In vivo Role of NAD(P)H:Quinone Oxidoreductase 1 in Metabolic Activation of Mitomycin C and Bone Marrow Cytotoxicity

Anbu Karani Adikesavan,1 Roberto Barrios,2 and Anil K. Jaiswal1

Abstract
NAD(P)H:quinone oxidoreductase 1−/− (NQO1−/−), NQO1+/− along with NHR:quinone oxidoreductase 2−/− (NQO2−/−), and wild-type (WT) mice were exposed to five once weekly doses of mitomycin C. The mice were euthanized 15 weeks after the first dose. Blood cell counts and histologic analyses were done. WT and NQO2−/− mice showed hypocellularity and a significant increase in adipocytes in bone marrow. They also showed anemia because of the loss of RBC and hemoglobin. The neutrophils and platelets were reduced, whereas other blood cell types and tissues were normal. Interestingly, NQO1−/− mice showed a complete resistance to mitomycin C–induced bone marrow cytotoxicity and reduction in RBC, hemoglobin, and neutrophils. NQO1+/− mice also showed limited resistance to mitomycin C–induced bone marrow cytotoxicity. These data show a major in vivo role of NQO1 in metabolic activation of mitomycin C with implications in mitomycin C chemotherapy. [Cancer Res 2007;67(17):7966–71]

Introduction
The bioreductive activation of drugs plays an important role in the efficacy of antitumor drugs (1). Bioreductive drug development is based on two major factors. The first is to develop drugs that are activated by specific reductase enzymes and the second is to identify tumor types that are rich in those enzymes (2). In addition, bioreductive drug development is also based on differences in oxygen content and cellular pH between normal and tumor tissues (1, 2). Several quinones occupy special place in bioreductive therapy because of their tendency to undergo reductive activation by different reductases in aerobic and hypoxic conditions. These include mitomycin C, indoloquinone EO9, diaziquone (AZQ), methyl-DZQ, and dinitrophenylaziridine CB1954 (3). Mitomycin C and its analogues are effective against several tumor tissue types, including colon, breast, head, and neck (4). Bioreductive metabolism leading to the formation of electrophilic metabolites, including 2,7-diaminomitosenes, capable of binding with DNA to cause its damage is the basic cause of cytotoxicity and antitumor activity of mitomycin C (5).

The cytosolic NAD(P)H:quinone oxidoreductase 1 (NQO1) and NHR:quinone oxidoreductase 2 (NQO2) are flavoproteins that catalyze the metabolic reduction of quinones (6). The cellular studies have provided evidence for and against the role of cytosolic NQO1 in metabolic activation of mitomycin C (2). The cellular studies using dicumarol as NQO1 enzyme inhibitor indicated that bioactivation by the obligatory two-electron reducing enzyme NQO1 might be especially important for the cytotoxicity of mitomycin C (2, 7, 8). On the other hand, the cells expressing higher levels of NQO1 failed to show increased sensitivity to mitomycin C compared with cells of similar origin containing normal levels of NQO1 (9, 10). The cellular studies have shown a role of NQO2 in metabolic activation of mitomycin C and other drugs leading to cytotoxicity and cell death (11, 12). Therefore, a further assessment of the role of NQO1 and NQO2 in mitomycin C activation especially in an in vivo model is warranted to understand the role of these reductions in mitomycin C activation. This is also important because NQO1 and NQO2 are reductases and generally present in higher levels in tumors than normal tissues of the same origin (13). High NQO1 levels have been reported in several cell types, including non–small cell lung cancer, colon cancer, breast cancer, ovarian cancer, and melanoma (2, 13). Among the human tumor materials received directly from the patients, high NQO1 levels have been observed in liver, lung, colon, and breast tumors (14, 15).

In the present studies, we investigated in vivo role of NQO1 and NQO2 in metabolic activation of mitomycin C and toxicity. Wild-type (WT) and NQO2−/− mice showed bone marrow hypocellularity, significant loss of RBC and hemoglobin, and decrease in neutrophils compared with control mice. Interestingly, NQO1−/− mice showed complete resistance to mitomycin C–induced bone marrow cytotoxicity, loss of RBC/hemoglobin and neutrophils, and morbidity. In addition, the NQO1+/− mice also showed limited resistance to mitomycin C–induced bone marrow toxicity and morbidity. These results combined suggest that NQO1 present in WT, NQO2−/−, and NQO1+/− mice activated mitomycin C to metabolites that caused bone marrow cytotoxicity, anemia, and morbidity.

Materials and Methods
Chemicals. Mitomycin C was purchased from Sigma-Aldrich. Neutral buffered formalin (10%) for tissue fixation was purchased from Fisher Scientific.

WT, NQO1−/−, NQO1+/−, and NQO2−/− mice. C57BL6 NQO1−/−, NQO1+/−, and NQO2−/− mice were generated previously in this laboratory as described (16, 17). These mice were bred and housed in the institutional animal facility. All animal studies were carried out with prior approval from the Institutional Animal Care and Use Committee and done in accordance with the animal ethics committee’s guidelines.

Mitomycin C injection. Groups of 20 mice (10 male and 10 female) of 8 weeks of age from each strain (WT, NQO1−/−, NQO1+/−, and NQO2−/−) were made for different dosage regimen of mitomycin C. Mitomycin C was dissolved in normal saline and injected into each group in each strain at a dose of 0, 1, and 2.5 mg/kg body weight, respectively, through i.p. route (0 mg control groups received only normal saline). Likewise, five doses of mitomycin C were given at a weekly interval. The mice were observed daily and the morbidity rate in each group was recorded. Ten weeks after final (fifth) injection, all mice were anesthetized using isoflurane and euthanized.

Requests for reprints: Anil K. Jaiswal, Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201. Phone: 410-706-2285; Fax: 410-706-0032; E-mail: ajaiswal@som.umd.edu.

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by cervical dislocation. The abdominal and thoracic cavity of the mice was surgically opened in an aseptical manner. Approximately 500 μL of whole blood were collected from each animal by direct heart puncture and transferred immediately into microtainer tubes containing EDTA and mixed gently and thoroughly.

**Hematologic analysis.** Whole blood collected from each animal was subjected to analysis for different blood variables, such as WBC, RBC, platelet count, differential count that included neutrophil, basophil, eosinophil, lymphocyte, and monocyte counts, and hemoglobin level, using Advia 120 hematology autoanalyzer.

**Serum chemistry.** Blood collected (without anticoagulant) from separate groups of WT, NQO1−/−, NQO1+/−, and NQO2−/− mice, each injected with 0 or 2.5 mg/kg body weight of mitomycin C, was used for obtaining serum. The sera samples were used for measuring the blood urea nitrogen (BUN) and creatinine levels to assess the kidney function status.

**Histopathologic analysis.** Different organs and tissues [i.e., liver, spleen, thymus, lymph nodes (salivary, inguinal, and mesenteric), lung, heart, kidney, and femur bones] were collected aseptically from these control and treated mice and washed in cold PBS. The organs were then fixed in 10% neutral buffered formalin for not less than 24 h. Decalcification of femur bones was done using TBD-2 decalcifier by previously described procedure (17). The tissues were subsequently embedded in paraffin and sections were made and stained with H&E stain. Kidney sections were also stained for iron to rule out hemosiderosis. The stained tissue sections were analyzed for histopathology under light microscope.

**Bone marrow analysis for NQO1 and NQO2.** Femur bones surgically collected from WT, NQO1−/−, NQO1+/−, and NQO2−/− mice were washed in cold PBS and snap frozen in liquid nitrogen. The two ends of the bone were cut with sterile blade and the bone marrow cells were flushed using a syringe and needle, with 100 μL of ice-cold lysate buffer into clean Eppendorf tubes (17). Total cell lysate of the bone marrow cells was made by homogenization using a micropestle followed by centrifugation at 12,000 × g for 10 min at 4°C. Protein samples (300 μg) from each were used for SDS-PAGE and Western blot analysis using antirabbit NQO1 antiserum raised in our laboratory and goat polyclonal NQO2 antibody (Santa Cruz Biotechnology) for NQO1 and NQO2, respectively.

**NQO1 enzyme activity assay.** Bone marrow cell lysates obtained from WT, NQO1−/−, NQO1+/−, and NQO2−/− mice (200 μg total protein each) were used in separate experiments in a final reaction mixture containing 25 mmol/L Tris-HCl (pH 7.4), 0.18 mg/mL bovine serum albumin (BSA), 5 mmol/L flavin adenine dinucleotide, 0.01% Tween 20, 200 mmol/L NADH, and 50 mmol/L 2,6-dichlorophenolindophenol. The reaction was monitored by measuring the decrease in absorbance at 600 nm due to reduction of 2,6-dichlorophenolindophenol. This gives the total NQO activity. The same experiment was repeated in the presence of 20 mmol/L dicumarol, a specific inhibitor of NQO1. The activity obtained in the presence

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**Figure 1.** Hematologic analysis and morbidity in mice untreated and treated with mitomycin C. WT, NQO1−/−, NQO1+/−, and NQO2−/− mice were i.p. injected with mitomycin C in concentrations as shown. Five once weekly doses were injected. The morbidity in mice was recorded. The mice were euthanized and sacrificed 15 wks after first injection of mitomycin C. The blood and tissues were collected. Blood was analyzed for RBC counts and hemoglobin content. A, RBC counts. B, blood hemoglobin content. C, mitomycin C–induced morbidity. D, weekly morbidity rate of mice.
of dicoumarol was subtracted from the total activity to obtain the dicumarol-inhibitable NQO1 activity. The experiments were repeated thrice independently.

Spectrophotometric analysis of mitomycin C metabolism. In vitro analysis of mitomycin C metabolism in the presence of WT, NQO1−/−, NQO1+/−, and NQO2−/− bone marrow cytosols was carried out with BSA as control in 10 mmol/L sodium phosphate buffer (pH 5.8) by previously described procedure (18). Protein samples (500 μg) were taken and the volume of each sample was equalized with the liver cytosol extraction buffer. NADH was used at 200 μmol/L final concentrations to serve as cofactor for the metabolic reaction and 500 μmol/L mitomycin C final concentration was used for the assay. The sample tubes were mixed gently and incubated in 37°C water bath. Absorbance at 554 nm was recorded after 0, 30, 60, 120, and 180 min to measure the amount of formation of mitomycin C metabolites especially 2,7-diaminomitosene. The absorbance was also recorded at similar time intervals at 365 nm to measure the disappearance of mitomycin C. The experiments were repeated independently thrice. The fold increase in the amount of formation of mitomycin C metabolites between BSA control, WT, NQO1−/−−, NQO1+/−, and NQO2−/−− mice bone marrow cytosols was graphically depicted.

Results

The results of the various experiments are shown in Figs. 1 to 4. Hematologic analysis revealed that exposure of WT, NQO1−/−−, NQO1+/−−, and NQO2−/−− mice to lower dose (1 mg/kg body weight) of mitomycin C had no effect on RBC and hemoglobin content (Fig. 1A and B). In addition, WT and NQO1−/−− mice did not show morbidity (Fig. 1C). However, NQO2−/−− mice showed 10% morbidity compared with WT and NQO1−/−− mice (Fig. 1C). In contrast, the higher dose (2.5 mg/kg body weight) of mitomycin C showed cytotoxicity in WT and NQO2−/−− but not in NQO1−/−− and NQO1+/−− mice (Fig. 1). Hematologic analysis revealed >50% decrease in RBCs and blood hemoglobin content and 70% morbidity was observed in WT and NQO2−/−− mice exposed to higher dose of mitomycin C compared with respective saline-treated control mice (P > 0.001; Fig. 1A–C). In similar experiments, NQO1−/−− and NQO1+/−− mice showed resistance to higher dose of mitomycin C and did not show any significant reduction in RBCs and hemoglobin content (Fig. 1A and B). In addition, the morbidity was absent in NQO1−/−− mice, whereas only 30% morbidity was observed with NQO1+/−− mice exposed to higher dose of mitomycin C (Fig. 1C). The morbidity in higher-dose mitomycin C–exposed WT and NQO2−/−− mice was observed between weeks 10 and 13 after first exposure. The morbidity in higher-dose mitomycin C–exposed WT and NQO2−/−− mice was more sensitive than WT mice. In contrast, NQO1−/−− mice showed slight difference in morbidity at weeks 11 and 12 after first exposure to mitomycin C (Fig. 1D). The NQO2−/−− mice were more sensitive than WT mice. In contrast, NQO1−/−− mice failed to show morbidity until 15 weeks and NQO1+/−− mice showed delayed morbidity that started at week 14 after first exposure to mitomycin C.

Total WBC count increased in NQO1−/−−, NQO1+/−−, and NQO2−/−− mice exposed to mitomycin C compared with unexposed mice (Fig. 2A). The neutrophils showed mitomycin C dose-dependent decline in WT and NQO2−/−− but not in NQO1−/−− and NQO1+/−− mice. In contrast, the NQO1−/−− mice showed delayed increase in neutrophils that started at week 14 after first exposure to mitomycin C. In all experiments, the mice showed normal to high platelet counts that suggested absence of thrombocytopenia (Fig. 2C).

Figure 2. Analysis of blood CBCs, BUN, and creatinine in mice untreated and treated with mitomycin C. WT, NQO1−/−−, NQO1+/−−, and NQO2−/−− mice were i.p. injected with mitomycin C. Mice at week 15 of the study were analyzed for blood CBCs, BUN, and creatinine.
mice (Fig. 2B). Platelets showed mitomycin C–dependent decline in all four types of mice (Fig. 2C). Blood levels of BUN and creatinine were normal in untreated and mitomycin C–treated mice of different genotypes, indicating that kidneys were functioning normal (Fig. 2D).

Histologic analysis of bone marrow, spleen, thymus, lymph nodes, liver, lung, heart, and kidney was done. Except bone marrow, none of the tissues analyzed showed lesions in untreated and mitomycin C–treated mice. Histologic examination revealed that bone marrow from all the four genetic background mice untreated and treated with lower dose of mitomycin C was normal. No lesions were detected. However, exposure of mice to high dose of mitomycin C showed differential bone marrow response to mitomycin C. The histology results on mice exposed to high dose of mitomycin C are shown in Fig. 3. WT and NQO2−/− mice showed bone marrow cytotoxicity, hypocellularity, and significant increase in adipocytes in mitomycin C–treated mice compared with untreated mice. Interestingly, NQO1-deficient NQO1−/− mice showed hypercellularity and resistance to mitomycin C cytotoxicity (Fig. 3). NQO1−/− mice, in the same experiment, showed only limited resistance to mitomycin C–induced bone marrow toxicity (Fig. 3).

Western blot analysis showed presence of NQO1 protein in WT, NQO2−/−, and NQO1+/− mice but absent in NQO1−/− mice (Fig. 4A). The amount of NQO1 protein in NQO1+/− mice was significantly lower compared with WT mice because of the presence of only one normal NQO1 allele. Similarly, NQO2 protein was present in WT, NQO1−/−, and NQO1+/− mice but absent in NQO2−/− mice. There was no significant alteration in the levels of NQO1 protein in NQO2−/− and NQO2 protein in NQO1−/− and NQO1+/− mice compared with WT mice bone marrow. However, NQO2 protein was significantly lower than NQO1 protein in bone marrow of WT mice. The NQO1 activity data supported the NQO1 protein results from Western blot analysis (compare Fig. 4A and B). The NQO1 activity in NQO2−/− mice was more or less

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**Figure 3.** Histology of bone marrow. WT, NQO1−/−, NQO1+/−, and NQO2−/− mice were treated with normal saline (control) or mitomycin C by procedures as described in Materials and Methods. Fifteen weeks after first mitomycin C treatment, the mice were sacrificed. Femur bones were collected and decalcified, and sections were cut and stained with H&E.

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similar as observed in NQO2−/− mice and lower in NQO1+/− mice (Fig. 4B). It is known that mitomycin C is metabolized by NQO1 to 2,7-diaminomitosene that has optimal absorption spectrum at 554 nm (18). The absorption maximum for mitomycin C is 365 nm (18). Therefore, we analyzed the capacity of bone marrow lysates from WT, NQO1−/−, NQO1+/−, and NQO2−/− mice to metabolize mitomycin C to 2,7-diaminomitosene. We treated lysates from mice bone marrow with mitomycin C for different time intervals and measured the conversion of mitomycin C to 2,7-diaminomitosene at 554 nm and disappearance of mitomycin C at 365 nm. The results are shown in Fig. 4C and D. The bone marrow lysates from WT and NQO2−/− mice bone marrow showed significant conversion of mitomycin C to 2,7-diaminomitosene compared with NQO1−/− mice. This was evident from rapid appearance of 2,7-diaminomitosene at 554 nm and disappearance of mitomycin C at 365 nm with increasing time. Interestingly, the conversion of mitomycin C to 2,7-diaminomitosene in case of NQO2−/− was slightly higher than WT mice. The NQO1−/− mice showed conversion of mitomycin C to 2,7-diaminomitosene that was halfway between WT and NQO1−/− mice.

Discussion

The results show that NQO1 is a major enzyme that catalyzes metabolic activation of mitomycin C to 2,7-diaminomitosene that causes bone marrow cytotoxicity, anemia, and morbidity in mice. This was clearly evident from hypocellularity and significant increase in bone marrow adipocytes and decrease in RBCs and hemoglobin in WT and NQO2−/− mice that contained substantial NQO1 protein and activity. Seventy percent of these mice did not survive during the study presumably because of anemia and related complications. On the other hand, mice deficient in NQO1 were resistant to mitomycin C–induced bone marrow toxicity. The studies also showed that prime target organ for mitomycin C toxicity is bone marrow, as histologic analysis of many other tissues did not show lesions. The mitomycin C–induced bone marrow toxicity was reported earlier (19). The mitomycin C–induced decrease in neutrophils in WT and NQO2−/− mice presumably is also related to NQO1-mediated mitomycin C–induced bone marrow toxicity. It is unknown if decrease in neutrophils contributed to lower survival of WT and NQO2−/− mice exposed to mitomycin C. The NQO1−/− mice did not show mitomycin C–induced decrease in neutrophils. The results also showed that NQO2−/− mice are slightly more susceptible to mitomycin C than WT mice. This was evident from increased morbidity and increased conversion of mitomycin C to 2,7-diaminomitosene. However, it remains unclear why NQO1 in NQO2−/− mice bone marrow is slightly more active against mitomycin C than NQO1 in WT mice.

The results from NQO1+/− mice also supported a role of NQO1 in metabolic activation of mitomycin C and bone marrow toxicity. NQO1+/− mice showed resistance to mitomycin C–induced bone marrow toxicity and morbidity compared with WT and NQO2−/− mice. However, the magnitude of resistance to mitomycin C–induced bone marrow toxicity and morbidity was significantly lower than NQO1−/− mice. Interestingly, the RBC and hemoglobin levels were more or less unaffected in mitomycin C–treated NQO1+/− mice. This was same as observed with NQO1−/− mice. The NQO1 protein and activity and NQO1-mediated metabolism of mitomycin C were reduced to half in NQO1−/− mice compared with WT mice. These results also revealed that alterations in NQO1 protein and activity lead to similar alterations in mitomycin C metabolic activation.

The observation of a major in vivo role of NQO1 in mitomycin C–induced toxicity is important for bioreductive chemotherapy, which is considered to be the most successful treatment for certain types of cancer cells (1–3). NQO1 is significantly overexpressed in certain tumor tissues compared with normal tissues of similar origin (14, 15). Therefore, effective dosages of drugs could be given with drastic damage to tumor cells and more or less no toxicity.

Figure 4. Western blot analysis, NQO1 activity, and metabolic activation of mitomycin C to 2,7-diaminomitosene. WT, NQO1−/−, NQO1+/−, and NQO2−/− mice were sacrificed and femurs were removed. The bone marrow was flushed out and lysed. A, Western blot analysis. Bone marrow lysates (300 μg) and liver lysate (75 μg) were resolved on SDS-PAGE, Western blotted, and probed with NQO1, NQO2, and actin antibodies. TCL, total cell lysate. B, NQO1 activity. WT, NQO1−/−, NQO1+/−, and NQO2−/− bone marrow lysates and BSA (control) were analyzed for dicumarol-inhibitable NQO1 activity. DCPIP, 2,6-dichlorophenolindophenol. Columns, mean of three independent assays; bars, SE. C and D, relationship between NQO1 and mitomycin C activation. Bone marrow lysates (500 μg) were incubated with mitomycin C for different time intervals and absorbance was read at 554 nm (appearance of 2,7-diaminomitosene) and 365 nm (disappearance of mitomycin C). Points, mean absorbance of three independent experiments; bars, SE.
to normal cells. This was evident from observations that higher doses (2.5 mg/kg body weight) of mitomycin C and not lower dose (1 mg/kg body weight) were cytotoxic in the presence of NQO1. Therefore, it is expected that higher concentration of NQO1 in many tumors will activate mitomycin C to cause tumor cell death and no toxicity in normal cells containing lower levels of NQO1. In addition, the existence of hypoxic regions is a major problem in radiation therapy, as oxygen is a radiation sensitizer. Therefore, hypoxic parts of the tumors often survive radiation treatment and may be center for a recurrent tumor. Bioreductive alkylation agents, such as mitomycin C, may be more active in these hypoxic regions of the tumors. The administration of such bioreductive agents as adjuvant to radiation may be favorable for killing solid tumor cells. The studies also suggest that mitomycin C therapy might not be suitable for humans that carry a cytosine to thymidine (C → T) polymorphism in exon 6 of human NQO1 gene, which produces a proline to serine (P187S) substitution that destabilizes and inactivates the enzyme (20). Human individuals (2–4%) are homozygous and 22% to 25% individuals are heterozygous for NQ01P187S mutant allele. Individuals carrying both mutated genomic alleles are completely lacking in NQO1 activity, whereas individuals who are heterozygous with one mutated allele have low to intermediate NQO1 activity compared with WT individuals (20).

The cellular studies have shown a role of NQO2 in mitomycin C activation and cytotoxicity (12). However, the current studies in mice showed that NQO2 plays a minimal role in in vivo activation of mitomycin C in bone marrow. This was evident from two observations. First, NQO2−/− mice showed slightly higher and not lower susceptibility to mitomycin C compared with WT mice. Second, the NQO2 is a weak metabolizer of mitomycin C to 2,7-diaminomitosenoe, as very little of later product was observed with NQO1−/− bone marrow lysates. The reasons for the discrepancy between cell and mouse models remain unknown. It is possibly related to the lower levels of NQO2 compared with NQO1 in bone marrow and/or lower concentration of NQO2 cofactor NRH in the bone marrow. It is noteworthy that cellular studies on the role of NQO2 in toxicity of mitomycin C used exogenously added cofacter NRH (17).

In conclusion, the studies showed a major role of NQO1 in in vivo metabolic activation and bone marrow cytotoxicity. The results also showed that alterations in NQO1 protein and activity cause similar alterations in metabolic activation of mitomycin C and toxicity. The results further revealed that NQO2 plays a minimal role in in vivo activation of mitomycin C in bone marrow. These results are highly valuable in mitomycin C therapy and development of mitomycin C–related and other chemotherapeutic drugs.

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