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Suppression of alkylating agent induced cell transformation and gastric ulceration by low-dose alkylating agent pretreatment

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ABSTRACT

Exposure to mild stress by chemicals and radiation causes DNA damage and leads to acquired stress resistance. Although the linear no-threshold (LNT) model of safety assessment assumes risk from any dose, evidence from radiological research demonstrates a conflicting hormetic phenomenon known as the hormesis effect. However, the mechanisms underlying radiation hormesis have not yet been clarified, and little is known about the effects of low doses of chemical carcinogens. We analyzed the efficacy of pretreatment with low doses of the alkylating agent N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) on the subsequent induction of cell transformation and gastric ulceration by high-dose MNNG. We used an *in vitro* Balb/3T3 A31-1-1 cell transformation test and monitored the formation of gastric ulcers in 5-week-old male ICR mice that were administered MNNG in drinking water. The treatment concentrations of MNNG were determined by the cell survival rate and past reports. For low-dose *in vitro* and *in vivo* experiments, MNNG was used at 0.028 μM, and 2.8 μg/mL, respectively. The frequency of cell transformation induced by 10 μm MNNG was decreased by low-dose MNNG pretreatment to levels similar to that of spontaneous transformation. In addition, reactive oxygen species (ROS) and mutation frequencies induced by 10 μm MNNG were decreased by low-dose MNNG pretreatment. Importantly, low-dose MNNG pretreatment had no effect on cell proliferation. *In vivo* studies showed that the number of gastric ulcers induced by 1 mg/mL MNNG decreased after low-dose MNNG pretreatment. These data indicate that low-dose pretreatment with carcinogens may play a beneficial role in the prevention of chemical toxicity under specified conditions.

1. Introduction

A fundamental misconception in toxicology is that no matter how low the dose, carcinogens can cause DNA damage and mutations that increase the risk of cancer. This concept is established by toxicity tests in bacteria, cultured cells, and animal no-threshold models and is based on linear dose–response relationships that are extrapolated to the origin for use in human safety assessments. However, previous reports have demonstrated the efficacy of low-dose treatments, which conflicts with these concepts. These observations are widely recognized as radiation hormesis or hormetic effects [1–3]. The hormetic effect is defined as "high dose is harmful, but low dose stimulates biological activity" or "low dose pretreatment leads to resistance to subsequent high doses" and it has been reported since the 1980s [4]. Clinical applications of this knowledge used single low-dose treatments or low-dose pretreatments [5]. Mechanisms of radiation hormesis have also been reported, and several lines of evidence show activation of antioxidants, DNA repair systems, and bystander effects [6–8]. Although these observations make it increasingly difficult to refute radiation hormesis, verification of hormesis is hampered by varied physicochemical properties of radiation and species differences. As such, the mechanisms underlying radiation hormesis have not yet been clarified, and safety assessments of radiation and carcinogens continue using the linear no-threshold (LNT) model.

Problems defining such a "threshold" have been demonstrated using a carcinogen with clear mechanisms of toxicity. Although research on the radiation hormesis-like effects of chemical carcinogens is scarce, we previously reported the specific efficacy of low doses of heavy metal [9]. Moreover, at low doses, the nongenotoxic carcinogens phenobarbital and alpha-benzene hexachloride exerted hormetic effects in N-diethylnitrosamine-initiated hepatocarcinogenesis. Indeed, the thresholds of 2-amino-3,8-dimethylimidazo

Abbreviations: MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; LNT, linear no-threshold; 6-TG, 6-thio-guanine; GST-P, glutathione S-transferase placental; ROS, reactive oxygen species; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; DMSO, dimethyl sulfoxide.

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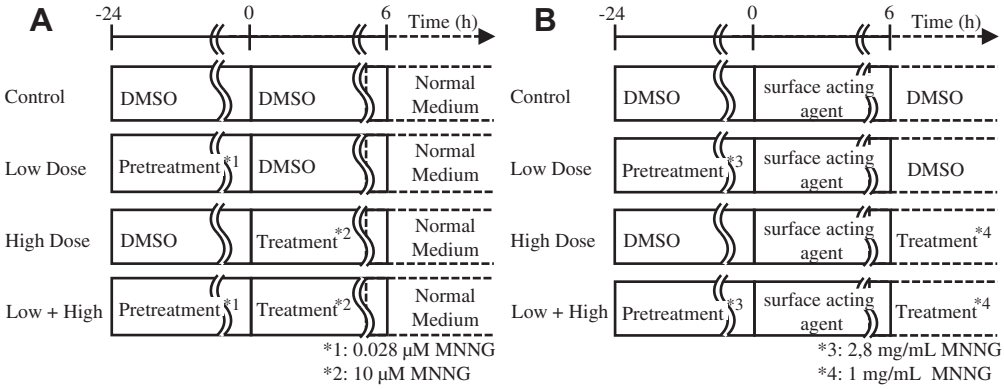

Fig. 1. Schematic representation of the MNNG pretreatment protocol.

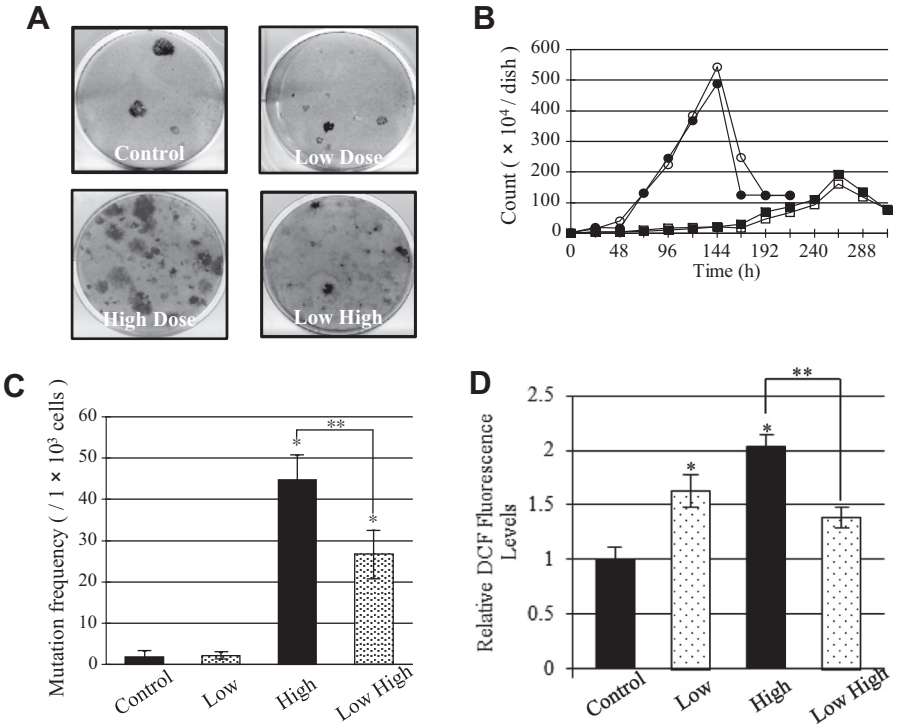

Fig. 2. Typical images of cell transformation show that low-dose MNNG mitigates induction of mutations and ROS by high-dose MNNG but does not affect cell proliferation. (A) Cells were treated with low- and high-dose MNNG according to experimental schema in Fig. 1A. After 4–5 weeks, the cells were stained with Giemsa and scanned, (B) the effect of low-dose MNNG on cell proliferation was indicated by the trypan blue dye exclusion test. For details refer to Materials and methods in Section 2.6. Control Group, open circle (○); Low Group, closed circle (●); High Group, open square (□); and Low High Group, closed square (■). (C, D) The effects of low-dose MNNG on induction of mutations and ROS by high-dose MNNG were assessed using 6-TG mutation resistance and DCFH-DA assays. For details refer to Materials and methods section 2.7–8. $^{*}P < 0.001$ vs control; $^{**}P < 0.001$ vs indicated sample (ANOVA and Tukey's HSD tests).

[4,5-f] quinoxaline and 2-amino-1-methyl-6-phenolimidazo [4,5-b] pyridine were demonstrated using aberrant crypt foci, glutathione S-transferase placental (GST-P)-positive foci, and 8-hydroxy-2′-deoxyguanosine levels as indexes [10–13].

Toxicology based on the carcinogenic threshold concept has frequently prompted reconsideration of the fundamental LNT concept [14]. In terms of human safety assessments, application of the LNT model might be suitable for extrapolation to humans. However, given the successes of radiation hormesis, full disclosure of its benefits is still required to serve the spirit of toxicological inquiry.

To analyze low-dose chemical effects, this report demonstrates the efficacy of low-dose MNNG, similar to radiation hormesis, using the *in vitro* transformation assay, the 6-thioguanine (6-TG)-resistant mutation assay, and assessments of gastric ulceration *in vivo* as indexes.

2. Materials and methods

2.1. Cell culture

Cryopreserved BALB/3T3 A31-1-1 cells were obtained from the RIKEN Bioresource Center (Ibaraki, Japan). The cells were thawed and used for experiments after varying periods. The cells were maintained in a state of continuous subconfluent growth by subculturing 3 times per week using trypsin. They were cultured in Eagle's minimum essential medium (Wako Chemical) containing 5% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2 in air.

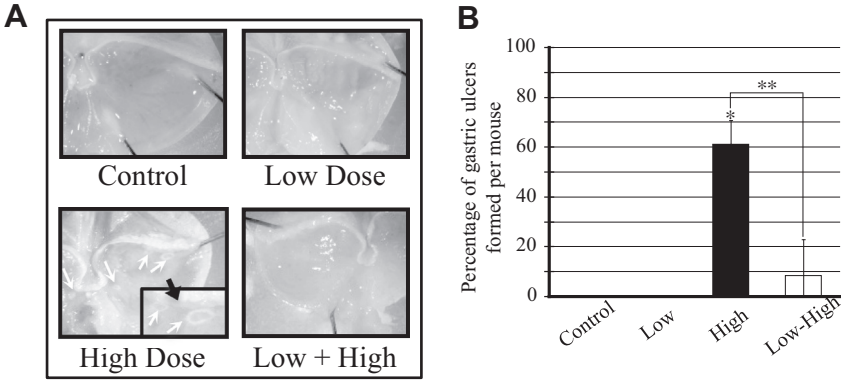

Fig. 3. Suppression of MNNG-induced gastric ulcers by low-dose MNNG pretreatment Mice were treated with low- and high-dose MNNG according to the experimental schema in Fig. 1B. After the high-dose MNNG treatment for 3 weeks, all male mice were sacrificed and their stomachs surgically removed. After 4% PFA fixation, images of gastric surface mucosa were acquired using a digital camera (A), and the number of ulcers were evaluated for each animal (B). $^{*}P < 0.001$ vs. control; $^{**}P < 0.001$ vs. indicated sample (ANOVA and Tukey's HSD tests).

Table 1
Effects of low-dose MNNG pretreatment on high-dose MNNG-induced transformation.

	Experiment	Cell survival (%)	Mean number of transformed foci	Number of wells with transformed foci
Control	1st	100.00 ± 1.98‡	0.67 ± 0.58‡	2/3
	2nd	91.09 ± 7.92‡	0.00 ± 0.00‡	0/3
	3rd	87.89 ± 9.45‡	0.67 ± 0.58‡	2/3
Low dose	1st	101.00 ± 2.16‡	0.33 ± 0.58‡	1/3
	2nd	87.13 ± 2.00‡	0.33 ± 0.58‡	1/3
	3rd	97.69 ± 6.05‡	0.67 ± 1.16‡	1/3
High dose	1st	12.87 ± 8.32†	3.33 ± 1.16†	3/3
	2nd	14.52 ± 1.14†	9.33 ± 2.31†	3/3
	3rd	13.20 ± 1.14†	12.7 ± 0.58†	3/3
Low + high	1st	7.43 ± 3.38†,‡	0.33 ± 0.58‡	1/3
	2nd	5.28 ± 1.14†,‡	0.33 ± 0.57‡	1/3
	3rd	5.28 ± 1.14†,‡	3.33 ± 1.53‡	3/3

† *P* value <0.05 vs each experimental Control Group.
‡ *P* value <0.05 vs each experimental High Dose Group.

2.2. Animals

Fifty 4-week-old specific-pathogen-free male ICR mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and were housed under specific pathogen-free conditions. The mice were maintained with a 12 h light/12 h dark cycle and were fed a CE-2 (CLEA Japan) diet.

2.3. Chemicals and treatment scheme

N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was purchased from Wako Chemical (Osaka, Japan). Polyoxyethylene hydrogenated castor oil 100 (HCO-100) was purchased from Nikko Chemicals (Tokyo, Japan). HCO-100 was dissolved in physiological saline. MNNG treatment regimens in 4 experimental groups were as follows (Table 1): no-treatment (Control Group), low-dose treatment (Low Group), high-dose treatment (High Group), and low-dose pretreatment and high-dose treatment (Low High Group).

2.4. Cell cytotoxicity assay

The cytotoxicity of MNNG was determined using a colony formation assay. BALB/3T3 A31-1-1 cells were seeded at 100 cells/dish (60 mm φ) in culture medium. After incubation for 24 h, the Low Group and Low High Group were pretreated with 0.028 μm MNNG for 24 h. The High Group and Low High Group were then treated with 10 μm MNNG for 6 h, while other plates received an equal quantity of the vehicle dimethyl sulfoxide (DMSO) [15,16]. After 2 weeks, the cells were fixed with methanol and stained with Giemsa solution, and the colonies were counted. Colonies comprising more than 50 cells were scored [17].

2.5. In vitro transformation assay

To analyze the ability of the compounds to transform BALB/3T3 A31-1-1 cells, we used the protocol recommended by the IARC/NCI/EPA working group [18] with slight modifications and a previously described plating method [16]. Cells were seeded at 3×10^5 cells/dish (100 mm φ) in culture medium. After 24-h incubation, the Low Group and Low High Group were pretreated with 0.028 μm MNNG for 2 h. After another 24-h incubation, the cells were treated with 10 μm MNNG for 6 h. After 1 week, 1×10^5 treated cells were plated into 6-well plates. These cells were then grown to subconfluence following calculation of the survival rate. Plated transformed cells were generally cultured for 4–5 weeks, and the medium was replaced once per week. After 4–5 weeks, the cells were fixed with methanol and stained with Giemsa solution. Scoring of transformed foci, which appeared as dark stained areas on the culture dish, was performed according to previously described criteria [19]. These foci are indicative of malignant transformation by chemical carcinogens [19].

2.6. Cell proliferation assay

Cells were seeded at 5×10^4 cells/dish (100 mm φ) in culture medium. After incubation for 24 h, the Low Group and Low High

Group were pretreated with 0.028 μm MNNG for 24 h. Subsequently, the High Group and Low High Group were treated with 10 μm MNNG for 6 h, while other plates received an equal quantity of vehicle (DMSO). Twenty dishes per group were then prepared, and 1 dish was analyzed per day. For analysis, the cells were harvested and stained with 0.4% trypan blue every 24 h for 14 days, and viable and nonviable cells were counted.

2.7. 6-TG resistance mutation assay

The 6-TG resistance mutation assay is an *in vitro* mammalian cell gene mutation test. Cells free of mutations were poisoned by 6-TG, whereas HPRT gene mutants survived and formed colonies. The cells were seeded at 1×10^5 cells/dish (60 mm φ) in culture medium. After incubation for 24 h at 37 °C, the Low Group and Low High Group were pretreated with 0.028 μm MNNG for 24 h. The High Group and Low High Group were then treated with 10 μm MNNG for 6 h, while other plates received an equal quantity of vehicle (DMSO). To assess the frequency of mutations, all groups were treated with 10 ng/mL of 6-TG in culture medium for 14 days. The cells were fixed with methanol and stained with Giemsa solution, and colonies were counted.

2.8. Assay of reactive oxygen species (ROS)

ROS were assayed using the intracellular fluorescent marker DCFH-DA, which is cleaved by nonspecific esterification to form DCFH and then quantitatively oxidized by ROS to form fluorescent DCF. Cells were seeded at 1×10^3 cells/96-well dish. After incubation at 37 °C for 24 h, the Low Group and Low High Group were pretreated with 0.028 μm MNNG for 24 h. To measure ROS, all groups were treated with 50 μm DCFH-DA for 30 min at 37 °C and then washed once. The High Group and Low High Group were treated with 10 μm MNNG for 3 h, other plates received an equal quantity of vehicle (DMSO). Fluorescence of DCF was determined using a fluorometric plate reader at 485 nm (excitation) and 580 nm (emission).

2.9. MNNG-induced gastric ulceration test

All male mice were starved overnight, and the Low Group and Low High Group then received low-dose MNNG (2.8 μg/mL) in free-access drinking water for 24 h. All groups received a single intragastric injection of HCO-100 at doses of 10 mg/kg body weight. After 6 h, the High Group and Low High Group received a high dose of MNNG (1 mg/mL) in free-access drinking water for 3 weeks. High-dose MNNG water was exchanged once per week. After 3 weeks, all the mice were sacrificed by anesthesia with diethyl ether and their stomachs were surgically removed. Stomachs from all animals were fixed in 4% paraformaldehyde and examined under a microscope.

2.10. Statistical analysis

Analysis of data was performed using ANOVA and Tukey's HSD tests with Kaleidagraph statistical software. *In vitro* assays were performed in 3 independent triplicate experiments.

3. Results and discussion

The biological efficacy of low-dose radiation or chemical carcinogens is demonstrated by noncarcinogenic and hormetic effects [13,20–22]. However, because of limitations of detection sensitivity and poor definition of target sites and molecules, these studies do not overturn the fundamental concepts of human safety assessments for carcinogenicity. In addition, reports that examine MNNG using *in vitro* carcinogenicity tests defined by ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) have not interpreted the data as nongenotoxic [23] because in analyses using traditional toxicity tests, identification of a threshold is difficult. Thus, we designed an experimental scheme that combined low- and high-dose treatments to demonstrate an MNNG threshold (Fig. 1). Using the model carcinogenic alkylating agent MNNG, which is widely used for research on carcinogenesis, we conducted *in vitro* transformation and gastric ulceration studies as indexes and investigated the efficacy of low-dose treatments with this genotoxic carcinogen.

Initially, to determine appropriate low-dose concentrations of MNNG, we analyzed cell survival following exposure to various concentrations of MNNG using a colony formation assay (Supplementary Fig. 1). We then determined the MNNG concentrations that allowed 98% cell survival, which were calculated using the Probit method to be 0.028 μM (Supplementary Table 1). In contrast, high-dose MNNG treatment with 10 μM for 6 h significantly induced *in vitro* cell transformation in accordance with previous reports [17,23]. Thus, low-dose MNNG efficacy was analyzed using these doses, as described in experimental Scheme 1-A. The survival rate was about 90% in the Control and Low Groups and was decreased to about 13% in the High Group (Table 1). In the Low High Group, the survival rate was improved by about 6%, although this tendency was not statistically significant (Table 1). The number of transformation foci were 0 in the Control and Low Groups and about 8 in the High Group (Table 1, Fig. 2A). On the other hand, the number of transformation foci in the Low High Group was drastically decreased (to about 1) compared with that in the High Group (Table 1, Fig. 2A). These data strongly suggest that pretreatment with a low-dose alkylating agent suppresses the *in vitro* induction of cell transformation by a high-dose alkylating agent.

Subsequently, we determined whether this result was based on apoptosis/cell death or stress resistance. Previous studies have shown that MNNG induces apoptosis through p53 or FAS/CD95/Apo-1 pathways, and hence, it was assumed that pretransformed cells were selectively removed [24,25]. According to the results of a previous study of hormesis, low-dose-radiation–induced apoptosis selectively removes transformed cells [26]. Thus, in this study, we assumed a similar mechanism and determined doubling times as a proxy for cell proliferation. The cytotoxicity of MNNG is related to dose-dependent inhibition of cell growth via induction of G2/M cell cycle arrest and subsequent apoptosis [27]. The doubling time of cells in the Low Group was not significantly different from that in the Control Group over 24 h (Fig. 2B, open and closed circles). The duration of cell cycle arrest was not significantly different between cells of the Control and Low Groups at around 144 h (Fig. 2B, open and closed circle). In comparison, the duration of cell cycle arrest in cells of the High and Low High Groups was around 168 h (Fig. 2B, open and closed squares). The doubling time of these recovered cells was around 24 h, and cell proliferation was arrested before the cells reached confluence on the culture dish (around 264 h later; Fig. 2B, open and closed squares). These findings indicate that low-dose MNNG pretreatment was not related to selective removal of transformed cells via apoptosis/cell death. On the other hand, the mutation frequency of the Low High Group was significantly lesser than that of the High Group (Fig. 2C). Furthermore, ROS production in the Low High Group was also significantly lesser than that in the High Group (Fig. 2D). These results suggested that low-dose MNNG pretreatment activates an adaptive response.

Given these *in vitro* data, we demonstrated the efficacy of low-dose MNNG *in vivo* by monitoring the induction of gastric ulcers by high-dose MNNG. Based on pre-examinations, 1 mg/mL MNNG

was added to the drinking water for the High Group in these *in vivo* experiments. Murine stomachs have generally been found to be relatively resistant to MNNG but are susceptible to MNNG treatment in combination with surface-active agents [28,29]. For low-dose *in vivo* experiments, 2.8 μg/mL MNNG was added to the drinking water. This concentration was consistent with the low and high doses used *in vitro*. In these experiments, gastric ulcers were far more prevalent in the High Group than in the other groups (Fig. 3A). Indeed, the frequency of gastric ulcers induced in the High and Low High Groups was about 60% and 10%, respectively(Fig. 3B). These data indicate the efficacy of this low-dose alkylating agent and provide compelling evidence of hormetic effects of a genotoxic chemical carcinogen. Potentially, application of this principle may mitigate the side effects of other oral anticancer drugs such as temozolomide, which has similar toxicity to MNNG [30].

In subsequent experiments, we demonstrated the effects of low-dose MNNG using an *in vitro* invasion assay in 2 different tumor cell lines [31,32]. The resulting data show no change in invasion ability in the Low High Group, suggesting that the effects of low-dose MNNG are specific to normal cells (Supplementary Fig. 2).

The experimental evidence of low-dose hormetic effects in this report, as with radiation hormesis, fails to identify mechanisms and define dependence on specific treatment doses or times. To investigate radiation hormesis, analyses of DNA repair, antioxidant, and immune activation hypotheses are being performed. [33–36]. The repair of radiation-induced DNA double-strand breaks reportedly depends on p53BP, which is induced by low-dose X-ray (0.1 Gy). Apoptosis induced by subsequent high-dose (1–2 Gy) X-rays was decreased by preirradiation with 0.075 Gy X-ray in a PARP-1-dependent manner [33]. Although this report did not show involvement of DNA repair genes, the decreased mutagenicity of MNNG following low-dose pretreatment in this study strongly suggests activation of DNA repair. In the context of the cellular antioxidant theory, a low dose of γ-radiation (2–50 cGy) reportedly activated SOD2 via TNF-NFκB signaling and initiated a SOD2 feedback response [34,35]. Although this report did not show activation of other antioxidant genes, decreased MNNG-induced ROS following pretreatment with low-dose MNNG indicates a multifaceted antioxidant response. Finally, a low dose of γ-radiation (0.2 Gy) triggered alterations in the expression of a large number of cytokines, which facilitated myeloid differentiation and transformed naïve T-cells into T-helper 2 but not T-helper 1 cells [36]. Low-dose MNNG treatment may produce similar cytokine responses, involving the cytokines IL-4, IL-13, IL-17, INF-γ, and VEGF, which are associated with gastric ulcers and carcinomas. In addition to the above classic explanations, epigenetic research provides important mechanistic possibilities that require further investigation [37]. For example, epigenetic regulation of O6-methylguanine-DNA methyltransferase (MGMT), a key enzyme for repair of MNNG-induced DNA methylation repair, was strongly implicated [38]. MGMT knockdown cell lines had hypermethylated CpG islands in the 5′ region of the MGMT gene [39]. Hence, the role of MGMT in low-dose MNNG efficacy is an important subject of future studies. Radiation hormesis has been observed at specific doses and at specific times after irradiation. In this study, 0.015 μm MNNG pretreatment suppressed *in vitro* cell transformation by the subsequent high dose (data not shown). Given that MNNG administered via drinking water may have resulted in variability of treatment doses and duration of effectiveness, these preliminary data show that this effect may not require such specific conditions.

In conclusion, acquisition of carcinogen resistance from low-dose carcinogen pretreatment indicates an ingenious defense mechanism. The present data support the hormetic effects of chemical carcinogens and warrant reconsideration of the threshold concept.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.049.

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