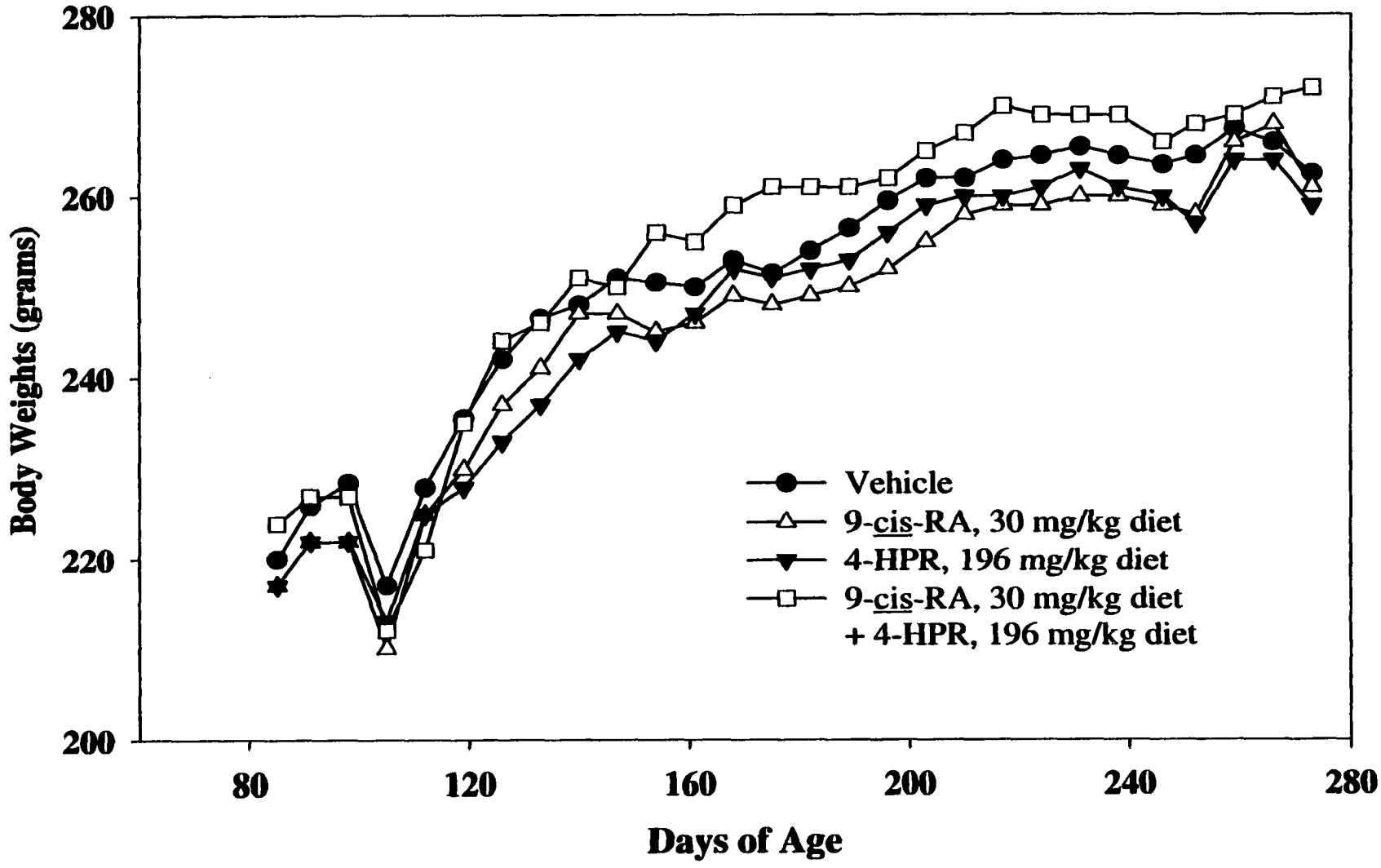


Figure 12. Percentage of mammary cancer incidence in rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 4-HPR: 196 mg/kg diet (Group 3), and 9-cis-RA: 30 mg/kg diet and 4-HPR: 196 mg/kg diet (Group 4). Treatment with chemopreventive agents was initiated at 93 days of age. MNU (50 mg/kg body weight) was administered at 100 days of age. 9-cis-RA = 9-cis retinoic acid; 4-HPR= 4-hydroxyphenylretinamide; MNU = N-methyl-N-nitrosourea.

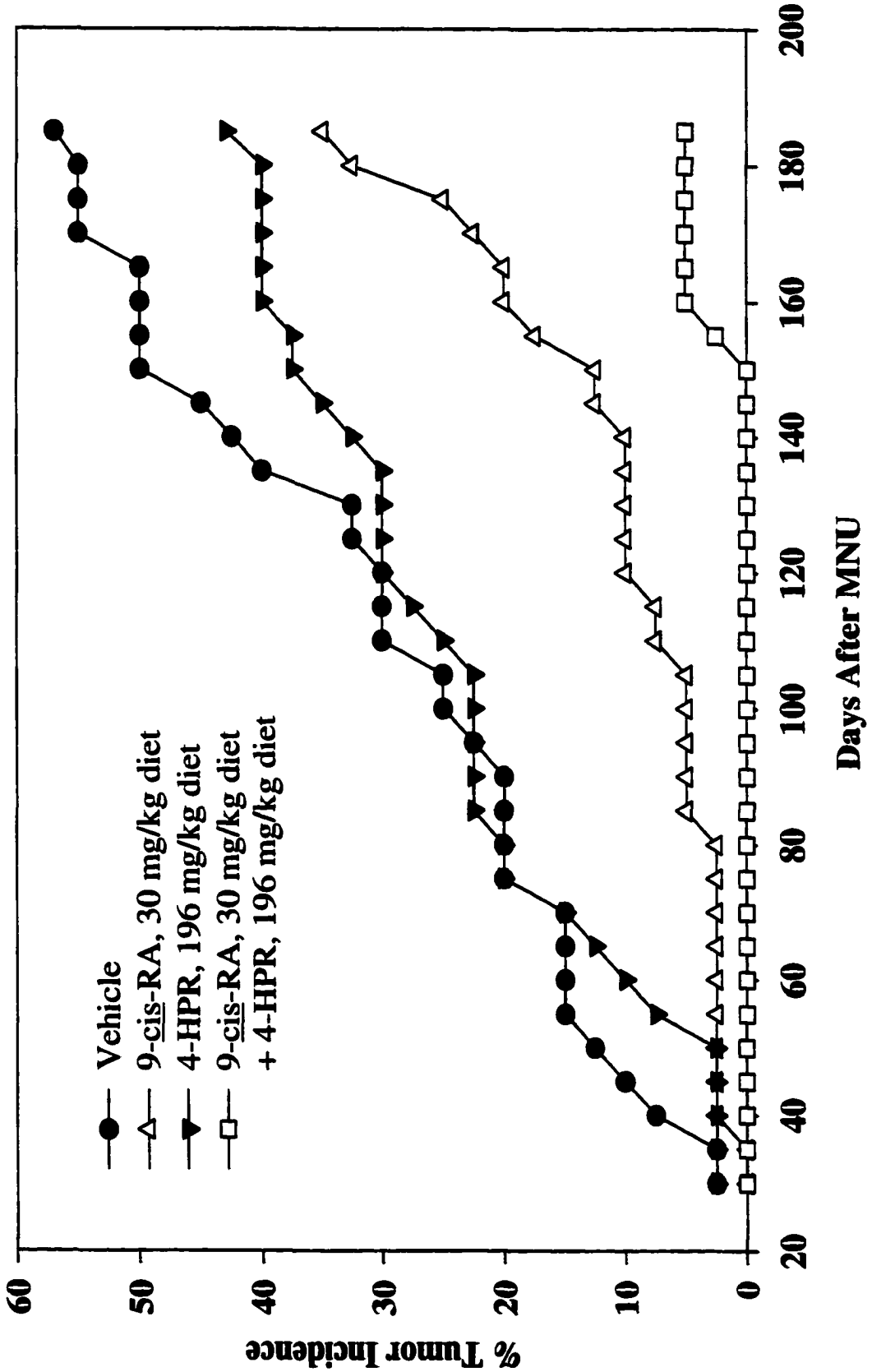


Figure 13. Average number of mammary cancers per rat receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 30 mg/kg diet (Group 2), 4-HPR: 196 mg/kg diet (Group 3), and 9-cis-RA: 30 mg/kg diet and 4-HPR: 196 mg/kg diet (Group 4). Treatment with chemopreventive agents was initiated at 93 days of age. MNU (50 mg/kg body weight) was administered at 100 days of age. 9-cis-RA = 9-cis retinoic acid; 4-HPR= 4-hydroxyphenylretinamide; MNU = N-methyl-N-nitrosourea.

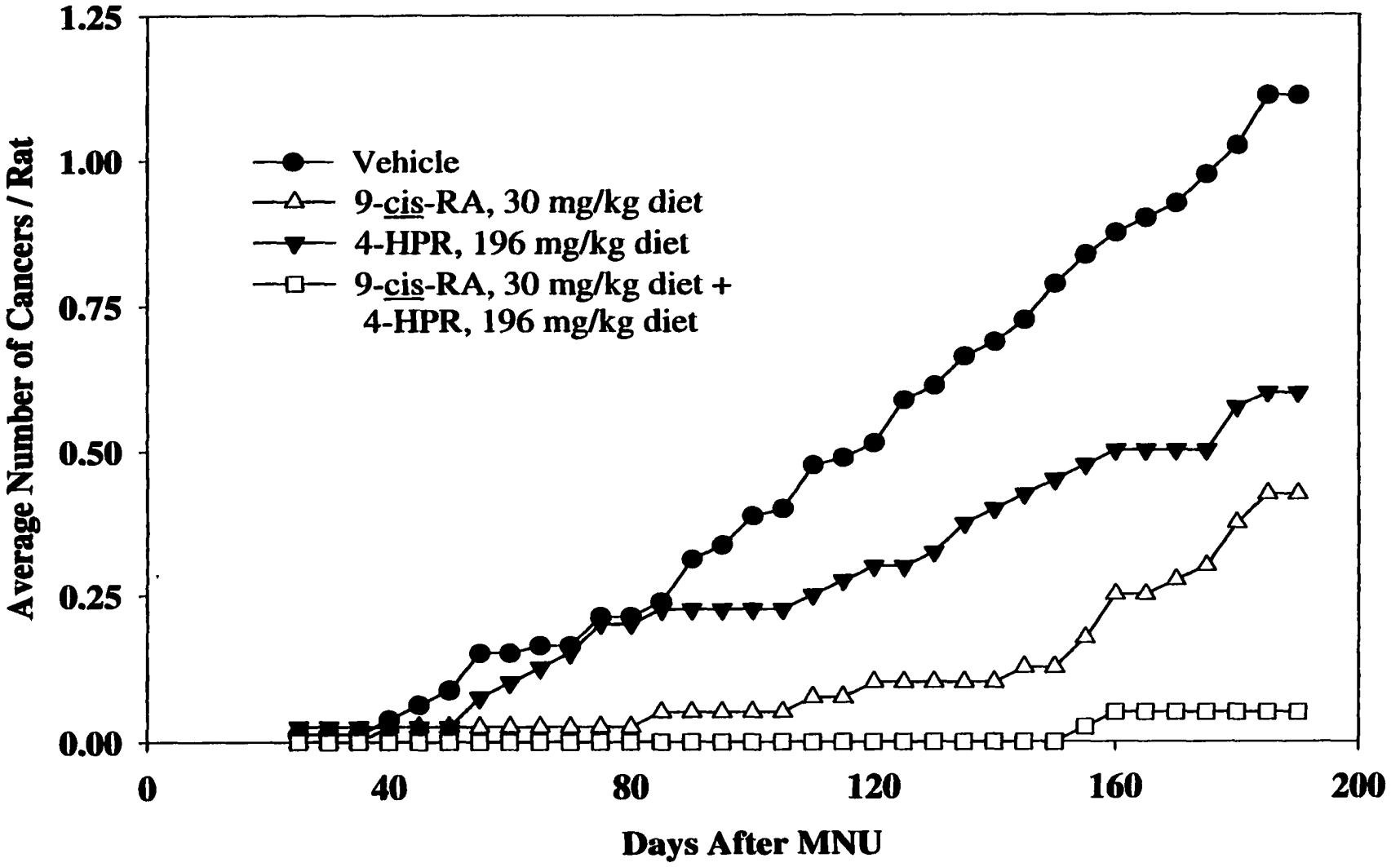


Figure 14. Average body weight (in grams) of rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 3.3 µg/kg diet (Group 4), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 3.3 µg/kg diet (Group 6). Treatment with chemo-preventive agents was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

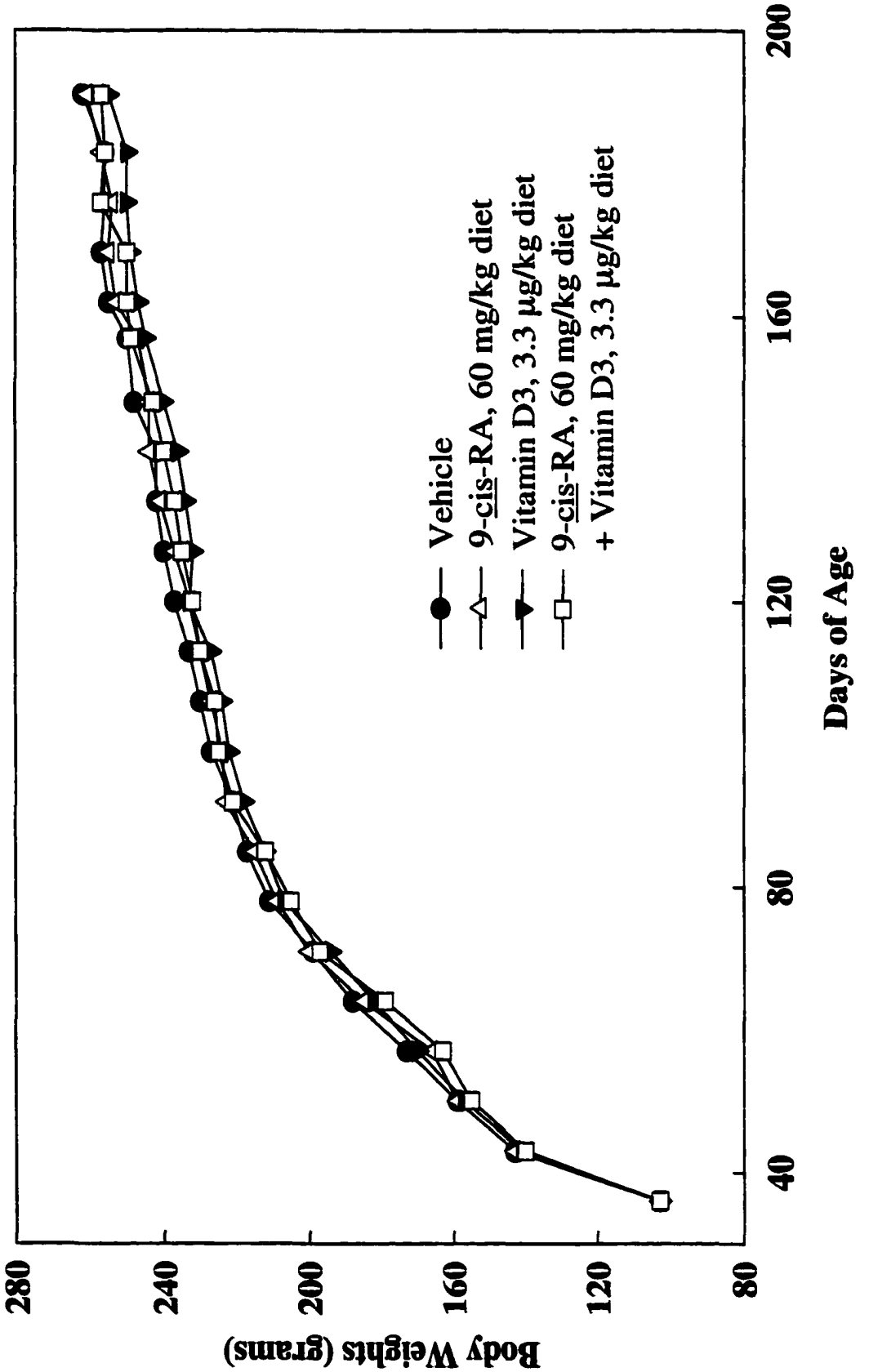


Figure 15. Average body weight (in grams) of rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 10 μg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 10 μg/kg diet (Group 5). Treatment with chemo-preventive agents was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

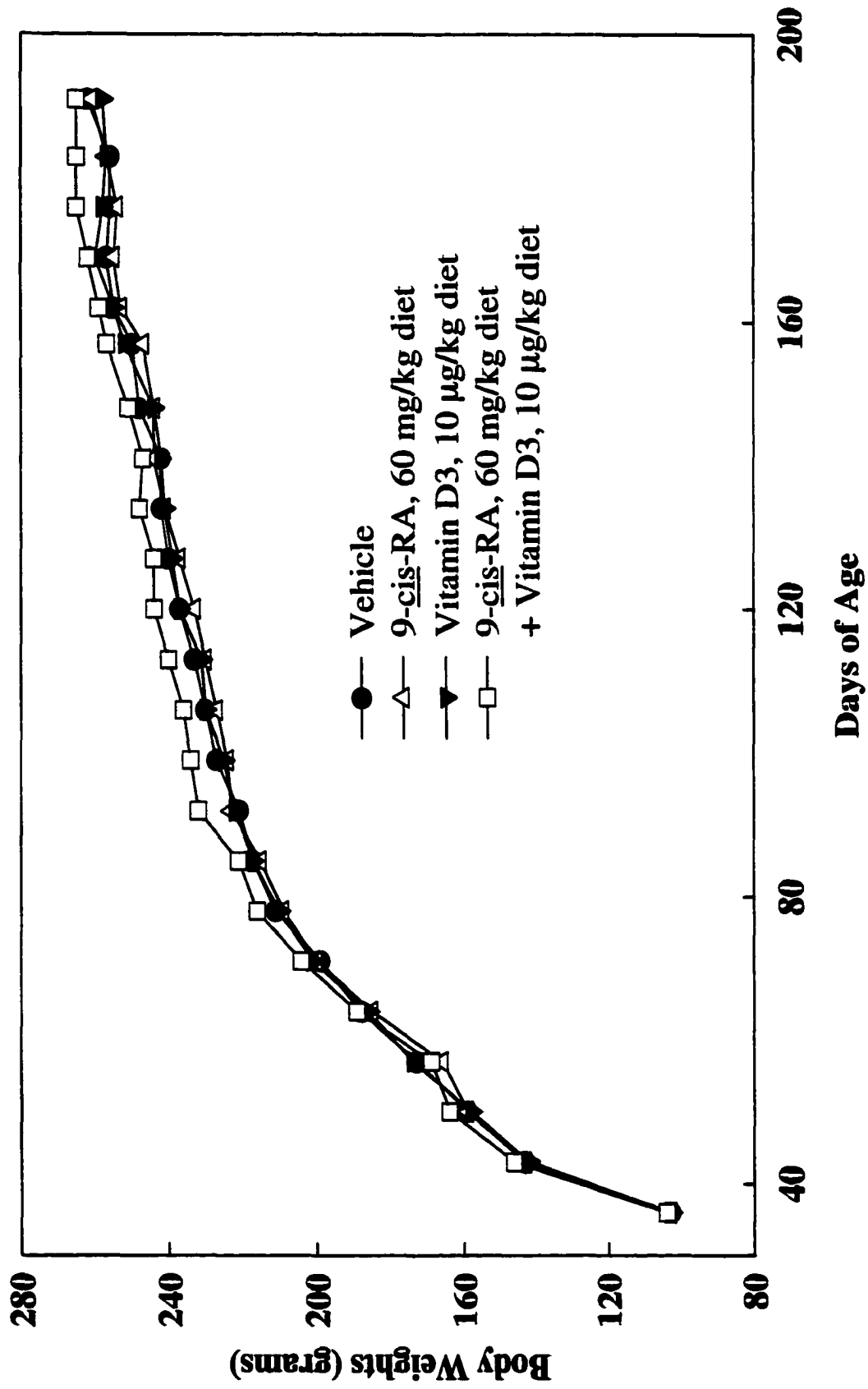


Figure 16. Percentage of mammary cancer incidence in rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 3.3 µg/kg diet (Group 4), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 3.3 µg/kg diet (Group 6). Treatment with chemopreventive agents was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

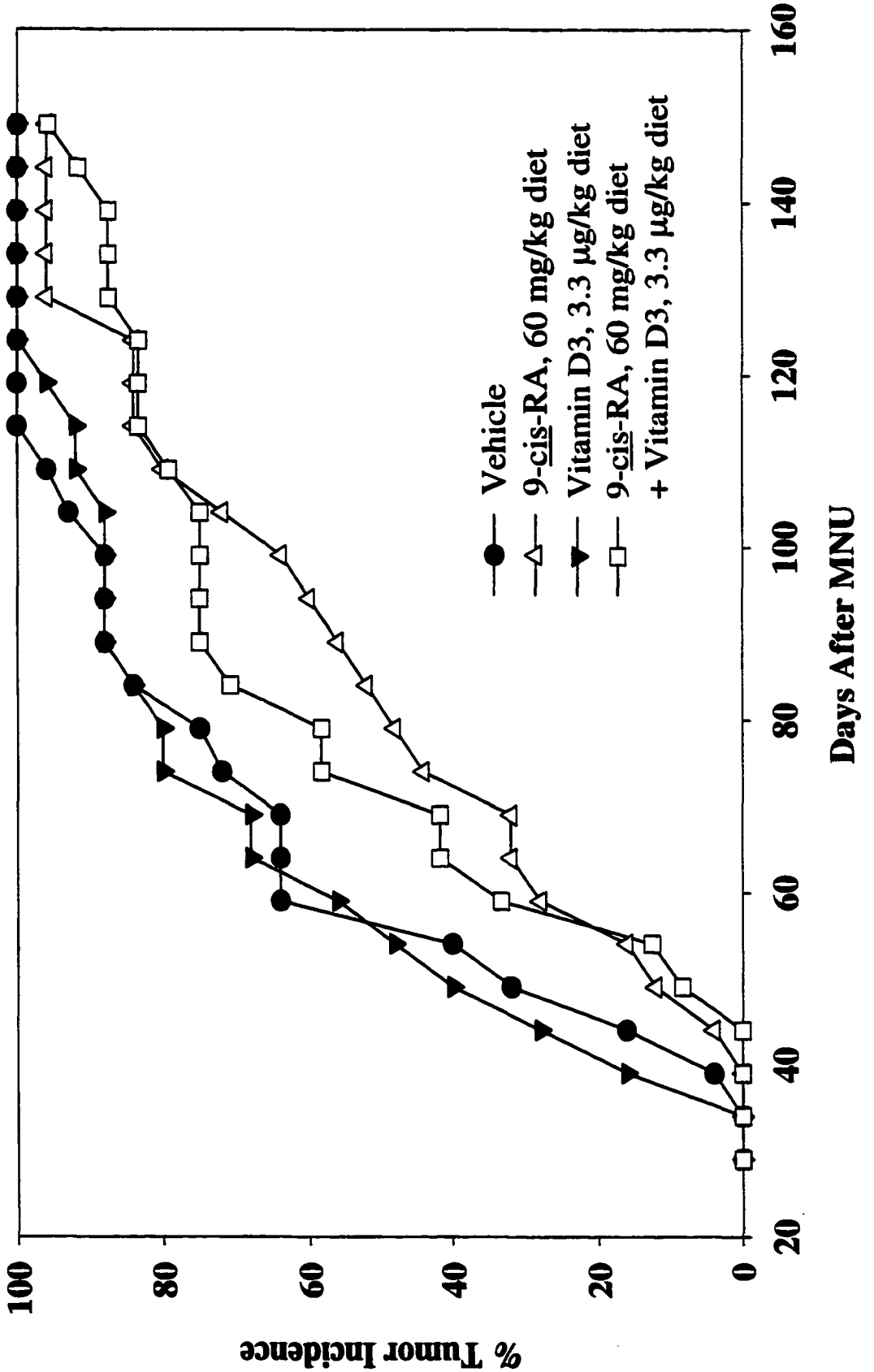


Figure 17. Percentage of mammary cancer incidence in rats receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 10 µg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 10 µg/kg diet (Group 5). Treatment with chemopreventive agents was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

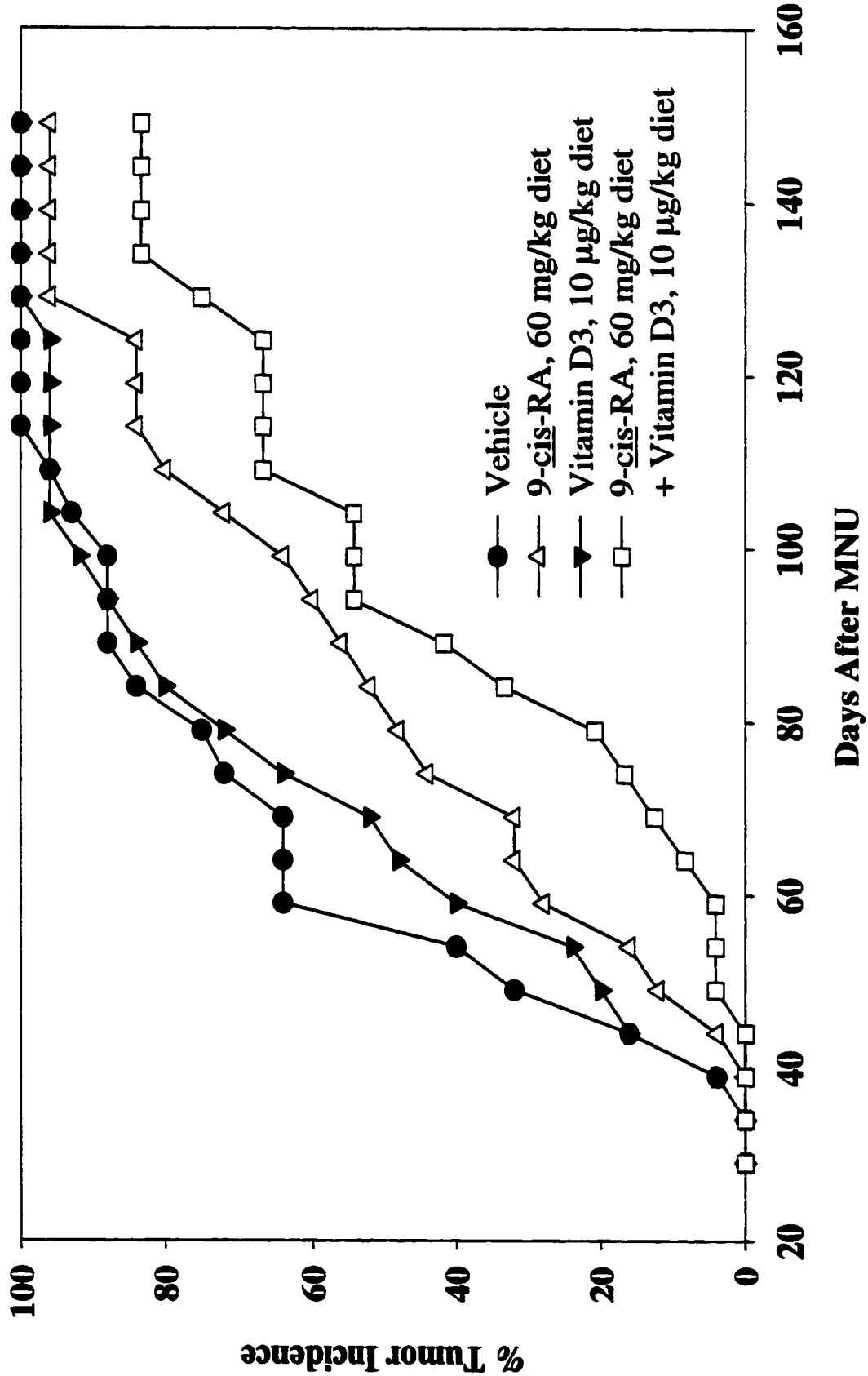


Figure 18. Average number of mammary cancers per rat receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 3.3 µg/kg diet (Group 4), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 3.3 µg/kg diet (Group 6). Treatment with chemopreventive agents was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

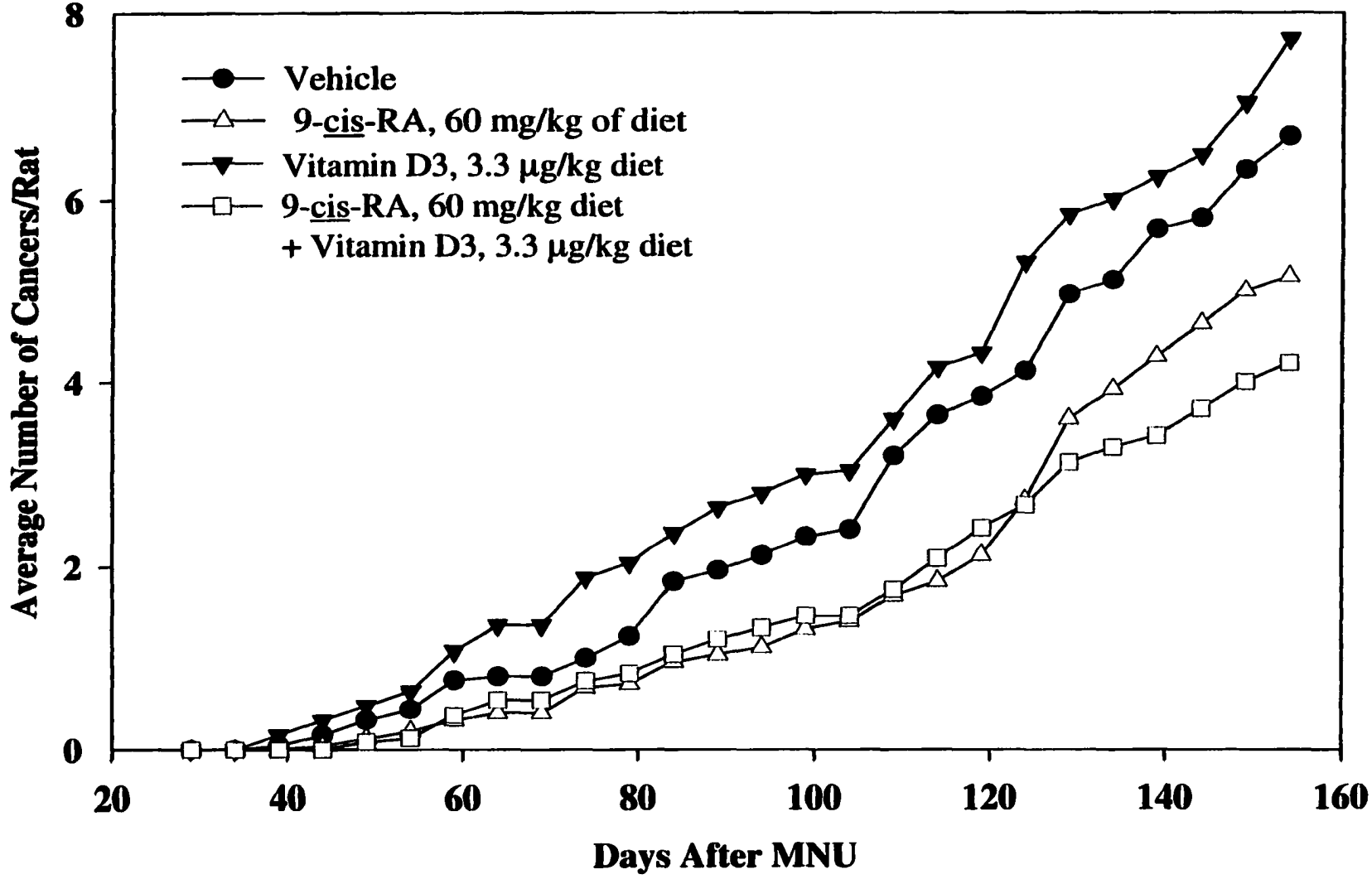


Figure 19. Average number of mammary cancers per rat receiving vehicle diet ad libitum (Group 1), 9-cis-RA: 60 mg/kg diet (Group 2), vitamin D3: 10 μg/kg diet (Group 3), and 9-cis-RA: 60 mg/kg diet and vitamin D3: 10 μg/kg diet (Group 5). Treatment with chemopreventive agents was initiated at 53 days of age. MNU (50 mg/kg body weight) was administered at 50 days of age. 9-cis-RA = 9-cis retinoic acid; MNU = N-methyl-N-nitrosourea.

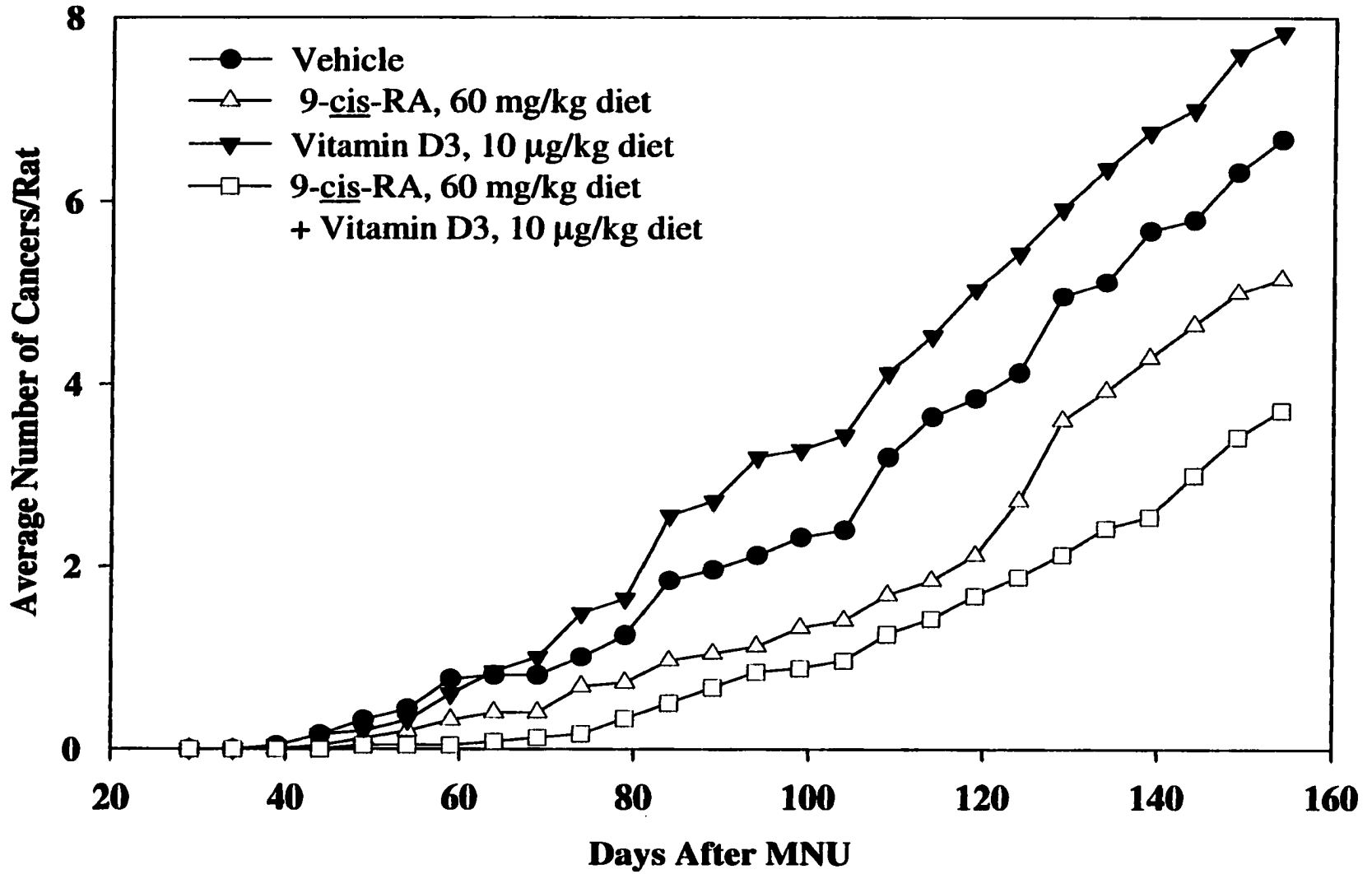
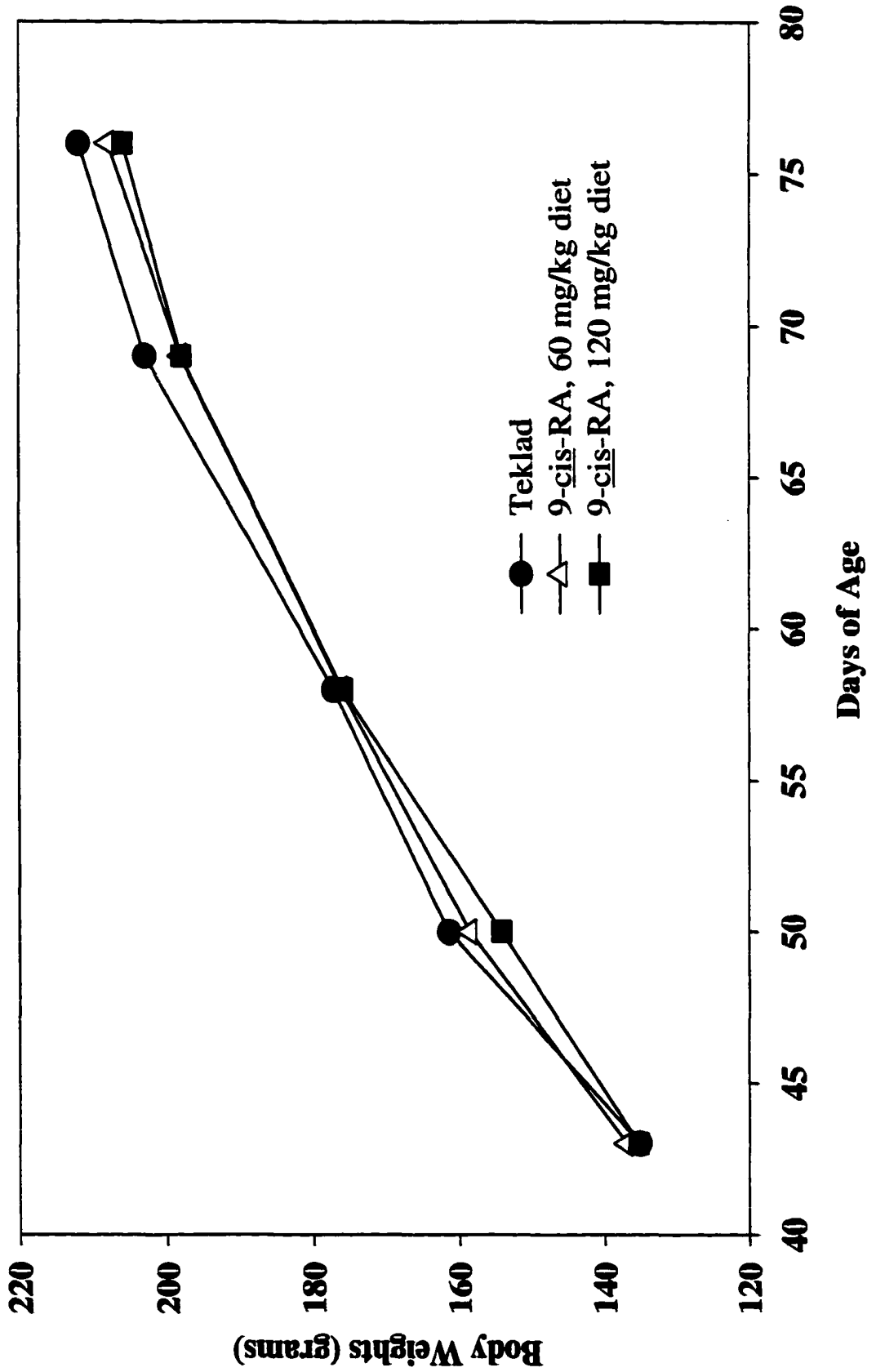


Figure 20. Average body weight (in grams) of rats receiving Teklad diet ad libitum (Group 4), 9-cis-RA: 60 mg/kg diet (Group 5), and 120 mg/kg diet (Group 6). Treatment with 9-cis-RA was initiated at 47 days of age and continued for 4 weeks. 9-cis-RA = 9-cis retinoic acid.



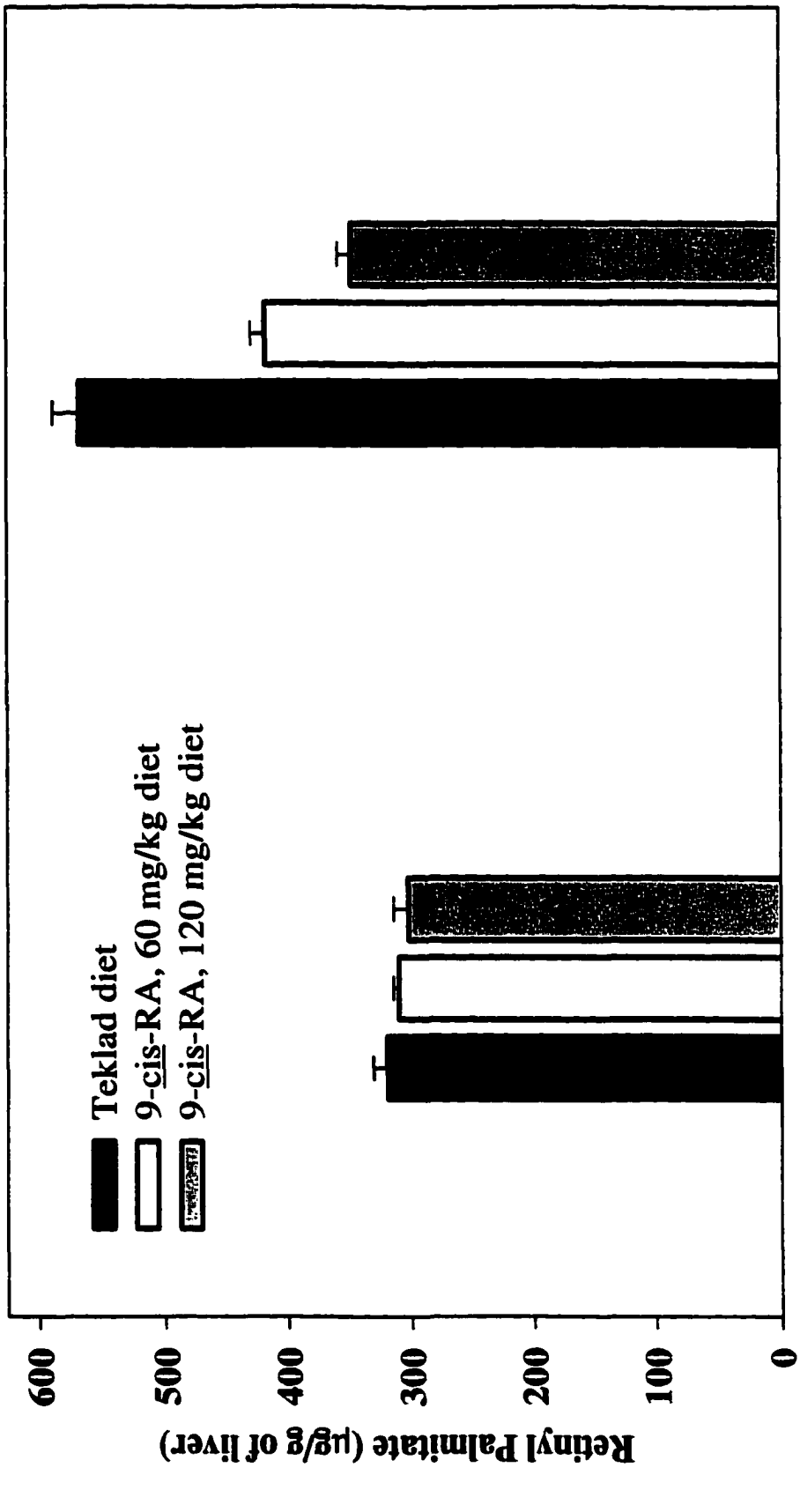
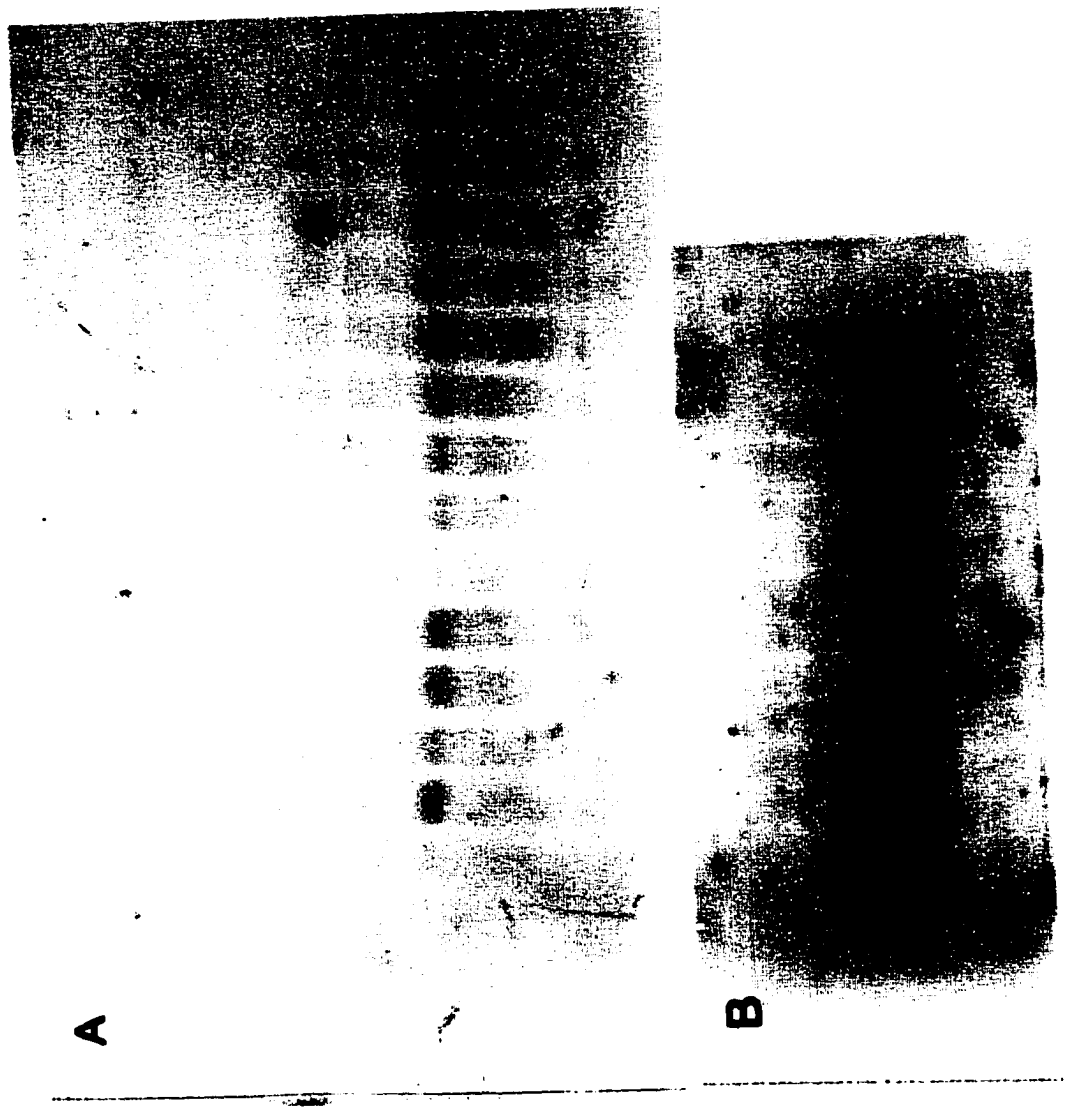


Figure 21. Levels of retinyl palmitate in the livers after 3 days or 4 weeks of feeding 9-cis-RA.

Figure 22. Autoradiographs showing retinoic acid receptor β mRNA expression levels in mammary epithelial cells from rats fed 9-cis retinoic acid for 3 days (A) or 4 weeks (B).



DISCUSSION

Although incidence rates of breast cancer have leveled off over the past decade, there are few promising preventive agents available to the public. 9-cis-RA could be used as a chemopreventive agent if its effects were better understood and its toxicity could be controlled. However, side effects at the maximum tolerated dose have been detected, and these negative findings make it difficult to pursue clinical trials using 9-cis-RA alone in humans. However, the combination of 9-cis-RA with other chemopreventive agents at low doses shows great promise as a means of overcoming the long-term toxicity.

The effectiveness of 9-cis-RA has been shown in the MNU-induced mammary cancer model for young rats (50 days of age when carcinogen is administered); (Anzano, Byers, et al., 1994; Anzano et al., 1996). Anzano, Byers, et al. (1994) reported that female Sprague-Dawley rats administered MNU at 50 days of age and then given 9-cis-RA in the diet (60 and 120 mg/kg) 1 week later for 5 months had reduced incidence and number of cancers compared to control animals. This study established that 9-cis-RA, when administered in the diet to young rats, will reduce the carcinogenic process during the promotional stage.

The incidence of breast cancer increases in humans as they age; however, no previous studies have investigated the effects of 9-cis-RA in older rats (100 days of age when carcinogen is administered). The mammary tissue of a 100-day-old rat is similar to that of a postmenopausal woman. Many cases of breast cancer occur in postmenopausal

women; therefore, it is important to investigate the effectiveness of 9-cis-RA in an animal model that can mimic this population.

In Experiment 1, MNU was administered to 100-day-old female Sprague-Dawley rats that were supplemented with various dose levels of 9-cis-RA at 93 days of age. The results indicated that none of the dose levels caused body weight reductions and that there was a dose dependent response to the 9-cis-RA. The four treated groups had significant reductions in the percentage of incidence and multiplicity of mammary cancers. This study established the effectiveness of 9-cis-RA in older rats. Because 9-cis-RA was supplemented 1 week prior to the carcinogen, the inhibitory effects related to 9-cis-RA might be involved with the initiation and/or promotional phases of carcinogenesis. Follow-up studies to investigate possible mechanisms are needed in order to determine how 9-cis-RA causes inhibition of mammary cancer in older rats.

There have been no previous studies to determine whether 9-cis-RA will be effective if supplemented for a limited time or if continuous treatment is required after the carcinogenic process has been initiated. Experiment 2 was done to investigate the efficacy of 9-cis-RA when given as a limited or as a continuous treatment in the MNU-induced mammary cancer model in young rats. To ensure that only the effects of 9-cis-RA on the promotional stage of carcinogenesis was determined, supplementation of the diet did not start until 3 days following the MNU injection.

Anzano, Byers, et al. and Anzano et al. (1994, 1996) showed that 9-cis-RA inhibited mammary cancer development in young rats when it was administered for 5 months following carcinogen treatment. In Experiment 2, it was shown that by removing 9-cis-RA from the diet of rats after 10 weeks of treatment mammary cancer development

progressively increased compared to animals that remained on 9-cis-RA for the entire study. Ten weeks after the MNU injection, the two groups supplemented with 9-cis-RA did not have differences in the average number of mammary cancers per rat. During the following 10 weeks, however, the two groups became significantly different in cancer multiplicity. The group of animals that received the limited (10 week) treatment had 6.95 mammary cancers per rat and the group supplemented with 9-cis-RA for the entire study had 4.30 mammary cancers per rat. This relationship should be considered for future studies. In order to achieve maximum effects, 9-cis-RA will be required continuously rather than for a limited time after initiation of carcinogenesis.

The first two experiments have provided new insight into the effects of 9-cis-RA in the MNU-induced model for mammary cancer. First, we have shown that the effects of 9-cis-RA are dose dependent and that it is effective in the older rat model. Second, we have provided data that show, for young rats, the advantage of continued treatment compared to limited treatment of 9-cis-RA. Both studies indicate the effectiveness of 9-cis-RA when given alone to young and old animals. Because clinical trials have revealed side effects in humans, however, this agent needs to be given at the lowest effective dose. One possible means of achieving this is to combine 9-cis-RA with other chemopreventive agents like 4-HPR and vitamin D3.

A number of in vitro studies have investigated the effects of 4-HPR on cell growth, cell differentiation, and apoptosis. Rat tracheal epithelial cells treated with benzo[a]pyrene to induce growth were suppressed by 4-HPR (Steele et al., 1990). HL-60 (Delia et al., 1993) and neuroblastoma cells (Ponzoni et al., 1995) that can be differentiated by most retinoids had no induction of differentiation after being treated

with 4-HPR. However, apoptosis occurred in head and neck (Oridate et al., 1996; Sun, Li, et al., 1999), ovarian (Sabichi et al., 1998; Supino et al., 1996), prostate (Roberson et al., 1997; Sun, Yue, & Lotan, 1999), and breast (Pellegrini et al., 1995; Sheikh et al., 1994, 1995; Swisshelm et al., 1994) cancers. Numerous animal models have shown the effectiveness of 4-HPR in cancer prevention. Welsch et al. (1983) reported that 4-HPR reduced the incidence and number of cancers in C3H mice. 4-HPR was also effective in the MNU-induced mammary model in Sprague-Dawley rats (Grubbs et al., 1990; Moon et al., 1979).

With regard to the animal models used in Experiments 3 and 4, differences in the ages of rats at the time of carcinogen administration can affect the induction of mammary cancers. In the mammary glands of the younger animals (Experiment 3), the carcinogen binds more readily to the DNA of replicating cells, which permits a greater induction of mammary cancer. In the older animals (Experiment 4), the mammary gland has differentiated to a point where there is a decreased risk of mammary carcinogenesis. Such observations have been made for 3-methylcholanthrene in Sprague-Dawley rats (Huggins et al., 1961); for 7,12-dimethylbenzanthracene in Sprague-Dawley (Janss & Hadaway, 1977; Moon, 1969; Nagasawa, Yanai, & Taniguchi, 1976; Russo & Russo, 1978), Wistar (Dao, 1969; Meranze, Gruenstein, & Shimkin, 1969) or Lewis (Haslam, 1979) rats; and for MNU in Sprague-Dawley rats (Anisimov, 1988; Grubbs, Peckham, & Cato, 1983;). These reports have indicated that dosing of rats with a carcinogenic aromatic hydrocarbon or nitrosamine after 50 days of age results in a decrease in the incidence and number of mammary cancers per rat. For rats dosed with MNU at 35, 50, 80, 140, and

200 days of age, the incidence of mammary cancer was inversely proportional to age (Grubbs et al., 1983).

Young rats, about 50 days old, are typically used to determine chemopreventive efficacy. In a preliminary report, however, we noted that 9-cis-RA, vorozole, dehydroepiandrosterone, and difluoromethylornithine were effective in preventing mammary cancers in older rats dosed with MNU (Steele et al., 1997). An additional goal of the present effort was to determine if combinations of retinoids, at doses causing little or no overt toxicity, could be effective in older rats in which the mammary glands are fully developed.

The application of combinations of agents has been a useful approach in the treatment of established cancer (Frei & Antman, 1993). Concerning retinoids, combinations of 4-HPR and glucarate are effective in inhibiting growth of carcinogen-induced mammary cancers in rats and human breast cancers implanted into athymic mice (Abou-Issa, Webb, Minton, & Moeschberger, 1989). In combination, glucarate and 13-cis-retinoic acid, at concentrations that have essentially no effect alone, increase mammary tumor latency and decrease tumor numbers in rats dosed with DMBA (Abou-Issa, Koolemans-Beynen, Minton, & Webb, 1989). In rats receiving MNU, the combination of 4-HPR and tamoxifen reduces tumor incidence to a greater extent than either drug alone (Moon et al., 1992), as does the combination of 9-cis-RA with tamoxifen (Anzano, Byers, et al., 1994) and raloxifene (Anzano et al., 1996). Recently, we demonstrated that coadministration of 9-cis-RA with suboptimal doses of toremifene, which is a tamoxifen analog, or vorozole, which is an aromatase inhibitor, yielded chemopreventive effects that appeared to be more than additive (Lubet, Steele, Kelloff,

Eto, & Grubbs, 1998). A goal of Experiments 3 (young animals) and 4 (older animals) was to determine if combinations of 9-cis-RA and 4-HPR, given at suboptimal doses, would be effective in preventing mammary cancer in rats dosed with MNU. Because toxic side effects are likely to be reduced, combinations of retinoids and other chemo-preventive agents, especially those with different mechanisms of action, may be effectively applied for chemoprevention of human cancers.

Neither of the groups treated with the retinoids alone in Experiment 3 had a significant reduction in incidence or number of mammary cancers. By combining the two retinoids, however, significant reductions were found. Experiment 4 had significant reductions in mammary cancer incidence and number per animal for the agents alone and especially in combination. These results indicate that 9-cis-RA and 4-HPR are effective at inhibiting carcinogenesis in both age groups but that the combination of these agents is more effective in both sets of animals studied.

Mehta et al. (1991) showed that 4-HPR accumulated in breast epithelial cells; however, intracellular mechanisms that allow 4-HPR to exert chemopreventive activity have not been fully elucidated. 4-HPR has not been shown to bind substantially to the nuclear RARs (Sheikh et al., 1995); therefore, other pathways may be involved with its chemopreventive effects. As for 9-cis-RA, the mechanism of action most likely involves RXR binding followed by heterodimerization with another member of the superfamily of steroid receptors and then DNA binding on a response element that signals the transcription of a gene involved with differentiation or apoptosis.

In Experiment 5, involving the MNU-induced mammary cancer model for young animals, the combination of vitamin D3 and 9-cis-RA caused reductions in the

multiplicity of mammary cancers and extended the latency period of the cancers. While 9-cis-RA was effective as a single agent, vitamin D3 alone caused a slight increase in mammary cancer number when compared to the control group. Related observations are that a human breast cancer cell line (T47D) specifically bound the natural form of vitamin D3 and stimulated growth (Freake, Marcocci, Iwasaki, & MacIntyre, 1981) and that vitamin D3 enhanced the chemically induced transformation of BALB 3T3 cells (Sasaki et al., 1986). Nevertheless, vitamin D3 at non-toxic dose levels inhibited the proliferation of breast tumor (BT-20) and MCF-7 cells (Saez et al., 1993).

Mammary tumors induced by MNU in female Wistar rats contain high-affinity VDR, which implies that mammary tissue has the potential of being responsive to vitamin D3 (K. W. Colston, Wilkinson, & Coombes, 1986). Vitamin D3 suppressed the growth of human colon cancer cell-derived xenografts in immune-suppressed mice (J. A. Eisman et al., 1987) and reduced the tumor incidence in female Sprague-Dawley rats given DMBA (Oinam, Karmakar, Roy, Indira, & Chatterjee, 1999). 1-α25-dihydroxy-16 ene-23-yne-26,27-hexafluorocholecalciferol, which is a vitamin D analogue, given in the diet (2.5 nmol/kg) caused increased mammary tumor latency, lower tumor incidence, and decreased tumor number in rats dosed with MNU without causing hypercalcemia (Anzano, Smith, et al., 1994). K. W. Colston et al. (1992) used a synthetic analog of vitamin D3 [1-α(OH)D3] to inhibit mammary cancers in the MNU model, but hypercalcemia developed. Although some animal studies investigating the effectiveness of vitamin D3 on carcinogenesis have shown inhibitory activity, most have also had hypercalcemia as a side effect. Decreasing the dose level of vitamin D3 can alleviate this side effect.

9-cis-RA has previously been shown to inhibit mammary cancers in the MNU-induced mammary model when supplemented in the diet 1 week following carcinogen treatment (Anzano, Byers, et al., 1994). The present study showed the chemopreventive effects of 9-cis-RA when rats were fed the agent 3 days after MNU injection. Because MNU is a carcinogen with a short half-life, 9-cis-RA is probably inhibiting the promotional phase of carcinogenesis in this animal model.

Numerous in vitro studies have shown that vitamin D3 and 9-cis-RA can cause a decrease in growth of mammary cancer cells (Chouvet et al., 1986; K. Colston et al., 1989; Dawson, Chao, Hobbs, & Zhang, 1998; J. Eisman et al., 1989; Isnardi, Raffo, Emionite, Chardraratna, & Toma, 1999). In another in vitro study, vitamin D3 analogs, EB1089 and KH1060, alone and combined with 9-cis-RA, caused an increase in differentiation of U937 and HL-60 leukemia cells (James, Williams, Kelsey, Newland, & Colston, 1997). Similarly, treatment of U937 cells with 9-cis-RA combined with vitamin D3 caused these leukemia cells to differentiate to a mature stage; RXRα mRNA expression was upregulated as well (Nakajima et al., 1996).

Retinoid and vitamin D receptors are members of the steroid/thyroid retinoic acid superfamily of receptors (Haussler, Pike, Chandler, Manolagas, & Deftos, 1981; Mangelsdorf et al., 1994; Pike, 1991). These receptors can heterodimerize in the presence of their ligands, and specific genes can be modified by the response elements in the DNA after the heterodimer binds to the DNA. Some of these genes may be involved with cell growth, differentiation, and/or apoptosis. Because 9-cis-RA is a ligand for the RARs and RXRs and vitamin D3 is the ligand for VDR, the combination of these agents

in the diet could influence one (or more) of the mechanisms involved with the carcinogenic process.

In Experiment 5, we determined the effects of 9-cis-RA and vitamin D3, alone and in combination, during the promotional phase of carcinogenesis (the agents were administered 3 days after MNU treatment). Compared to the controls, 9-cis-RA alone caused a 23% decrease in mammary cancers, while vitamin D3 (10 and 3.3 μg/kg diet) alone increased mammary cancer multiplicity by 17% and 16%, respectively.

Because mammary epithelial cells in the 50-day-old animals were rapidly dividing, any process that can inhibit proliferation or induce differentiation in these cells could decrease cancer formation (Russo et al., 1979). Previously, 9-cis-RA, given for 120 days beginning 1 week after MNU administration to 50-day-old animals, showed chemopreventive activity in the MNU model (Anzano, Byers, et al., 1994). In Experiment 5, 9-cis-RA was supplemented in the diet for 150 days beginning 3 days after MNU administration. Our data further confirm that 9-cis-RA is an inhibitor of the promotional phase of the carcinogenic process.

As mentioned earlier, vitamin D3 is effective at inhibiting growth of human breast cancer cell lines (Chouvet et al., 1986; K. Colston et al., 1989; Dawson et al., 1998; J. Eisman et al., 1989; Isnardi et al., 1999), and more than two thirds of human breast cancers have VDR expressed (Berger et al., 1987). The dose levels of vitamin D3 in our study were low in order to bypass toxic side effects normally caused by high doses of the agent. The data presented, however, revealed that these dose levels of vitamin D3 were not effective at inhibiting cancer, and there was perhaps a slight enhancement of cancer multiplicity. Previous findings (Freake et al., 1981; Sasaki et al., 1986) have shown

similar effects of vitamin D3, that is promotion of the carcinogenic process. The mechanism for this enhancement, however, has not been elucidated.

MNU may alter the expression levels of genes such as the RARs, RXRs, and VDR in mammary epithelial cells during the initiation phase of carcinogenesis. With decreased levels of these receptors, expression of genes that control cell growth, differentiation, and apoptosis may be blocked from signaling pathways controlled by these receptors. RXR is thought to play a role not only in retinoid receptor signaling but also in signaling for all members of the hormone superfamily of receptors, including VDR and thyroid receptor (Kliewer et al., 1992). 9-cis-RA is ligand for RXR (Mak, Fuernkranz, Ge, & Karathanasis, 1994; Schimerlik, Peterson, Hobbs, Dawson, & Leid, 1999). VDR may need RXRα or other retinoid receptors activated by 9-cis-RA in order to heterodimerize and cause transcription of genes that are involved with the prevention of carcinogenesis. This scenario may account for the increased effectiveness of 9-cis-RA combined with vitamin D3 compared to 9-cis-RA or vitamin D3 alone.

By combining the lower dose levels of vitamin D3 and 9-cis-RA in the diet, we have shown that these two agents can cause an enhanced reduction of mammary cancer in the MNU-induced rat model. The mechanisms causing this enhancement are not known. Perhaps, 9-cis-RA and vitamin D3 regulate genes involved with cell proliferation, differentiation, and/or apoptosis by binding the RARs, RXRs, and VDR in the mammary epithelial cells.

Xu et al. (1997) reported that ductal carcinoma in situ and invasive carcinomas from human breast cancer patients had significantly lower RARβ mRNA compared to normal adjacent breast tissue. Transfection of the RARβ gene into cervical, breast, and

lung cancer cells lacking RARβ caused growth inhibition and induced apoptosis when retinoids were administered (Y. Li et al., 1998; Liu et al., 1996; Seewaldt et al., 1995; Si et al., 1996; Weber et al., 1999). These studies suggest that the expression of RARβ is needed in order for cancer cells to be sensitive to retinoids. Experiments 2, 3, and 5 show that 9-cis-RA can inhibit the carcinogenic process in young rats after the mammary epithelial cells have been exposed to MNU. The mechanism for this chemopreventive activity is unknown.

Experiment 6 was designed to investigate how 9-cis-RA affects the expression of RARβ mRNA in mammary epithelial cells from young rats. The diets for rats at 47 days of age were supplemented with 9-cis-RA for either 3 days or 4 weeks. At the end of each feeding period, epithelial cells were isolated from the mammary glands. The RNA from these cells was extracted and analyzed for RARβ mRNA expression.

Our results from the Northern blot analysis did not reveal any significant differences in RARβ mRNA expression levels between the three groups of animals after the 3-day feeding. Retinyl palmitate levels in the liver did not differ between groups, and liver weights were not affected. Also, after the 4-week feeding, rat mammary epithelial cells did not have any modification in the expression of RARβ mRNA. Liver weights, however, were significantly increased in the group fed 9-cis-RA (120 mg/kg diet), and retinyl palmitate levels were significantly lower in both groups supplemented with 9-cis-RA. It is possible that 9-cis-RA bypasses liver incorporation, thus leading to the decreased levels of retinyl palmitate. The high dose of 9-cis-RA caused a significant decrease in uterus weights, but neither dose level affected ovarian weights. Mammary

gland morphology was not changed by the administration of 9-cis-RA for 3 days or 4 weeks at either dose level.

Overall, we did not identify an interaction between 9-cis-RA and RARβ expression in the normal mammary epithelial cells from young rats. The loss of RARβ expression most likely occurs during the promotional stage of carcinogenesis because studies have shown that only the malignant cells from human subjects exhibit loss of expression and that adjacent normal tissue continues to express the RARβ gene (Xu et al., 1997). An enhancement of RARβ in the mammary epithelial cells after 9-cis-RA treatment was expected, but this was not found. After MNU administration to the young rats, the target epithelial cells were initiated and can potentially enter the promotional stage of carcinogenesis. Follow-up studies measuring RARβ in mammary epithelial cells immediately after MNU injections need to be performed. It may be that only after RARβ expression is inhibited during the promotional stage will 9-cis-RA induce an enhancement of the RARβ gene.

The capacity of 9-cis-RA to bind to both the RARs and RXRs makes it difficult to find the pathways involved with its chemopreventive action, especially in vivo. 9-cis-RA activity is even more complex because other steroid receptors can heterodimerize with RXR after it is bound by 9-cis-RA. Coactivators and corepressors are also involved with the regulatory actions of 9-cis-RA, and they may help bridge the receptors and transcriptional machinery in place. The number of receptors, the distinct receptor functions, the expression patterns, ligand specificities, and the ability to activate and/or repress a number of genes involved with cell proliferation, differentiation, and/or apoptosis make retinoid signaling extremely complex.

More investigations are needed to find the mechanism(s) involved with the chemopreventive activity of 9-cis-RA, but overall the findings from these experiments evaluating 9-cis-RA are promising. Experiment 1 established the effectiveness of 9-cis-RA in the older animal model. Continued treatment of 9-cis-RA appears to be more beneficial than a limited treatment. In young animals, 9-cis-RA was an effective inhibitor of mammary carcinogenesis at the promotional stage. Possibly the most interesting findings were that the combination of 4-HPR and vitamin D3 with 9-cis-RA was more effective than 9-cis-RA alone. Combination chemopreventive trials using suboptimal doses of 9-cis-RA, 4-HPR, and vitamin D3 need to be evaluated in women who have increased risks of developing breast cancer.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE DOCUMENTATION

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Vice President for Health Affairs
Institutional Animal Care and Use Committee

NOTICE OF APPROVAL

INVESTIGATOR: GRUBBS, CLINTON J.

TITLE: EFFECTS OF VARIOUS CHEMOPREVENTIVE AGENTS ON CARCINOGEN-INDUCED RAT MAMMARY TUMORS--WORKSTATEMENT NO. 49

AGENCY: NCI

APPROVAL DATE: May 1, 1996

APPROVAL NUMBER: 9602809

The above application was reviewed and approved by this institution's Institutional Animal Care and Use Committee (IACUC). Effective February 1, 1987 an approval number for the above project became mandatory for placing an animal order with the Animal Resources Program.

The University of Alabama at Birmingham has an Animal Welfare Assurance on file with the Office for Protection from Research Risks. The Assurance Number is A3255-01. UAB is a fully accredited AAALAC institution. Please forward this notice to the appropriate granting agency.

Dr. Clinton J. Grubbs
Chairman,
Institutional Animal Care and
Use Committee (IACUC)

SPECIES: RAT
STRESS LEVEL: B

The University of Alabama at Birmingham
B10 Volker Hall • 1717 Seventh Avenue South
Birmingham, Alabama 35294-0019 • (205) 934-3553 • FAX (205) 934-1188

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Executive Vice President and Provost
Institutional Animal Care and Use Committee

NOTICE OF APPROVAL

DATE: May 26, 1999

TO: Clinton J. Grubbs, PhD
WEBB-316 3360
FAX: 975-5082

FROM: ~~Clinton J. Grubbs, PhD, Chairman~~ Gene A. Hines, Ph.D., Director GAH
~~Institutional Animal Care and Use Committee~~

SUBJECT: Efficacy Studies of Chemopreventive Agents in Animal Models - Workstatement Number 70, (NCI), 990503643

On May 26, 1999, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Rats	B	850

Animal use is scheduled for review one year from May 26, 1999. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 990503643 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

The University of Alabama at Birmingham
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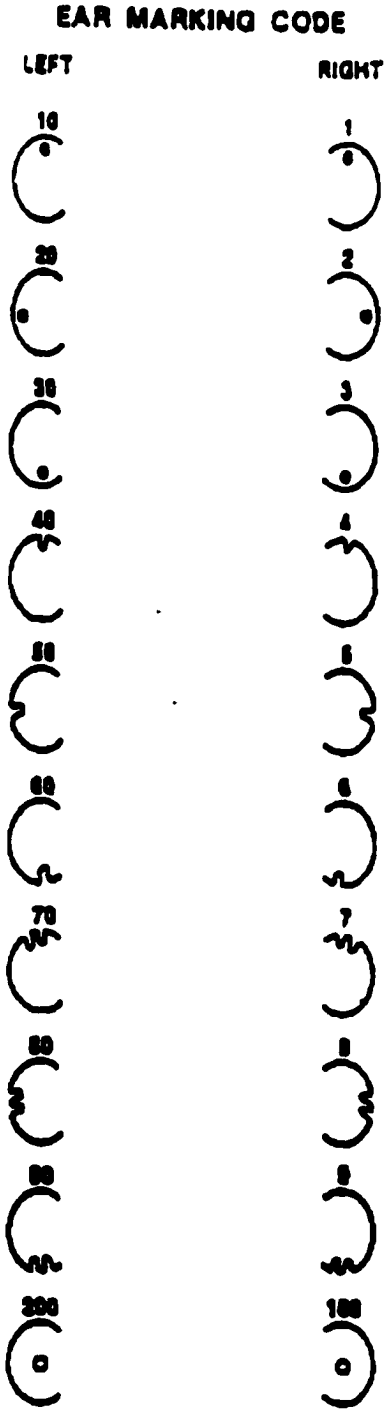
APPENDIX B

UNIVERSAL NUMBERING SYSTEM FOR THE INDENTIFICATION OF RODENTS



Note. From the Manual for Assistant Laboratory Animal Technicians (Figure 13-1), by W. B. Sapunki and J. E. Harkness (Eds.), 1984, Joliet, Il: The American Association for Laboratory Animal Science. Copyright 1984 by the American Association for Laboratory Animal Science. Reprinted with permission.

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Mark B. Cope

Graduate Program Nutrition Science

Title of Dissertation Efficacy of 9-cis-Retinoic Acid (9-cis-RA) in Preventing Methylnitrosourea (MNU)-Induced Mammary Cancers

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name	Signature
Clinton J. Grubbs, **Chair**	
Isao Eto	
Gary L. Johanning	
Donald Muccio	
Heidi L. Weiss	

Director of Graduate Program

Dean, UAB Graduate School

Date 8/20/01