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Review

Animal Models of *N*-Methyl-*N*-nitrosourea-induced Mammary Cancer and Retinal Degeneration with Special Emphasis on Therapeutic Trials

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Abstract. *N-Methyl-N-nitrosourea (MNU) is a direct-acting alkylating agent that interacts with DNA. Accumulation of mutations may enhance cancer risk in target organs or cause cell death in susceptible tissues or cells when excessive DNA damage is not repaired. MNU targets various organs in a variety of animal species. MNU-induced carcinogenesis can be used as organ-specific animal models for human cancer, and MNU has been most extensively utilized for the induction of mammary cancer in rats. MNU-induced rat mammary tumors possess many similarities to those of human breast cancer, and the model is utilized for screening cancer modulators. MNU-induced cell disruption is also seen in several organs and tissues, especially when MNU is applied before maturity. However, photoreceptor cells in adults are highly sensitive to MNU, which causes cell death due to apoptosis. MNU-induced photoreceptor apoptosis mimics human retinitis pigmentosa and can be used for studies of therapeutic intervention. In this review, the targets of MNU in various animal species are described, and special emphasis is given to therapeutic trials against MNU-induced mammary cancer and retinal degeneration in animal models.*

N-Methyl-*N*-nitrosourea (MNU; Figure 1) is an *N*-nitroso compound and a direct-acting alkylating agent that was originally tested as a chemotherapeutic agent. It was an effective therapy for mice that had been intraperitoneally (*i.p.*) or intracerebrally implanted with L1210 leukemia cells (1). Alterations in DNA structure that are left unrepaired may accumulate mutations and eventually enhance cancer risk. Many chemotherapeutic agents, including MNU, are also potent mutagens and carcinogens (2). MNU has never been produced in commercial quantities; therefore, no human case reports or epidemiological studies are available. However, MNU is anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals [International Agency for Research on Cancer (IARC) Group 2A carcinogen]. On the other hand, when the DNA damage is very severe, MNU acts as a cell-disrupting agent and causes cell death in susceptible organs and tissues (3). MNU targets multiple animal organs to cause cancer (uncontrolled cell proliferation) and/or degenerative disease (cell death). MNU-induced mammary cancer and retinal degeneration models are relevant to human disease and can be used for therapeutic trials. In this review, MNU-induced abnormalities in animals are described, and therapeutic trials on mammary cancer and retinal degeneration receive special emphasis.

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Carcinogenicity of MNU in Animals

The different organotropic effects of MNU on animals depend on dose, frequency, route, and age at administration. MNU is carcinogenic in various animals including rodents, pigs, dogs, and rabbits (Table I). In rodents, multiple organs are targets of MNU. From the phylogenetic point of view, the Insectivora order is considered to contain the most primitive placental mammals and to be much closer to the early primates than are rodents. Among the Insectivora, the house musk shrew (*Suncus murinus*, family Soracidae) has been used for carcinogenicity studies (7). Several chemicals that are carcinogenic in rodents produce tumors in shrews but the target organs are often different, and some chemicals are not carcinogenic. When MNU was administered *i.p.* to 35-day-old female shrews, ≥25 mg/kg body weight (bw) MNU evoked acute toxicity and was lethal. However, MNU at doses of 5 and 10 mg/kg resulted in the development of a few tumors in the large intestine when

Table I. *Organ-specific carcinogenicity of N-methyl-N-nitrosourea in various animal species of different orders.*

Order	Species	Site
Primates	Monkey	Upper gastrointestinal tract
Artiodactyla	Pig	Stomach
Carnivora	Dog	Brain
Lagomorpha	Rabbit	Nerve tissue, intestine, blood vessel
Rodentia	Mouse, rat, gerbil, hamster, guinea-pig	Nerve tissue, stomach, esophagus, respiratory tract, intestine, lymphoreticular tissue, skin, kidney
Insectivora	Shrew	Large intestine

Summary quoted from references 4, 5, and 6.

the shrews were older than 44 weeks. Intrarectal infusion of a 1.5-mg MNU solution biweekly 16 times or 0.5-mg MNU solution weekly 24 times starting from 6 weeks of age was given to female shrews; moribund animals were killed and survivors observed until 37 weeks of age (5). All MNU-treated shrews developed exophytic and endophytic types of large intestinal cancer (mean age of sacrifice 30.8 and 36.2 weeks of age, respectively), with a high frequency of mesenteric lymph node metastasis (31%, 4/13 and 23%, 3/13, respectively) while the intestines of MNU-untreated shrew were free of tumors.

Nonhuman primates are a valuable experimental model for the evaluation of human carcinogenic risk due to their close phylogenetic relationship to humans. However, the use of nonhuman primates in carcinogenicity studies has been restricted by their large body size and long lifespan, which increase the time and cost of research. After 10 mg/kg bw MNU was administered *per os* (*p.o.*) each day for 10 years, 26 monkeys died without tumors, and the remaining 18 monkeys developed malignant tumors (mean cumulative dose of 140.4 g with latent period of 146.5 months; range, 62-264 months) (6). Squamous cell carcinoma of the esophagus (34%, 15/44) was accompanied by dysplastic epithelium. Tumors in the oral cavity, pharynx, larynx, and stomach indicated the direct action of MNU. Therefore, although the target of MNU differs among species, MNU acts as carcinogen across species, from primitive primates to nonhuman primates. The carcinogenic action of MNU can be applicable for the establishment of organ-specific models for human cancer. MNU has most extensively been utilized for the induction of mammary cancer in rats.

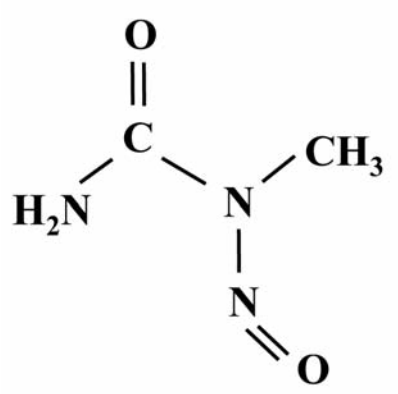

Figure 1. *Chemical structure of N-methyl-N-nitrosourea (MNU).*

Mammary Carcinogenesis in Rodents

Rats and mice, the two major rodent species used in the laboratory, have been extensively studied to obtain fundamental information on the biology, prevention, and treatment of human breast cancer. In mice, the murine mammary tumor virus (MMTV) is heavily involved in the genesis of so-called spontaneous mammary tumors, and a majority of tumors are hormone-independent and acinar in origin, which is distinct from human breast cancer (8). In contrast, rats are free of MMTV and are highly susceptible to chemical carcinogens and radiation. Rats have a high frequency of hormone-dependent tumors that are ductal in origin (9, 10). Mammary tumors can be easily induced by 7-12-dimethylbenz[α]anthracene (DMBA) or MNU, which are the two major mammotrophic carcinogens (11), with no need for irradiation. It is easy to prepare an injectable MNU solution because it is water soluble, whereas DMBA is more difficult to use because it is oil soluble. MNU purchased from the manufacturer should be kept at –20˚C in the dark and then dissolved in physiologic saline containing 0.05% acetic acid just before use. The *i.p.* route is the simplest way to administer MNU to animals and yields a high incidence of estrogen and/or progesterone receptor (ER/PgR)-positive mammary tumors (Figure 2). MNU-induced mammary cancer is age dependent; rats that are between 4 and 7 weeks of age are most susceptible to MNU. When animals are sacrificed, a histological analysis of the tumors is essential because benign and malignant tumors of stromal and epithelial origin develop in the mammary gland, and the histological characteristics of the tumors have implications for the interpretation of the results (12). The MNU-induced mammary cancer model is widely used to screen and evaluate the potency of cancer- suppressing/promoting agents.

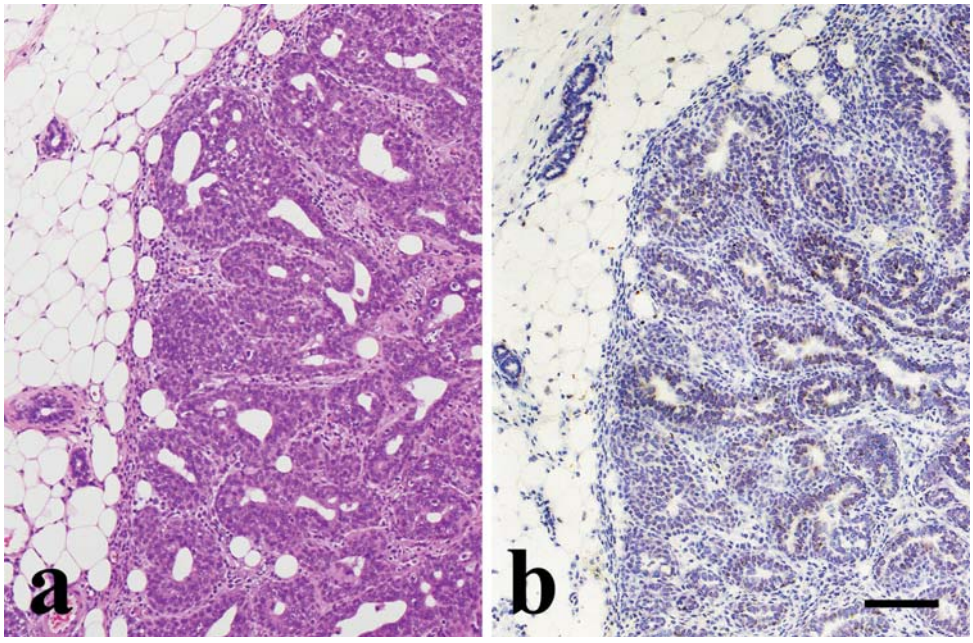

Figure 2. *N-Methyl-N-nitrosourea (MNU)-induced mammary cancer in female Lewis rats. a: Adenocarcinoma with papillary pattern. b: Estrogen receptor is highly positively stained in the nuclei (bar=100 μm).*

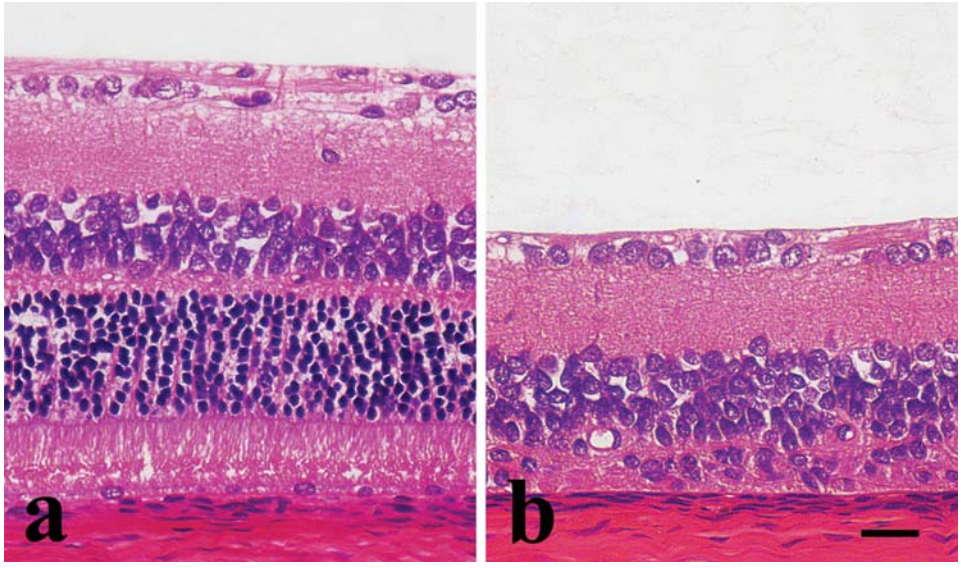

Figure 3. *N-Methyl-N-nitrosourea (MNU)-induced retinal degeneration in Sprague-Dawley rats. a: Age-matched normal control retina. b: Retina from rat 7 days after MNU injection. Almost all photoreceptor cells are lost (bar=20 μm).*

MNU-induced Mammary Cancer and Cancer Modulators

Epidemiological studies have found that lifestyle factors, especially the ingestion of a diet rich in n-3 polyunsaturated fatty acids (PUFA), and reproductive factors, such as a young maternal age at first birth, provide protection against the development of human breast cancer. Although the incidence of breast cancer has traditionally been low in the Inuit population, an increase in its incidence has been observed in recent years (13). This increase may be caused by changes in the traditional Inuit diet rich in n-3 PUFA from marine products to a more Westernized diet and an increase in the maternal age at first birth.

Dietary fat. The amounts and types of dietary fat have different effects on breast carcinogenesis (14). In human breast cancer cells in culture, n-6 PUFA, especially linoleic acid (LA), stimulates their growth, whereas n-3 PUFA, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), suppresses their growth (15-17). Conjugated fatty acids are positional and geometrical isomers with conjugated double bonds. While LA increases the risk of breast cancer, LA-derived conjugated LA (CLA) has an anticarcinogenic effect (18). Thus, conjugated fatty acids derived from n-3 PUFA may have a more potent anticarcinogenic effect. Among n-3 PUFAs, DHA suppressed KPL-1 human breast cancer cell growth *in vitro* more effectively than EPA, and conjugated DHA (CDHA) was a more effective suppressor than the parent DHA (IC_{50} for 72 h: EPA, 669 μM; DHA, 270 μM; CDHA, 97 μM) (19). However, *in vivo* studies are necessary to determine the optimal timing and duration of administration.

In one study, 7-week-old female Sprague-Dawley (S-D) rats were treated with MNU and fed diets containing 10% fatty acid consisting of EPA, DHA, or a 1:1 mixture of EPA and DHA until the rat developed a mammary tumor ≥1 cm or reached 27 weeks of age (20). Mammary cancer incidence and multiplicity was significantly suppressed in the DHA diet group (Table II). In another study, 7-week-old female S-D rats were given MNU and received a diet containing 0%, 0.2% or 1.0% CDHA, and the experiment was terminated when the animals reached 40 weeks of age (21). Compared with the 0% CDHA diet, the ≥0.2% CDHA diets significantly suppressed mammary cancer incidence and multiplicity. Conjugated fatty acids (CLA and CDHA) are powerful anticancer agents because a dietary dose of 1.0% or less is sufficient to achieve an anticancer effect, while n-3 PUFA such as EPA and DHA requires a dose of 5%-10% to obtain comparable effects (15, 20, 22). Thus, the *in vitro* culture data was confirmed in the MNU-induced animal model of mammary cancer. However, when potent CDHA was administered prior to MNU for a short period, suppression of mammary cancer was not observed (21). Animal model evidence indicates that dietary fat exerts its effect on the promotion and progression stages of carcinogenesis (23); a certain fatty acid must be present in the diet for a long duration during these stages to suppress mammary carcinogenesis. In this respect, agents that provide lifelong breast cancer protection with a short duration of treatment may be more desirable.

Parity and pregnancy hormone. Women that give birth to their first child before 20 years of age have a 50% reduction in their lifetime breast cancer risk when compared with nulliparous women (24). Parity protection from breast cancer is seen in Lewis rats either before or after MNU exposure (25) and can also be achieved by short-term treatment (approximately equivalent to the gestational period of rats; 21 days or shorter) with estrogen and progesterone mimicking pregnancy (26, 27). In rats, pregnancy or estrogen and progesterone treatment at a young age (≤3 months) is highly effective, but it is less effective in older animals (27). The mechanisms that have been proposed for parity protection include general factors, such as changes in hormone levels, and local factors, such as changes in mammary gland architecture (27). The mammary glands of parous rats treated with MNU exhibited up-regulation of differentiation-related genes and down-regulation of proliferation-related genes, indicating blockage of carcinogen-induced cell proliferation (28). A low n-6/n-3 PUFA ratio of serum phospholipids in parous rats (29) may also be involved in parity protection from mammary cancer. The timing of short-term exposure to pregnancy hormone treatment appears critical for mammary cancer protection, and chemicals with estrogenic properties may show the same protective effects.

Phytoestrogens. Genistein (4',5,7-trihydroxyisoflavone) and resveratrol (*trans*-3,4',5-trihydroxystilbene) are classified as phytoestrogens (30). Genistein is present at high levels in soybeans and soy products. Although it stimulates ER-positive human breast cancer cells at low concentrations (3.7-37 μM; MCF-7 cells in culture for 72 h), regardless of hormone receptor status, it inhibits the growth of human breast cancer cell lines at high concentrations (IC_{50} for 72 h: MCF-7, 274 μM; and MDA-MB-231, 131 μM) (31). Genistein causes G_2/M arrest and apoptosis through the up-regulation of Bax protein, down-regulation of Bcl-X_L protein, and activation of caspase-3.

Epidemiological studies indicate that high soy intake during adolescence reduces breast cancer risk; while there are conflicting results regarding adulthood exposure (32). The timing (stage of development) of exposure to estrogenic chemical is an important factor in physiological outcome, especially when the endogenous estrogen activity is low. Childhood and adolescence are the periods of life when the breast is most sensitive to dietary influence. Effects on prepubertal genistein exposure were evaluated in the MNU-induced mammary cancer model in S-D rats (33). Daily subcutaneous (*s.c.*) injection of 1.5 or 30 mg/kg bw of genistein (daily intake in Asian countries is 1.0 mg/kg) from 15 to 19 days of age followed by the administration of MNU at 28 days of age tended to suppress the incidence and multiplicity of MNU-induced mammary cancer, as compared to genistein-untreated rats (Table III). To evaluate the effects of genistein exposure in adults, MNU was administered to 45-day-old rats, followed by daily *s.c.* injection of 1 mg/kg genistein or vehicle for 20 weeks (37). Genistein administered to adult female S-D rats significantly increased the multiplicity of MNU-induced mammary carcinogenesis. In the DMBA-induced model, mammary carcinogenesis was decreased by prepubertal genistein exposure, whereas prenatal (*in utero*) and adult exposure showed no effect or stimulated mammary carcinogenesis (38-40). Therefore, short-term exposure to

Table II. *Effect of dietary administration of eicosapentaenoic acid, docosahexaenoic acid and conjugated docosahexaenoic acid on the development of N-methyl-N-nitrosourea-induced mammary cancer in female Sprague-Dawley rats.*

Treatment	Duration[1]	MNU[1]	Sacrifice[1]	≥1 cm MC (%)	MC/rat	Reference
10% EPA diet	7-27	7	27	11/15 (73)a	1.7[a]	20
5% EPA+5%DHA diet	7-27	7	27	12/17 (65)[b]	1.6[a]	
10% DHA diet	7-27	7	27	3/13 (23)	0.2	
0% CDHA diet	7-40	7	40	22/23 (96)	3.1	21
0.2% CDHA diet	7-40	7	40	16/24 (67)[c]	1.7[c]	
1.0% CDHA diet	7-40	7	40	17/25 (68)[c]	1.9[c]	

[1]Age (weeks); MC, mammary cancer; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; CDHA, conjugated DHA; [a]$p<0.01$ and [b]$p<0.05$ compared with 10% DHA group; [c]$p<0.05$ compared with 0% CDHA group.

Table III. *Effects of phytoestrogens and mycoestrogens on N-methyl-N-nitrosourea-induced mammary cancer in female Sprague-Dawley rats.*

Treatment	MNU[1]	Sacrifice[1]	≥1 cm MC (%)	MC/rat	Reference
Control	4	26	14/20 (70)	1.6	33
1.5 mg/kg Genistein			10/24 (42)[a]	1.2	
3.0 mg/kg Genistein			11/23 (48)	1.3	
Control	7	39	7/24 (29)	0.9	34
10 mg/kg Resveratrol			7/24 (29)	1.0	
100 mg/kg Resveratrol			14/24 (58)a	1.7[a]	
Control	4	37	19/24 (79)	3.1	35
0.1 mg/kg Zearalenone			11/24 (46)[a]	2.1	
10 mg/kg Zearalenone			12/24 (50)[a]	1.4[a]	
Control	4	37	18/22 (82)	1.5	36
0.1 mg/kg Zeranol			14/22 (64)	2.3	
10 mg/kg Zeranol			16/23 (70)	1.5	

Phytoestrogen and mycoestrogen, respectively, were subcutaneously injected daily from 15 to 19 days of age. [1]Age (weeks); MC, mammary cancer; [a]$p<0.05$ *vs*. Control.

genistein during the prepubertal period can provide rats with lifelong protection from chemically-induced mammary carcinogenesis. The timing of exposure seems to be essential.

Resveratrol is highly concentrated in grape skin and is also abundant in red wine (41). Epidemiologically, a significant inverse relationship exists between resveratrol from grape consumption and breast cancer risk (42). Low concentrations of resveratrol cause proliferation of ER-positive human breast cancer cells (MCF-7, ≤4 μM; KPL-1, ≤22 μM), whereas high concentrations inhibit the growth of both ER-positive (MCF-7 and KPL-1) and ER-negative (MKL-F) cells (IC_{50} of 137, 149, and 105 μM, respectively, after incubation for 72 h) (43). Growth suppression was due to apoptosis through up-regulation of Bax and Bak protein, down-regulation of Bcl-X_L, and activation of caspase-3. In another study, MNU-treated female S-D rats were exposed to resveratrol at the adult stage (44). MNU was administered to 49-day-old rats, and 10 or 100 mg/kg bw resveratrol was administered by gavage 5 days per week from 42 days of age until 169 days of age. Although 10 mg/kg bw resveratrol showed no effect, the 100 mg/kg dose prolonged the latency and reduced the multiplicity of mammary cancer. In another study, prepubertal female S-D rats were given daily *s.c.* injections of 10 or 100 mg/kg resveratrol or vehicle for 5 days starting at 15 days of age, and MNU was administered at 49 days of age (34). In contrast to adult exposure, short-term prepubertal exposure to 100 mg/kg bw resveratrol significantly accelerated mammary cancer incidence and multiplicity at 39 weeks of age, while 10 mg/kg resveratrol showed no adverse effects on mammary carcinogenesis (Table III). The mean dietary intake of naturally occurring resveratrol in the United States population is 0.08 mg/day (0.001 mg/kg bw/day), while commercial dietary supplements contain 50 to 2000 mg/day (0.8-33 mg/kg bw/day for a 60-kg human) (45). Although 700 mg/kg bw/day of resveratrol in a 90-day study was non-toxic to rats, foods containing high levels of resveratrol should not be consumed by young children.

Mycoestrogens. Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resocyclic acid-lactone] synthesized by *Fusarium* molds and its natural metabolic product zeranol

(α-zearalanol) [6-6,10-dihydroxyundecyl-β-resocyclic acid lactone] have estrogenic activity (46). Zeranol accelerates ER-positive MCF-7 and KPL-1 cells at low concentrations but suppresses ER-positive MCF-7 and KPL-1 and ER-negative MDA-MB-231 human breast cancer cells at a higher concentration (≥50 μM) (47). Zearalenone and zeranol have been widely used to promote the growth of livestock in the United States due to their anabolic effects (48). Thus, zearalenone and zeranol can enter the human food chain directly *via* ingestion of contaminated grain or indirectly *via* consumption of meat products from animals fed mold-infected grain or injected with zearalenone or zeranol for growth stimulation. People living in the United States are exposed to an estimated 1 to 5 mg (0.02-0.1 mg/kg bw) of zearalenone (49). Prenatal or neonatal zearalenone exposure at a dose of 0.2 μg/kg bw leads to phenotypic alterations in mammary glands of the adult female Wistar rats (50). In one study, female S-D rats received a daily *s.c.* injections of 0.1 or 10 mg/kg of zearalenone between 15 and 19 days of age and received MNU at 28 days of age (35). At 37 weeks of age, the mammary cancer incidence was significantly reduced in rats treated with 0.1 or 10 mg/kg zearalenone as compared to untreated controls, and mammary cancer multiplicity was significantly reduced in rats treated with 10 mg/kg zearalenone (Table III). In another study, female S-D rats received a daily *s.c.* injections of 0.1 or 10 mg/kg of zeranol between 15 and 19 days of age and received MNU at 28 days of age (36). As a result, mammary carcinogenesis was not affected at the termination of the experiment (Table III). Prepubertal exposure to zearalenone or zeranol at 0.1 and 10 mg/kg did not affect body weight gain, but vaginal opening was accelerated and the estrous cycle was disrupted, and the frequency of rats with no newly formed corpora lutea in the ovaries (indicating anovulation) increased in parallel to the dose of zearalenone or zeranol (35, 36). Thus, although the development of mammary cancer was lowered or unchanged by prepubertal zearalenone or zeranol treatment (both high- and low-dose), endocrine function and ovarian structure in adulthood was severely disrupted. Beginning on gestational day 15, pregnant CD-1 mice were given four daily *s.c.* injections of 0.5 or 10 mg/kg/day of genistein, resveratrol, or zearalenone, and their female offspring were examined at 4, 8, 12, and 16 weeks of age (51). A lack of corpora lutea was transiently seen in the high-dose genistein and resveratrol groups at 4 weeks of age, and was continuously seen at 4, 8, 12, and 16 weeks of age in the high-dose zearalenone group; again, zearalenone exerted prolonged deleterious effects on the ovaries. The severe effects on ovaries indicate that consumption of food containing zearalenone or zeranol in the developmental stage should be avoided.

Cell Disruption Caused by MNU in Animals

MNU causes alterations in DNA structure. When these alterations are left unrepaired, the accumulation of mutations will increase cancer risk (3). However, when excessive DNA damage is fixed in certain tissues or cells that are highly sensitive to MNU, the cells become disrupted by an apoptotic mechanism. Hematopoietic cells are the targets of MNU, and the resulting myelosuppression can cause death in laboratory animals. In addition to hematopoietic cells, cells in tissues such as the brain, eye, and hair are also affected by MNU exposure (Table IV). Prenatal *i.p.* exposure of 10 mg/kg bw MNU on day 13.5 or 15.5 of gestation causes excessive apoptotic cell death of neuronal precursors/stem cells in the dorsal telencephalon that results in microcephaly in the fetuses of ICR mice (52).

Chemotherapy-induced alopecia has been well documented. Alopecia can be induced in male and female neonatal C57BL mice by a single *i.p.* injection of 60 mg/kg bw MNU. MNU exposure is most effective in 8-day-old mice, which are in the active stage of the first hair cycle, and less effective in 5-day-old mice, which are in the early anagen stage of the first hair cycle; MNU exposure does not cause alopecia in 14-day-old mice, which are in the telogen stage of the first hair cycle (53). In 8-day-old MNU-treated mice, hair matrix cells undergo apoptosis due to up-regulation of Bax without the down-regulation of Bcl-2. Hair loss was evident at 14 days of age and persisted for a maximum of 22 days; and the hair growth resumed at the end of the second hair cycle. In chemically induced alopecia in adult animals, depilation is essential for adjusting the hair cycle to the anagen stage, while depilation is unnecessary when utilizing the first hair cycle of neonatal animals. Thus, the MNU-induced 8-day-old alopecia model may be useful in the search for drugs effective for hair growth.

Cataract, defined as opacification of the lens, can be caused by many factors (61). MNU damages the DNA of lens epithelial cells in S-D rats, leading to apoptosis and ultimately to lens opacity by up-regulation of Bax, down-modulation of Bcl-2, and activation of caspase-3 (54). Sensitivity of the lens epithelial cells to MNU is inversely related to the age of the animals. When a single *i.p.* injection of 100 mg/kg bw MNU was given to 0-, 5-, 10-, and 15-day-old male and female S-D rats, cataracts developed very rapidly. Gross lenticular opacities, usually bilateral, occurred in all rats by 7 days after MNU treatment of 0-day-old rats and by 14 days after MNU treatment of 5- or 10-day-old rats; whereas lens opacity was not evident 30 days after MNU treatment of 20-day-old rats. Mortality was inversely related to the age at MNU exposure. The 14-day mortality rate was 21% (10/48) for 0-day-old MNU-treated rats and 8% (2/26) for 5-day-old MNU-treated rats, whereas 10-, 15-, and 20-day-old MNU-treated rats (16 rats per group)

Table IV. *Disease states caused by cell disruption induced by intraperitoneal injection of N-methyl-N-nitrosourea in rodents.*

Pathology	Species	MNU		Age at sacrifice (days)	Reference
		Age (days)	Dose		
Microcephaly	Mouse	E13.5-E15.5	10	0	52
Alopecia	Mouse	8	60	14-36	53
Cataract	Rat	15	70	43	54, 55
Retinal dysplasia	Mouse, rat	0-3	50-60	7-8	56, 57
Retinal degeneration	Mouse, rat, hamster	49	60-90	56	58, 59, 60

MNU, *N*-Methyl-*N*-nitrosourea; E, embryonic day.

survived for 30 days. A 70 mg/kg dose of MNU given to 15-day-old rats seems to be a reliable model for cataract induction in 4 weeks with no mortality (55).

Retinal dysplasia is a developmental abnormality present at birth. It is morphologically characterized by the anomalous development of the retina with dysplastic rosettes, which are composed of neuronal retinal cells (62). Retinal dysplasia is caused by a variety of extrinsic causes as well as genetic factors. When 50 mg/kg bw of MNU was administered to 0-day-old S-D rats, retinal dysplasia characterized by rosette formation was found in the outer neuroblastic/nuclear layer above the continuous single layer of pigment epithelial cells (56). In C57BL mice, 60 mg/kg bw of MNU administered at 0 or 3 days of age caused retinal dysplasia, while MNU administered to mice older than 11 days of age caused retinal degeneration characterized by photoreceptor cell loss (57).

Retinitis pigmentosa is characterized by the loss of photoreceptor cells leading to the loss of peripheral vision and eventually to total blindness. Retinitis pigmentosa comprises a heterologous group of inherited human disorders that involve primary degeneration of photoreceptor cells following the detachment of retinal pigment epithelial cells from Bruch's membrane; these detached cells accumulate around the retinal blood vessel, resulting in bone-spicule pigmentation (63). More than 160 different mutations in genes that encode proteins with remarkably diverse functions result in rod photoreceptor degeneration (www.sph.uth.tmc.edu/retnet). However, the final common pathway of the disease is apoptotic cell death of photoreceptor cells. A single *i.p.* administration of MNU to adult animals induces photoreceptor apoptosis not only in rodents (mouse, rat, and hamster), but also in Insectivora (shrew), Lagomorphia (rabbit), Carnivora (cat), and non-human primates (monkey) (64). Thus, phylogenetically, MNU-induced retinal degeneration is a universal phenomenon. In monkeys, the lesion originates from the equatorial zone similar to human retinitis pigmentosa, whereas the lesion originates from the posterior pole in shrews, mice, rats, and hamsters (65). Fundus images of retinitis pigmentosa patients show perivascular retinal pigment epithelial cell deposition in a bone-spicule configuration. MNU-induced photoreceptor cell loss is followed by intraretinal migration of melanin-containing retinal pigment epithelial cells in rats and hamsters, and retinal pigment epithelial cells in direct contact with intraretinal blood vessels are seen only in hamsters. In shrews, mice, and monkeys, the retinal pigment epithelial cells do not migrate. Although the retinal pigment epithelial cell movement differs among species, photoreceptor apoptosis is a final common phenomenon; thus, MNU-induced photoreceptor apoptosis is a good model for human retinitis pigmentosa.

Retinal Degeneration in Animals

rd mice carrying the *Pde6b* gene have a reduced number of photoreceptor cells from 11 days of age, and the photoreceptor cells are completely lost or reduced to a single layer of cells by 20 days of age (66). *rds* (retinal degeneration slow) mice carrying the *Prpf2* gene exhibit photoreceptor cell loss starting at 2 weeks of age, and complete loss occurs 1 year after birth (65). In RCS (Royal College of Surgeons) rats with the *Mertk* gene, photoreceptor degeneration begins at 20 days of age, and complete loss occurs by 60 days of age. The *rd, rds,* and RCS animals have heterogeneous genetic defects that cause photoreceptor apoptosis; mutations in these genes have been detected in retinitis pigmentosa patients. Mammalian eyes are highly sensitive to toxic substances, and MNU may be a good candidate for the rapid induction of photoreceptor cell loss. The suppression of MNU-induced photoreceptor apoptosis can be utilized to identify therapeutic strategies for human retinitis pigmentosa.

Mechanisms of MNU-induced Photoreceptor Cell Loss

Herrold (67) first reported that MNU causes retinal degeneration (loss of photoreceptor cells) in Syrian golden hamsters. Later, apoptosis was identified as the cause of MNU-induced photoreceptor cell loss (58). Although the dose of MNU required for photoreceptor cell death from a single systemic administration differs in different animal species, the photoreceptor cell loss is completed in one week in both male

and female animals (65). MNU selectively damages photoreceptor cells, and no other cells in the retina are damaged. In rat studies, MNU interacts with DNA and yields the 7-methyldeoxyguanosine (7-medGua) DNA adduct selectively in photoreceptor nuclei 6, 12 and 24 h after MNU treatment, and the level of DNA adduct gradually decreases at days 3 and 7 (68, 69). DNA damage induced by MNU activates poly (ADP-ribose) polymerase (PARP) involved in DNA repair using nicotinamide adeninedinucleotide (NAD^+) as a substrate. PARP activity as evaluated by poly (ADP-ribose) expression (the product of PARP) increases at 12 and 24 h and peaks at day 3 (70). PARP regulates transcription by interacting with transcription factors. Decreased NF-κB activity (71), increased Jun-*N*-terminal kinase (JNK) activity, and the induction of AP-1 (c-Jun and c-Fos) (70) are involved in photoreceptor apoptosis. In the apoptosis cascade, Bcl-2 was down-regulated 12 h after MNU, Bax was up-regulated at 24 h, and caspase-3/CCP32, caspase-6/Mch2, and caspase-8/FLICE protease activities peaked 72 h after MNU (68). MNU administration results in increased calcium content in the retina leading to calpain activation at 1 and 3 days; the calpain activation is decreased at day 7 (72). Calpain and PARP are involved in the release of apoptosis-inducing factor (AIF) and cause neuronal cell death (73). These changes in molecular events occur concurrently and/or consecutively resulting in photoreceptor cell loss due to apoptosis. However, MNU-induced photoreceptor cell loss is independent of the *p53* gene (74). Twenty-four hours after MNU treatment, the first evidence of histological alterations was detected, and these alterations were the pyknosis and karyorrhexis of the photoreceptor nuclei, the shortening of the photoreceptor inner segment and the disorientation of the outer segment (58). At 48 h, the destruction of photoreceptor nuclei was most prominent, and at 72 h the degenerative nuclear component vanished due to phagocytosis by neighboring cells. At day 7, the active signs of nuclear degeneration became indistinct, reflecting almost completely the loss of photoreceptor cells (Figure 3). There are currently no effective therapies for retinitis pigmentosa. Many animal models of retinitis pigmentosa are available and have been used to identify therapeutic strategies (75). Based on the signaling pathway of MNU-induced photoreceptor cell apoptosis, we can speculate that the inhibition of PARP, caspase, or calpain may ameliorate photoreceptor cell loss.

Therapeutic Trials Against MNU-induced Retinal Degeneration

PARP inhibitors nicotinamide and 3-aminobenzamide. Nicotinamide, water-soluble vitamin B3, is a PARP inhibitor and a NAD^+ precursor. PARP hyperactivation results in the depletion of cellular NAD^+ pools, leading to ATP deficiency, energy loss, and subsequent cell death. Photoreceptor cell loss was suppressed when an *s.c.* injection of 10 or 25 mg/kg of nicotinamide was concomitantly administered with MNU (69). Seven days after MNU was administered to S-D rats, the photoreceptor cell ratio [(photoreceptor cell thickness/total retinal thickness) ×100] at the central retina (approximately 150 μm from either side of the optic nerve) was 5.6%, while rats treated with 10 or 25 mg/kg bw nicotinamide had a photoreceptor cell ratio of 22.4% and 33.6%, respectively (photoreceptor cell ratio of MNU-untreated control rat retina was 34.5%; Figure 4a). Nicotinamide dose-dependently ameliorated MNU-induced retinal damage; a 25 mg/kg dose provided complete protection, and a 10 mg/kg dose provided partial protection. In rats, nicotinamide at a dose of 1000 mg/kg yielded no side-effects and suppressed MNU-induced retinal damage both morphologically and functionally (76). An *s.c.* injection of 1000 mg/kg bw of nicotinamide was given to S-D rats 2, 4, 6, and 12 h after MNU treatment (the onset of retinal injury), and the photoreceptor cell thickness was monitored. Nicotinamide administered ≤4 h after MNU resulted in complete retinal protection, while nicotinamide administered 6 h after MNU provided partial retinal protection, and MNU administered 12 h after MNU was not protective. Mice were more resistant to nicotinamide as compared to rats. In C57BL mice, a 1000 mg/kg dose provided complete protection, and a 100 mg/kg dose provided partial protection. Seven days after MNU treatment, the photoreceptor cell ratio in mice concomitantly treated with 1000, 100, or 0 mg/kg bw nicotinamide was 29.7%, 11.8%, and 5.9%, respectively (MNU-untreated control retina, 32.2%). Nicotinamide may block the depletion of NAD^+ and/or inhibit PARP activation. PARP activation was diminished by nicotinamide treatment through the JNK/AP-1 signaling pathway (70).

3-Aminobenzamide is a PARP inhibitor but not an NAD^+ precursor. 3-Aminobenzamide at a dose of 50 or 30 mg/kg administered concomitantly with MNU suppressed photoreceptor cell loss in S-D rats (complete and partial retinal protection, respectively) *via* the preservation of NF-κB activity (71). The photoreceptor cell ratio 7 days after MNU administered concomitantly with 50, 30, or 0 mg/kg bw 3-aminobenzamide was 48.4%, 38.4%, and 17.8%, respectively (MNU-untreated control retina, 53.1%) (Figure 4b). Thus, 3-aminobenzamide dose-dependently rescued MNU-induced retinal damage when concomitantly administered with MNU. However, 3-aminobenzamide administered 12 h before or 4 h after MNU was ineffective.

Caspase-3 inhibitor Ac-DEVD-CHO. In MNU-induced photoreceptor cell apoptosis, caspase-3, -6, and -8 were activated (68). Caspase-8, which is an apoptotic initiator, acts upstream of caspase-3 and -6, which are effector caspases. Caspase-3 inhibition was selected for therapeutic trial because caspase-3 is a major contributor to the apoptotic machinery. Intravitreal administration of caspase-3 inhibitor (two 4000-

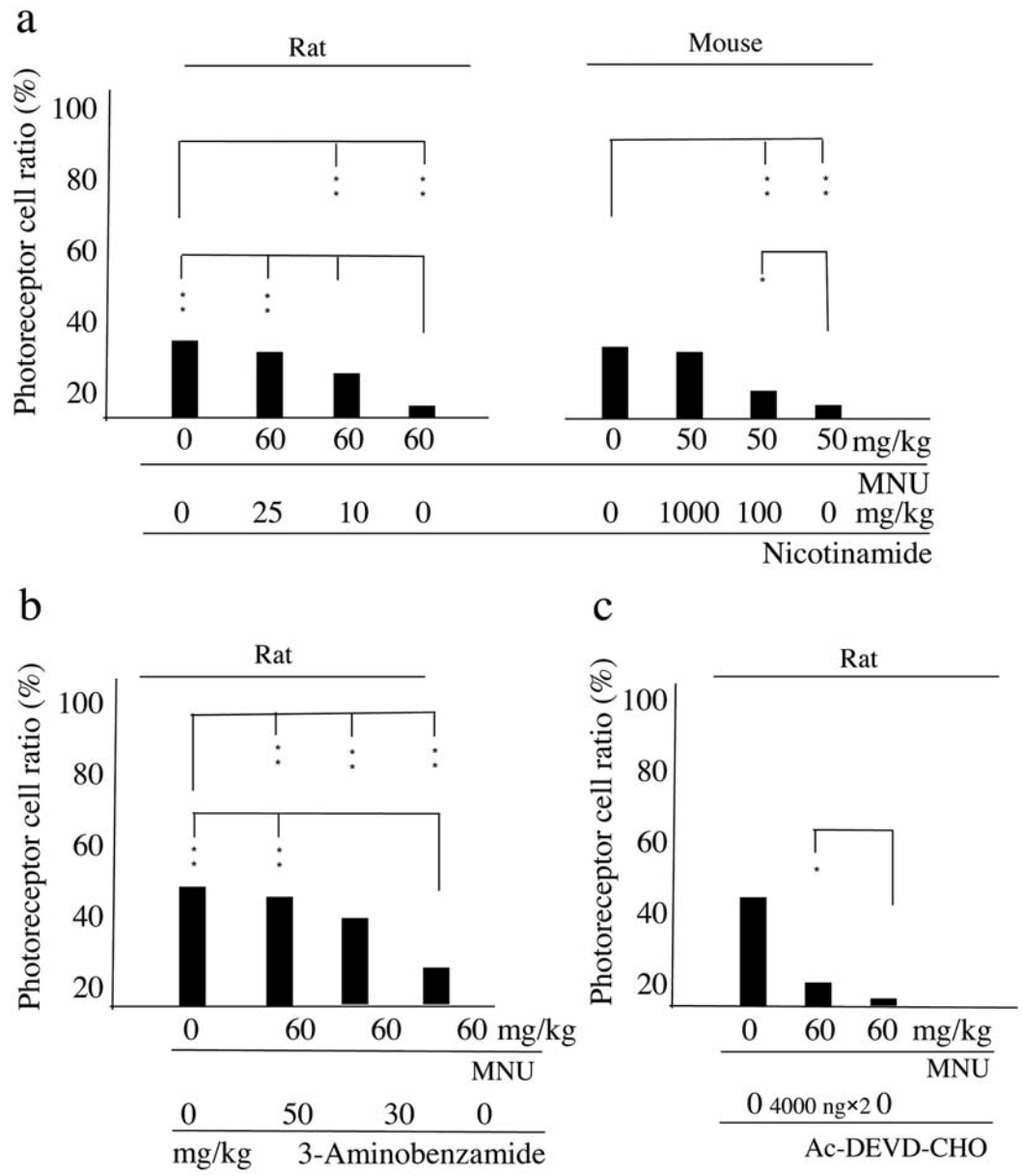

Figure 4. *Rescue capacities of photoreceptor cell loss against N-methyl-N-nitrosourea (MNU)-induced retinal damage by nicotinamide, 3-aminobenzamide, and Ac-DEVD-CHO, as evaluated by the photoreceptor cell ratio. The photoreceptor cell ratio was calculated as [(photoreceptor cell thickness/total retinal thickness) ×100] measured at the central retina (approximately 150 μm from either side of the optic nerve). MNU, N-Methyl-N-nitrosourea; **p<0.01; *p<0.05. Summarized from references 69, 71, and 77.*

ng doses of Ac-DEVD-CHO) suppressed MNU-induced photoreceptor apoptosis (77). When retinal damage was measured 7 days after MNU treatment, the photoreceptor cell ratio (Figure 4c) was 2.7% without Ac-DEVD-CHO and 11.7% with Ac-DEVD-CHO (MNU-untreated control retina, 48.4%). MNU-induced retinal damage proceeds from the central retina to the peripheral retina. Thus, when the retinal damage ratio [(length of damaged retina/whole retinal length) ×100] was used for evaluation (damage to the retina was defined as the presence of less than four rows of photoreceptor cell nuclei in the outer nuclear layer), the retinal damage ratio in MNU-treated rat retina (98.5%) was significantly reduced to 54.4% after Ac-DEVD-CHO treatment. Ac-DEVD-CHO significantly preserved the photoreceptor cell ratio in the central retina and significantly reduced the retinal damage ratio that Ac-DEVD-CHO suppressed and/or delayed the progression of photoreceptor cell damage.

Calpain inhibitor SNJ-1945. Calpain inhibitor ((1S)-1-((((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl) amino)carbonyl-3-methylbutyl) carbamic acid 5-methoxy-3-oxapentyl ester (SNJ-1945) suspended at 5% (w/v) in distilled water containing 0.5% carboxymethyl cellulose was administered *p.o*. at a dose of 100 or 200 mg/kg bw within 30 min after MNU and once daily for 7 days thereafter (72). SNJ-1945 at a dose of 200 mg/kg bw significantly reduced the severity of photoreceptor cell loss by 46% seven days after MNU treatment (as determined by outer nuclear thickness), while 100 mg/kg SNJ-1945 was ineffective. SNJ-1945 caused no side-effects, and a dose of 1000 mg/kg/day for 14 days produced no obvious toxic signs or abnormalities in rats (78).

Concluding Remarks

MNU targets various organs, and MNU-induced mammary cancer and retinal degeneration in rats are useful animal models for human disease. These organ-specific models can be used to identify therapeutic interventions. Fats and phytochemicals modulate breast cancer. n-3 PUFA protects against MNU-induced mammary cancer. CDHA was more potent than DHA, and the DHA effect was stronger than EPA. However, n-3 PUFA must be present in the diet for a long duration. Parity protection (high levels of estrogen or estrogen along with progesterone for short duration) against breast cancer is a well-known phenomenon. Short-term exposure to phytochemicals (phytoestrogen and mycoestrogen) at the prepubertal period exerts a lifelong effect on mammary cancer. Short-term genistein exposure compatible to Asian intake suppresses mammary cancer, while resveratrol exposure at higher than natural consumption levels enhances mammary cancer. Short-term zearalenone and zeranol exposure do not enhance mammary cancer yield but do cause anovulatory ovaries later in life. No effective therapies for retinitis pigmentosa currently exist. Retinitis pigmentosa is defined by the loss of photoreceptor cells by an apoptotic mechanism. During the course of MNU-induced photoreceptor apoptosis, the activation of PARP, caspase, and calpain occurred. PARP inhibitors (nicotinamide and 3-aminobenzamide), caspase-3 inhibitor (Ac-DEVD-CHO), and calpain inhibitor (SNJ-1945) were effective at suppressing photoreceptor cell loss in the MNU-induced retinal degeneration model. These therapeutic strategies may be adopted for humans.

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