PARP Inhibitors Accelerate N-Methyl-N-Nitrosourea-induced Cataractogenesis in Sprague-Dawley Rats

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Abstract. Background: There have been no previous studies of the effects of poly(ADP-ribose) polymerase (PARP) inhibitors on N-methyl-N-nitrosourea (MNU)-induced cataractogenesis in rats. Materials and Methods: A single intraperitoneal injection of 70 mg/kg MNU was administered to 15-day-old male and female Sprague-Dawley rats. In Experiment 1, rats were then subcutaneously injected with 1000 mg/kg nicotinamide, either once or 3 times at 1-week intervals. In Experiment 2, rats were subcutaneously injected once with 50 mg/kg 3-aminobenzamide. For comparison, the following age-matched controls were included: MNU-untreated nicotinamide-injected rats, MNU-untreated 3-aminobenzamide-injected rats, and MNU-untreated PARP-inhibitor-untreated rats. Rats were examined for lens opacity. At 28 days after MNU injection, 10 to 20 rats per group were sacrificed. In Experiment 1, at 3 days after MNU injection, 10 rats per group were sacrificed for apoptosis and cell proliferation detection. Results: MNU caused lens epithelial cell apoptosis in the germinative zone, as indicated by TUNEL staining. However, regardless of MNU treatment, lens epithelial cell proliferation was consistently seen in the germinative zone and sporadically seen in the central zone. At 28 days after MNU, mature cataracts were observed. Nicotinamide significantly accelerated lens opacity and cataractogenesis, as indicated by a cataract index. 3-Aminobenzamide significantly accelerated the development of lens opacity and tended to accelerate cataractogenesis. Conclusion: The PARP inhibitors nicotinamide and 3-aminobenzamide accelerated MNU-induced cataractogenesis.

Cataracts are defined as lens opacity associated with visual impairment and are the leading cause of blindness worldwide, accounting for 50% of all blindness (1). Animal models are indispensable for the study of cataracts and various animal models of human cataracts have been established (2). When N-methyl-N-nitrosourea (MNU), an alkylating agent, was administered to 15-day-old rats over a 4-week period, both nuclear and cortical cataracts developed, at equal rates in males and females; the cataracts developed rapidly and constantly (3). DNA damage induced by various agents can give rise to cataracts and it has been proposed that apoptosis of lens epithelial cells contributes to various models of cataracts such as ultraviolet (UV)-induced (4), selenite-induced (5) and MNU-induced cataracts (6). MNU-induced cataracts are associated with the formation of 7-methyldeoxyguanosine DNA adducts in lens epithelial cell nuclei, leading to apoptosis by up-regulation of Bax protein, down-modulation of Bcl-2 protein and activation of caspase-3 (6).

Cellular DNA damage can activate repair mechanisms or signal transduction pathways that lead to cell death. Poly(ADP-ribose) polymerase (PARP) is a nuclear, zinc-finger, DNA-binding protein that specifically detects DNA strand breaks (7). PARP is a key enzyme mediating the cellular response to DNA strand breaks and plays a critical role in genomic stability and the survival of proliferating cells (8). PARP-null mice exhibit extreme radiosensitivity and high genomic instability, and die rapidly from radiation toxicity or after exposure to MNU (9, 10). Embryonic fibroblasts from PARP-null mice exhibit high sensitivity to MNU (9). These findings indicate that PARP is involved in DNA base-excision repair and that PARP acts as a survival factor for proliferating cells.

A remarkable feature of lens epithelial cells is their capacity to divide and differentiate throughout most of the life of an individual (11). Despite the fact that PARP appears to play a critical role in genomic stability and survival of proliferating cells undergoing DNA damage, there have been no reported studies of whether PARP inhibitors act as regulators of cataractogenesis. Thus, in the present study, we examined the effects of the PARP inhibitors nicotinamide and 3-aminobenzamide on MNU-induced cataractogenesis in rats.
Materials and Methods

Animals. Twelve-day-old Sprague-Dawley rats (Jcl:SD), together with their mothers (5 male and 5 female pups per mother), were purchased from Clea Japan, Osaka. Rats were housed in plastic cages with wood-chip bedding in an air-conditioned animal room at 20±2°C and relative humidity of 60±10% with a 12-h light/dark cycle. The illumination intensity was below 60 lux at the cage level. Mothers were allowed to suckle their pups until weaning (21 days of age) and the animals were maintained on a basal diet (CMF; Oriental Yeast, Chiba, Japan) with free access to food and water. Throughout the experiments, the animals were cared for in accordance with the Guidelines for Animal Experimentation of Kansai Medical University, Japan.

Chemicals and dose formulation. MNU was purchased from Chem Service (West Chester, PA, USA) and kept at ~20°C in the dark. Immediately before use, MNU was dissolved in physiological saline containing 0.05% acetic acid and was kept on ice shielded from light. Nicotinamide was purchased from Nacalai Tesque (Kyoto, Japan) and was dissolved in physiological saline. 3-Aminobenzamide was purchased from Sigma (St. Louis, MO, USA) and was dissolved in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). The dose of nicotinamide and 3-aminobenzamide was based on results of our previously reported studies (12, 13).

Experimental procedures. The rats were divided into eight groups, and two experiments were conducted.

Experiment 1 (Groups 1-5): At 15 days of age, Groups 1 to 3 (30 rats each) received a single intraperitoneal (i.p.) injection of 70 mg/kg MNU. In Groups 2 and 3, the MNU injection was immediately followed by a subcutaneous (s.c.) injection of 1000 mg/kg nicotinamide. In Group 4 (20 rats), the same dose of nicotinamide was s.c. injected into age-matched MNU-untreated rats. Group 3 received 2 further injections of nicotinamide, at 1-week intervals. At 3 days after MNU injection, 10 rats in each group were killed and processed for apoptosis and cell proliferation evaluation. The remaining rats were observed daily for clinical signs, weighed once per week, examined for lens opacity, and killed 28 days after MNU injection (43 days of age). For comparison, age-matched MNU-untreated nicotinamide-untreated control rats (Group 5, 20 rats) were sacrificed at the same time points as for the other groups.

Experiment 2 (Groups 6-8): At 15 days of age, Groups 6 and 7 (10 and 20 rats, respectively) received a single i.p. injection of 70 mg/kg MNU. In Group 7, the MNU injection was immediately followed by s.c. injection of 50 mg/kg 3-aminobenzamide. For comparison, a group of age-matched MNU-untreated 3-aminobenzamide-treated rats (Group 8, 10 rats) were included. At 15 days of age, male rats weighed 30.5 g to 44.1 g (mean, 36.5±3.1 g) and female rats weighed 31.1 g to 36.2 g (mean, 34.2±1.9 g). Rats were observed daily for clinical signs, weighed once per week, examined for lens opacity, and killed 28 days after MNU injection (43 days of age).

In both experiments, at necropsy, bilateral eyeballs were macroscopically examined. The rats were killed by cervical dislocation, and the eyeballs were dissected immediately thereafter.

Tissue fixation and processing. One eye from each rat was fixed overnight in 10% neutral buffered formalin, while the other was fixed in methacarn. The formalin- and methacarn-fixed tissues were embedded in paraffin and cut into mid-sagittal sections, which were stained with hematoxylin and eosin (HE).

TUNEL assay and PCNA staining. Formalin-fixed sections from rats sacrificed 3 days after MNU injection (18 days of age) underwent the TUNEL assay to identify apoptotic cells using an apoptosis detection kit (Integrin ApopTag kit, Integrin, Purchase, NY, USA). Methacarn-fixed sections obtained 3 days after MNU injection were processed for proliferating cell nuclear antigen (PCNA) immunohistochemistry using mouse monoclonal antibody to PCNA (PC10, Novocastra, Newcastle upon Tyne, UK) and labeled streptavidin biotin (LSAB) kit (Dako, Carpinteria, CA, USA). Antigen retrieval was performed for the demonstration of PCNA. In both methods, 3,3′-diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen and hematoxylin was used as the counterstain.

Lens opacity. The severity of lens opacity, as revealed by inspection, was arbitrarily graded as none (Grade 0), mild (Grade 1; Figure 1a), or severe (Grade 2; Figure 1b). Each eye was separately evaluated and the grades of both eyes of each rat were averaged.

Cataract index. To determine the severity of cataracts in HE-stained tissue sections, a microscopic cataract scoring system described in detail elsewhere (2, 6) was used, with a slight modification. Briefly, the following eight items were independently scored 0 to 4: lens epithelial apoptosis, lens epithelial desquamation, multilayered spindle epithelium, lens fiber swelling, liquefaction/vacuolar change, extent of calcification, bizarre nuclei of lens fiber cells, and lens rupture. The scores of these eight items were then totalled and the sum was defined as the cataract index.

Statistical analysis. The percentages of rats with lens opacity and the data quantifying the severity of lens opacity were analyzed for independence using the Chi-square test. The cataract index and the data quantifying the severity of various forms of histological lens damage were analyzed using the 2-tailed, independent Student's t-test for unpaired samples, after assuring homogeneity of variances. A probability value of p<0.05 was considered to indicate statistical significance.

Results

General remarks. All the rats survived for at least 4 weeks (43 days of age) or until they were sacrificed. MNU-treated groups exhibited body weight gain (Groups 1, 2, 3, 6, and 7), but their weight gain was less than that of the MNU-untreated groups (Groups 4, 5, and 8). Nicotinamide and 3-aminobenzamide did not affect body weight gain. In all rats examined, there was no extracocular abnormality, and intraocular lesions were restricted to the lens. There was no gender difference in the incidence or severity of lens opacity; consequently, the data for males and females were combined.

Effects of nicotinamide. In MNU-treated groups (Groups 1, 2, and 3), 3 days after MNU injection, lens epithelial cell nuclei and fragmented nuclei in the germinative zone were TUNEL-positive (Figure 2a). In MNU-untreated groups
Figure 1. MNU-induced lens opacity in Sprague-Dawley rats. a) Mild (grade 1) lens opacity. b) Severe (grade 2) lens opacity.

Figure 2. Lens epithelial cell apoptosis detected by TUNEL staining. a) TUNEL-positive nuclei are distributed in the germinal zone (between arrows) 3 days after MNU treatment. b) No TUNEL signals are detected in lenses from age-matched untreated controls. Original magnification, x100.

Figure 3. Lens epithelial cell proliferation detected by PCNA immunoreactivity. a) Cells in germinative zone are consistently positive. b) Cells in the central zone are sporadically positive. Original magnification, x100.

Figure 4. Lenses of MNU-treated and -untreated rats (HE). a) A mature cataract can be seen in an MNU-treated nicotinamide-treated rat lens 28 days after MNU injection. b) Lens from an age-matched MNU-untreated control rat (HE). Original magnification, x20.
Table III shows the sequential effects of 3-aminobenzamide. Additional effect.

 genesis and that additional nicotinamide injections had no nicotinamide injection accelerated MNU-induced cataractogenesis. Among MNU-treated rats, the severity of the group treated 3 times with nicotinamide (Group 3) was significantly higher than that of the nicotinamide-untreated rats (Group 1), with the frequency and severity of lens opacity increasing 4 weeks after MNU injection. Nicotinamide tended to accelerate MNU-induced cataractogenesis, but MNU-untreated nicotinamide-treated rats (Group 4), like untreated control rats (Group 5), did not exhibit lens opacity.

 Histological samples were collected 4 weeks after MNU injection (at 43 days of age). In MNU-treated groups (Group 1, 2 and 3), mature cataracts were observed in all rats. Histologically, lens fibers were degenerated, swollen, vacuolated and liquefied, and Morgagni-like water vacuoles were observed (Figure 4a). However, the lenses of MNU-untreated nicotinamide-treated rats (Group 4) remained intact, and were comparable to lenses of untreated control rats (Group 5; Figure 4b).

 The effect of nicotinamide treatment on the severity of MNU-induced lens damage was quantitatively evaluated using the cataract index (Table II). The severity of the various forms of histological damage in the lens were scored and the cataract index was calculated. The MNU-treated nicotinamide-untreated group (Group 1) had a cataract index of 7.8, whereas the MNU-treated rats that received a single nicotinamide injection (Group 2) had a significantly higher index of 11.7, and MNU-treated rats that received 3 nicotinamide injections (Group 3) had a cataract index of 12.7 while the MNU-treated 3-aminobenzamide-untreated group (Group 6) had a cataract index of 13.4; the difference was not significant. These results indicate that a single nicotinamide injection (at 43 days of age). In MNU-treated groups (Groups 1, 2 and 3), no lenticular abnormality was observed 1 or 2 weeks after MNU injection. The first sign of lens opacity appeared as a faint opacity, 3 weeks after MNU injection. Among MNU-treated rats, the severity of the group treated 3 times with nicotinamide (Group 3) was significantly higher than that of the nicotinamide-untreated rats (Group 1), with the frequency and severity of lens opacity increasing 4 weeks after MNU injection. Nicotinamide tended to accelerate MNU-induced cataractogenesis, but MNU-untreated nicotinamide-treated rats (Group 4), like untreated control rats (Group 5), did not exhibit lens opacity.

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 Effects of 3-aminobenzamide. Table III shows the sequential occurrence and severity of lens opacity in Experiment 2. The first lenticular abnormality was observed in MNU-treated rats 3 weeks after MNU injection and 3-aminobenzamide significantly increased the severity of lens opacity (Group 7), compared with 3-aminobenzamide-untreated rats (Group 6). The frequency and severity of lens opacity increased 4 weeks after MNU injection. However, MNU-untreated 3-aminobenzamide-treated rats (Group 8) did not exhibit lens opacity. According to the cataract index (Table IV), MNU treatment caused more severe lens damage in Experiment 2 (Group 6) than in Experiment 1 (Group 1). In Experiment 2, the MNU-treated 3-aminobenzamide-untreated group (Group 6) had a cataract index of 12.7 while the MNU-treated 3-aminobenzamide-treated rats had a cataract index of 13.4; the difference was not significant.

 Discussion

 In the present study, a single i.p. injection of 70 mg/kg MNU into 15-day-old Sprague-Dawley rats rapidly induced cataracts in both males and females, with no other abnormalities within the eye or in extraocular tissues. This is similar to a previous report (3) in which 4 weeks after MNU injection, lens fibers showed degeneration, swelling, vacuolation, liquefaction and Morgagni-like water vacuole formation indicating mature cataracts. In the present study, 3 days after MNU injection, proliferative activity of lens epithelial cells as seen by PCNA labeling was not affected compared with MNU-untreated rat lenses. However, while no TUNEL-positive signal was seen in MNU-untreated rat lenses, TUNEL-positive cells were observed among lens epithelial cells located in the germinative zone, which is similar to previous TUNEL results for selenite-induced cataractogenesis (5). In a previous study of UV-induced cataracts (4), TUNEL-positive cells were detected throughout the entire epithelium, but not in the bow area, indicating that UVB irradiation specifically affected the surface of the lens. In the present study, nicotinamide did not noticeably affect the number or location of TUNEL-positive cells. However, the PARP inhibitors nicotinamide and 3-aminobenzamide accelerated cataractogenesis, indicating that PARP acted as a survival factor opposing MNU-induced cataractogenesis in rat lens epithelial cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>1</td>
<td>MNU</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>25 (0.3)</td>
<td>85 (1.7)</td>
</tr>
<tr>
<td>2</td>
<td>MNU+NAMx1</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>35 (0.6)</td>
<td>90 (1.3)</td>
</tr>
<tr>
<td>3</td>
<td>MNU+NAMx3</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>50 (0.9)*</td>
<td>100 (2.0)</td>
</tr>
<tr>
<td>4</td>
<td>NAMx1</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Untreated</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

MNU, N-methyl-N-nitrosourea; NAM, nicotinamide. Severity was graded as follows: Grade 0, no opacity; Grade 1, mild opacity; Grade 2, severe opacity. *p<0.05, compared to Group 1.
PARP plays a critical role in the survival and maintenance of the genomic stability of proliferating cells exposed to DNA-damaging agents. Studies of cell cultures and PARP-null mice indicate that PARP plays a significant biological role in the recovery of proliferating cells from DNA damage (9, 10). In selenite-induced lens epithelial apoptosis, PARP levels decreased in parallel with cataractogenesis (5). Nicotinamide is a PARP inhibitor and NAD+ precursor, while 3-aminobenzamide is a specific PARP inhibitor (14). In human leukocytes, 3-aminobenzamide has been found to accelerate damage caused by hydrogen peroxide (15). In the present study, both nicotinamide and 3-aminobenzamide accelerated MNU-induced cataractogenesis.

In striking contrast to the normally cytoprotective function of PARP, DNA damage can cause PARP hyperactivation due to depletion of NAD+, leading to cell death via energy failure (8). MNU can induce photoreceptor cell apoptosis and photoreceptor cell loss within 7 days when given to young adult rats (50 days old) (15). In non-proliferating cells, such as photoreceptor cells, MNU can cause acute cell death via PARP overactivation that leads to depletion of NAD+ pools (16), and this MNU-induced photoreceptor cell apoptosis can be suppressed by PARP inhibitors such as nicotinamide (3, 16, 17) and 3-aminobenzamide (13). Thus, PARP inhibitors appear to be able to reduce photoreceptor cell damage caused by MNU. In non-proliferating cells, PARP inhibition experiments result in DNA repair, cell survival and maintenance of genomic stability. Thus, PARP inhibition can induce both anti- and pro-apoptotic effects, depending on the cell type.

### Conclusion

The present results indicate that PARP functions as a survival factor against MNU-induced cataractogenesis in lens epithelial cells and that the PARP inhibitors

### Table II. Effects of nicotinamide on the cataract index and severity of histological damage in the lens after MNU injection in Sprague-Dawley rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mean scores of histological damage</th>
<th>Cataract index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lens epithelial apoposis</td>
<td>Lens epithelial desquamation</td>
</tr>
<tr>
<td>1</td>
<td>MNU</td>
<td>0.8±0.4</td>
<td>0.3±0.5</td>
</tr>
<tr>
<td>2</td>
<td>MNU+NAM x 1</td>
<td>1.1±0.2**</td>
<td>0.7±0.7*</td>
</tr>
<tr>
<td>3</td>
<td>MNU+NAM x 3</td>
<td>1.4±0.5**</td>
<td>1.1±0.7**</td>
</tr>
<tr>
<td>4</td>
<td>NAM x 1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Untreated</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

MNU, N-methyl-N-nitrosourea; NAM, nicotinamide. Each score is mean±SD from 10-20 rats. *p<0.05 and **p<0.01, compared with Group 1.

### Table III. Effect of 3-aminobenzamide on the frequency and severity of MNU-induced lens opacity in Sprague-Dawley rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>MNU</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>50 (0.3)</td>
<td>100 (2.0)</td>
</tr>
<tr>
<td>7</td>
<td>MNU+3AB x 1</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>60 (1.0)*</td>
<td>100 (2.0)</td>
</tr>
<tr>
<td>8</td>
<td>3AB x 1</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

MNU, N-methyl-N-nitrosourea; 3AB, 3-aminobenzamide. Severity was graded as follows: Grade 0, no opacity; Grade 1, mild opacity; Grade 2, severe opacity. *p<0.05, compared with Group 6.

### Nicotinamide and 3-aminobenzamide accelerate MNU-induced cataractogenesis.

### Acknowledgements

We would like to thank Ms. T. Akamatsu for her technical assistance and Ms S. Nakagawa for preparing this manuscript.

### References


Table IV. Effect of 3-aminobenzamide on the cataract index and severity of histological damage in the lens after MNU injection in Sprague-Dawley rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mean scores of histological damage</th>
<th>Cataract index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lens epithelial apoptosis</td>
<td>Lens epithelial desquamation</td>
</tr>
<tr>
<td>6</td>
<td>MNU</td>
<td>1.0±0.0</td>
<td>1.1±0.6</td>
</tr>
<tr>
<td>7</td>
<td>MNU +3AB x 1</td>
<td>1.1±0.4</td>
<td>0.8±0.6</td>
</tr>
<tr>
<td>8</td>
<td>Untreated</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

MNU, N-methyl-N-nitrosourea; 3AB, 3-aminobenzamide. Each score is mean±SD from 10-20 rats. *p<0.05 compared with Group 6.


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