Nuclear Overexpression of NAD(P)H:quinone oxidoreductase 1 in Chinese Hamster Ovary Cells Increases the Cytotoxicity of Mitomycin C under Aerobic and Hypoxic Conditions*

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The effects of the subcellular localization of overexpressed bioreductive enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1) on the activity of the antineoplastic agent mitomycin C (MC) under aerobic and hypoxic conditions were examined. Chinese hamster ovary (CHO-K1/ dhfr) cells were transfected with NQO1 cDNA to produce cells that overexpressed NQO1 activity in the nucleus (148-fold) or the cytosol (163-fold) over the constitutive level of the enzyme in parental cells. Subcellular localization of the enzyme was confirmed using antibody-assisted immunofluorescence. Nuclear localization of transfected NQO1 activity increased the cytotoxicity of MC over that produced by overexpression in the cytosol under both aerobic and hypoxic conditions, with greater cytotoxicity being produced under hypoxia. The greater cytotoxicity of nuclear localized NQO1 was not attributable to greater metabolic activation of MC but instead was the result of activation of the drug in close proximity to its target, nuclear DNA. A positive relationship existed between the degree of MC-induced cytotoxicity and the number of MC-DNA adducts produced. The findings indicate that activation of MC proximal to nuclear DNA by the nuclear localization of transfected NQO1 increases the cytotoxic effects of MC regardless of the degree of oxygenation and support the concept that the mechanism of action of MC involves alkylation of DNA.

Mitomycin C (MC) is a naturally occurring antibiotic that was isolated originally from the microorganism Streptomyces casptosus (1). MC exhibits a broad spectrum of antitumor activity and is an important component in the combination chemotherapy of malignancies such as early stage head and neck cancer, early stage cervical cancer, and intravesicle therapy of superficial bladder cancer. Specific MC-DNA lesions associated with the action of MC consist of both monofunctional and bifunctional alkylations (2–9). Monoaalkylations initially occur through the linkage of the C-1 position of MC to the amino function in the 2-position of guanine bases in DNA and may proceed to a DNA cross-link through the C-10 position of MC to an amino entity in the 2-position of an adjacent DNA guanine (6). Although monoaalkylations are potentially cytotoxic, compelling evidence in both bacterial and mammalian systems implicates MC-induced cross-links as the primary event responsible for cell death (10–12).

A salient feature of the molecular mechanism of action of MC is that this agent exists as a prodrug, and both its DNA cross-linking and monoaalkylating activities require the reduction of the quinone ring to a hydroquinone, which transforms MC into a highly reactive alkylating species (11). Enzymes known to activate MC to intermediates capable of alkylating DNA do so either by a one- or a two-electron reduction mechanism. One-electron reducing enzymes include NADPH:cytochrome P450 oxidoreductase (NPR; EC 1.6.2.4) (13–16); NADH:cytochrome b₅ oxidoreductase (NBR; EC 1.6.2.2) (17, 18); xanthine:oxygen oxidoreductase (EC 1.1.2.9) (19); and NADPH-ferredoxin reductase (EC 1.18.1.2) (20). One-electron reducing enzymes activate MC to a semiquinone anion radical, which is oxygen-sensitive. It is this property that leads to the preferential kill of hypoxic cells by MC. Thus, under aerobic conditions, the semiquinone anion radical reacts rapidly with molecular oxygen at a near diffusion-limited rate to regenerate the parent prodrug MC (21). However, under hypoxic conditions, the semiquinone is a longer lived species and participates in a disproportionation reaction to produce the MC hydroquinone (MCH₂) intermediate, which leads to the cross-linking of DNA (5). Two-electron reducing enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1; DT-diaphorase; EC 1.6.99.2) (22–24) and xanthine:NAD⁺ oxidoreductase (EC 1.1.1.204) (25, 26) activate MC directly through a single step to produce MCH₂.

The difference in the production of these two reactive species, MCH₂ and MC semiquinone anion radical, gives rise to the difference in the survival curves that are observed for cells treated with MC under aerobic and hypoxic conditions. This separation, known as the “aerobic/hypoxic differential,” is reflected in the cytotoxicity profiles for Chinese hamster ovary (CHO) cell transfectants overexpressing NPR (27) and NBR (28, 29) but not for those overexpressing NQO1 (30). Thus, although NPR and NBR are not important enzymes in the activation of MC under aerobic conditions, they contribute to the preferential activation of MC in hypoxia. Bioactivation of

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Mitomycin C (MC) is a naturally occurring antibiotic that was isolated originally from the microorganism Streptomyces casptosus (1). MC exhibits a broad spectrum of antitumor activity and is an important component in the combination chemotherapy of malignancies such as early stage head and neck cancer, early stage cervical cancer, and intravesicle therapy of superficial bladder cancer. Specific MC-DNA lesions associated with the action of MC consist of both monofunctional and bifunctional alkylations (2–9). Monoaalkylations initially occur through the linkage of the C-1 position of MC to the amino function in the 2-position of guanine bases in DNA and may proceed to a DNA cross-link through the C-10 position of MC to an amino entity in the 2-position of an adjacent DNA guanine (6). Although monoaalkylations are potentially cytotoxic, compelling evidence in both bacterial and mammalian systems implicates MC-induced cross-links as the primary event responsible for cell death (10–12).

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Nuclear Localization of NQO1 and MC Cytotoxicity

Directly generates MCH2 (4, 7, 10, 15, 22, 31) since NQO1, being a two-electron reducing system that occurs with overexpressed NBR localized predominantly in the nuclear localization of the enzyme resulted in greater DNA cross-links with CHO cells transfected with a cDNA encoding rat NLS-NQO1. This gene product was unidirectionally inserted into the cultures using a Hamilton syringe without compromisement coefficient of 21 mM

MC under both aerobic and hypoxic conditions. A number of factors are involved in modulating the therapeutically efficacious of MC. These include the levels of the individual reductase enzymes and the cofactors, NADH and NADPH, the extent of formation of the exceedingly cytotoxic DNA cross-link; and the damaging oxygen radicals, superoxide, hydrogen peroxide, and hydroxyl radicals that are formed by redox cycling reactions. Reductive activation of the MC present in the cytosol in air to form the highly reactive electrophile MCH2 minimizes the formation of the exceedingly lethal DNA cross-links, since MCH2 reacts with a variety of cellular nucleophiles, including water, during its diffusion into the nucleus and reaction with DNA. Thus, activation of MC in the nucleus close to the DNA target should result in a greater number of DNA cross-links and increased cell kill. Such a prediction was realized in studies with CHO cells transfected with a cDNA encoding rat NBR, in that nuclear localization of the enzyme resulted in greater cell kill and increased numbers of MC-DNA adducts over those occurring with overexpressed NBR localized predominantly in its normal mitochondrial and endoplasmic reticulum locations (29). Since NQO1, being a two-electron reducing system that directly generates MCH2 (4, 7, 10, 15, 22, 31), is considered to be a more important bioactivator of MC than the one-electron activating system, NBR, we have measured the cytotoxicity of MC and the number of MC-DNA adducts formed from this agent in CHO cells overexpressing NQO1 activity in the cytosolic and nuclear compartments under aerobic and hypoxic conditions.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The cDNA mammalian expression constructs of nuclear localized NQO1 (NLS-NQO1) and cytoplasmic localized NQO1 (CYTO-NQO1) were prepared by PCR using a plasmid encoding the cDNA for the rat NQO1 gene (30) as the template. To create the NLS-NQO1, the NQO1 gene was modified by PCR using the following upstream oligonucleotide primer: 5′-CGC GGA GCT AGC CCG TGT AGA GCC CTG TGT GTA 3′. This oligonucleotide featured the sequences for a unique NheI site (shown above in italics) upstream of the coding sequences for rat NQO1 truncated to remove the start codon. The NheI site was used to fuse NQO1 in frame behind the nuclear localization signal of the SV40 large T antigen (36) in the pBC-CMV-end (Invitrogen).

The 3′-end of the gene was modified to include the sequences for the 15 amino acids of the muscle actin epitope (highlighted by the double underline) (39), which is recognizable by the HUC 1-1 monoclonal antibody (ICN, Costa Mesa, CA), followed by a unique XbaI site (represented by the double underline) (39), which is recognizable by the HUC 1-1 monoclonal antibody (ICN, Costa Mesa, CA) to the muscle actin epitope was identical to the one employed for the CYTO-NQO1 construct. This oligonucleotide was generated using the following upstream oligonucleotide primer: 5′-CGC GGA GCT AGC CCG TGT AGA GCC CTG TGT GTA 3′. The reverse primer for the muscle actin epitope was identical to the one employed for the cytosolic NQO1 construct. The oligonucleotide was inserted into the NheI site of the NLS-NQO1 plasmid, which was digested with BamHI and HindIII. The resulting plasmid was named pBC-CMV-NLS-NQO1 (Invitrogen). This plasmid was linearized with NotI to generate a HindIII-BamHI-EcoRI digestion pattern and used as a template for the preparation of NLS-NQO1 and CYTO-NQO1 fusion constructs. The plasmid also contains the sequence for the nuclear localization signal from the SV40 large T antigen. Expression of both NLS-NQO1 and CYTO-NQO1 fusion constructs were driven by the cytomegalovirus promoter to produce fusion proteins.

Cell Culture—The cell line used in this study is the deficient variant of the CHO-K1 cell line called CHO-K1/dhfr-I (42) and was obtained from the American Type Culture Collection (Manassas, VA). These cells were maintained in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 2 mM glutamine, 0.1 mM hypoxanthine, 0.01 mM thymidine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Transfected cell lines were maintained in identical medium in the presence of 1 mg/ml 6-thioguanine (Geneticin; Invitrogen) to provide a positive selection pressure for the expression vector. Cells were grown and treated as monolayers in a variety of cell culture vessels in a humidified atmosphere of 95% air and 5% CO2 at 37 °C.

Transfections—CHO-K1/dhfr-I cells were transfected with either NLS-NQO1 or CYTO-NQO1 constructs using calcium phosphate methodology essentially as described by the manufacturer (Invitrogen). Cells containing NLS-NQO1 or CYTO-NQO1 were selected using G418, and individual positive populations were expanded and screened for NQO1 activity. Cell populations with elevated enzyme activity were subjected to single cell sorting by flow cytometry and expanded. Expanded clones were rescreened and selected for high levels of NQO1 activity and basal levels of NFR and NBR activities. Relatively matched clones with respect to NQO1 activity were selected for study.

Enzyme Activity Assays—Monolayers of exponentially growing cells incubated under aerobic (95% air and 5% CO2) conditions were harvested in phosphate-buffered saline and lysed by sonication, and NQO1 activity was measured spectrophotometrically as described by Ernster (41). NQO1 activity inhibitable by dicumarol (Sigma) was quantified by measuring the reduction of dichlorofluorodinolphenol (Sigma) at 600 nm with a Beckman model 25 UV-visible spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Activities were calculated using an extinction coefficient of 21 mM−1 cm−1 at 30 °C. NBR activity was quantified by the reduction of ferricytochrome c measured at 550 nm at 30 °C; activities were calculated using an extinction coefficient of 1.02 mM−1 cm−1 at 30 °C (42). NBR activity was quantified as NADH:ferricyanide reductase measured at 420 nm at 30 °C; activities were calculated using an extinction coefficient of 1.02 mM−1 cm−1 at 30 °C (42) using a final concentration of 0.34 mM NADH. Protein concentrations were determined by the bicinchoninic acid assay (Pierce) (45).

Indirect Immunofluorescence—Cells were seeded at 1000 cells/well on a poly-L-lysine-treated 8-chambered glass slide (BD Falcon, Boston, MA). Twenty-four h later, the cells were fixed in 20% formaldehyde for 15 min and were permeabilized with ice-cold acetone for 5 min. Samples were incubated with primary antibody, anti-muscle actin (HUC 1-1 monoclonal antibody (ICN, Costa Mesa, CA) to the muscle actin epitope at 1:120 dilution, washed, and then incubated with goat anti-mouse IgG-fluorescein isothiocyanate-conjugated antibody at a 1:128 dilution (Sigma). Each incubation was performed at 4 °C for 18 h. The samples were then treated with 50% glycerol in phosphate-buffered saline, pH 9.0, and examined using a Nikon Optiphot microscope equipped with a Nipkow Diaphragm and an Epifluorescence attachment EF-D and a Nikon UFX-IA MicroFocus Camera.

Aerobic and Hypoxic Cell Survival Experiments—Cells were seeded in glass milk dilution bottles at 2.5 × 105 cells/bottle and grown for 3 days in a humidified atmosphere of 95% air and 5% CO2. Hypoxia was established by gassing the cultures with a humidified mixture of 95% N2 plus 5% CO2 (containing <10 ppm O2) (AirGas, Cheshire, CT) at 37 °C for 2 h through a rubber septum fitted with 13-gauge (inflow) and 18-gauge (outflow) needles. MC at 2.5, 5.0, 10.12.5, and 15 μM was then introduced into the cultures using a Hamilton syringe without compromising the hypoxic environment, and cultures were incubated for 1 h. Cells under aerobic conditions were treated identically but gassed with a humidified atmosphere of 95% air plus 5% CO2. Cells were then washed, harvested by trypsinization, and assayed for survival using a clonogenic assay (27). Macrophage colonies consisting of more than 40 cells were counted, and the plating efficiencies, defined as the number of macroscopic colonies counted divided by the number of cells plated, was determined. The surviving fractions were calculated by normalizing the plating efficiencies of the drug-treated groups to those of the vehicle-treated control groups.

MC Metabolic Studies—Suspension cultures were treated with 12.5 μM MC under aerobic (1 × 105 cells/ml) or hypoxic (5.0 × 105 cells/ml) conditions as described above. Cell suspensions (0.75 ml) were collected at various times (0–4 h) and mixed with an equal part of acetonitrile. NQO1 activity was measured as NADPH:cytochrome c reductase activity (33) in 0.4-ml C18 reverse phase columns (Applied Biosystems, Foster City, CA) by elution with a 3–27% acetonitrile gradient in 0.03 M KH2PO4 (pH 5.4) at a flow rate of 0.8 ml/min. Absorbance was monitored at 360 nm using a Beckman 168 UV-visible spectrophotometer. Untrans-
Isolation of Stable CHO Cell Line Transfectants Overexpressing NQO1—To examine the effects of the overexpression of NQO1 activity in different subcellular compartments on the sensitivity of aerobic and hypoxic cells to MC, clonal populations of CHO cells were isolated and assayed for NQO1 activity. Clones that exhibited high levels of NQO1 activity relative to parental CHO cells, with no significant differences in the levels of expression of both NBR and NPR were chosen for these studies; the enzymatic activity profiles for NLS-NQO1, CYTO-NQO1, and parental CHO cells are summarized in Table I. To ensure that the nuclear localization signal included in the fusion construct of NLS-NQO1 directed the overexpressed enzyme to the nucleus, NQO1 was visually assessed by indirect immunofluorescence techniques in NLS-NQO1, CYTO-NQO1, and parental CHO-K1/dhfr cells (Fig. 3A). Since both the CYTO-NQO1 and NLS-NQO1 fusion proteins are epitope-tagged, the use of antibody-directed immunofluorescence resulted in visualization of only the fusion protein and not the endogenous NQO1 protein. Staining with the HUC1-1 monoclonal antibody for the epitope tag revealed intense nuclear staining in the overexpressing NLS-NQO1 cells, indicating that nuclear localization of NQO1 was achieved (Fig. 1A). In contrast, CYTO-NQO1 cells demonstrated intense staining, which was predominantly cytoplasmic, with minimal nuclear staining (Fig. 1B). As expected, parental cells exhibited minimal fluorescence staining, since the HUC1-1 antibody specifically recognized the fusion protein expressed by the transfectants (Fig. 1C).

Effects of MC under Aerobic and Hypoxic Conditions on the Survival of Stable CHO Cell Transfectants Overexpressing NQO1 in the Cytosol or the Nucleus—The effects of overexpression of NQO1 activity, either in the cytosol (Fig. 2A) or nucleus (Fig. 2B), on the cytotoxicity of MC under aerobic and hypoxic conditions were measured by comparing the survival curves for the CHO transfectants with that of parental cells. In the parental cell line, greater levels of cell kill were obtained in hypoxia than under aerobic conditions, a finding consistent with previous reports (27, 29, 30). Comparative survival curves for CHO-K1/dhfr parental and CYTO-NQO1 transfectants (Fig. 2A) and CYTO-NQO1 and NLS-NQO1 transfectants (Fig. 2B) exposed to MC under aerobic and hypoxic conditions are shown. Overexpression of cytosolic-localizing NQO1 activity in CHO cells resulted in a significant increase in the cytotoxicity of MC relative to parental cells at concentrations of MC of 2.5–10 µM under both aerobic and hypoxic conditions. Furthermore, a highly significant increase in MC-induced toxicity was observed in cells that overexpressed NQO1 activity in the nucleus over those overexpressing NQO1 activity in the cytosol under both aerobic and hypoxic conditions at a concentration of 10 µM MC (Fig. 2B). Exposure of CHO-K1/dhfr parental, CYTO-NQO1, and NLS-NQO1 cells to higher concentrations of MC in the range of 10–15 µM clearly demonstrated that overexpression of NQO1 in either the cytosol or nucleus increased cell kill under both aerobic (Fig. 3A) and hypoxic (Fig. 3B) conditions, with nuclear localization of the enzyme activity decreasing cell survival more than cytosolic localization, although cytosolic overexpression of NQO1 activity was increased by 163-fold, whereas nuclear overexpression was increased somewhat less by 148-fold.

Metabolic Activation of MC—Since metabolic activation of MC is required for the cytotoxic effects of this agent, differences in activation between CHO-K1/dhfr parental, CYTO-NQO1, and NLS-NQO1 cells may be responsible for the observed differences in the cytotoxicity produced by MC. To measure the bioreduction of MC in intact cells, the comparative ability of these cell lines to metabolically activate the drug was assessed by measuring the disappearance of MC from the cultures. Fig. 4, A and B, depicts the time course for the activation of MC under aerobic and hypoxic conditions, respectively, for CHO-K1/dhfr parental, CYTO-NQO1, and NLS-NQO1 cells treated with 12.5 µM MC. The aerobic studies (Fig. 4A) were performed using 1.0 × 10^7 cells/ml, whereas the hypoxic studies (Fig. 4B) were performed using 5.0 × 10^6 cells/ml. Thus, although not evident from the data presented in Fig. 4, A and B, the rate of disappearance of MC under hypoxia was 20 times greater than in air, requiring the use of 20 times fewer cells under hypoxia to generate the results shown. Nonetheless, it is clear that
overexpressed NQO1 activity.

result of greater activation of MC by the nuclear localized

NLS-NQO1 cells relative to CYTO-NQO1 cells was not the
tions, demonstrating that the greater cytotoxicity of MC to

NQO1 transfectants under both hypoxic and aerobic condi-

tions of cells to the cytotoxic actions of MC under both aerobic and

hypoxic conditions. Thus, although an increase in cell kill oc-
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treated with 12.5 μM MC under aerobic conditions an ~35-fold
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NQO1 activity in the nucleus compared with those overex-

pressing the enzyme in the cytosol. Similarly, in cells exposed to

the same concentration of this agent under hypoxic condi-
tions, an approximately 35-fold increase in cell kill was ob-
tained in cells that overexpressed nuclear localized NQO1 ac-
tivity compared with cytosolic localized NQO1. Interestingly,
significant increases were observed only at concentrations of 10
μM MC or higher. Since NQO1 activity was overexpressed at
levels in the CYTO-NQO1 and NLS-NQO1 transfectants that
were greater than 100-fold more than that of parental CHO
cells, it is conceivable that MC may be essentially completely
depleted by activation by CHO-K1/dhfr− parental cells when
used at relatively low concentrations, accounting for the fact
that differences in cytotoxicity were not observed at concentra-
tions below 10 μM in both aerobic and hypoxic conditions. In an
analogue shower that NBR was overexpressed in the nucleus
by 3-fold, greater differences between aerobic (5-fold) and hy-

poxic (10-fold) toxicities were observed than seen with mito-

chondrial/endoplasmic reticulum-localized NBR, overex-

pressed by 5-fold. The fact that aerobic cell toxicity is lower
than hypoxic toxicity is a reflection of the oxygen-sensitive
one-electron reductive activation of MC by NBR.

DISCUSSION

The effects of overexpressed NQO1 activity in different sub-
cellular locations on the sensitivity of CHO-K1/dhfr− cells to
the antineoplastic agent MC was ascertained to determine the
contribution of this activating enzyme to the cytotoxic action of
this agent. Overexpression of NQO1 activity by 163-fold in the
cytosol caused significant increases in sensitivity of these cells to
the cytotoxic effects of MC under both aerobic and hypoxic condi-
tions (Fig. 2). These results are consistent with the finding
that the intracellular concentration of NQO1 activity is
important to the cytotoxic action of MC, independent of the
degree of oxygenation, providing further evidence for a role for
NQO1 in the bioreductive activation of MC. In earlier studies
from our laboratory, in which dicumarol was used to protect
NQO1-rich cells from the cytotoxic actions of MC, a role for this
enzyme was assigned in activating MC under aerobic but not
hypoxic conditions (15, 24). However, it is now widely recog-
nized that dicumarol is not a specific inhibitor of NQO1 in that
it can inhibit other enzymes (48). Additionally, dicumarol has
been shown to induce the expression of xanthine:NAD+ oxid-
doreductase and xanthine:oxygen oxidoreductase (49), al-
though we do not believe that the induction of these enzymes
occurs in CHO cells. Therefore, it is likely that the increased
sensitivity in CHO cells treated with dicumarol under hypoxic conditions reflects contributions to the activa-
tion of MC by bioreductive enzymes other than NQO1.

A comparison of the survival curves for NLS-NQO1 and
CYTO-NQO1 cells revealed that nuclear overexpression of
NQO1 activity in CHO-K1/dhfr− cells results in greater cyto-
toxicity from exposure to MC. This finding is in agreement with
that reported for cytosolic (28) and nuclear expression of NBR
(29) in CHO-K1/dhfr− cells. Since one of the features of NQO1
that separates it from NPR and NBR is that NQO1 activates
MC by a two-electron reduction process to directly generate the
oxygen-insensitive MCH2 intermediate, we anticipated that
overexpression of NQO1 activity would increase the sensitivity
of cells to the cytotoxic actions of MC under both aerobic and
hypoxic conditions. Thus, although an increase in cell kill oc-
curred in both CYTO-NQO1 and NLS-NQO1 cells over that obtained in CHO-K1/dhfr− parental cells, when cells were

treated with 12.5 μM MC under aerobic conditions an ~35-fold
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than hypoxic toxicity is a reflection of the oxygen-sensitive
one-electron reductive activation of MC by NBR.

NQO1 activates MC to directly generate the oxygen-insensi-
tive MCH2-reactive intermediate. Therefore, we anticipated
the increase in the cytotoxic action of MC that occurred under
both aerobic and hypoxic conditions by the overexpression of
NQO1 activity. A 10-fold increase in cytotoxicity was observed
in cells that overexpressed NQO1 activity in the nucleus com-
pared with cells that overexpressed the enzyme in the cytosol,
when exposed to 10 μM MC under aerobic conditions. Similarly,
at the same concentration of this agent under hypoxic condi-
tions, a 7-fold increase in cytotoxicity was observed in cells that
overexpressed nuclear localized NQO1 activity compared with
cells with cytosolic localized NQO1 activity. Additionally, at higher concentrations of MC, comparison of the curves suggests a trend wherein nuclear overexpression of NQO1 activity increased cytotoxicity to MC to a greater extent than cytosolic overexpression of the enzyme under both aerobic (solid symbols) and hypoxic (open symbols) conditions. Parental (●, ○), CYTO-NQO1 (■, □), and NLS-NQO1 (▲, ▲) cells were treated as described under “Experimental Procedures.” Surviving fractions were calculated using the plating efficiencies of aerobic and hypoxic vehicle-treated controls. Points represent the geometric means of 3–7 independent determinations ± S.E.

In analogous studies where NPR is overexpressed in the nucleus by 9-fold, greater differences between aerobic (11-fold) and hypoxic (90-fold) toxicities were observed than were seen with endoplasmic recticulum-localized NPR overexpressed by 16-fold (45). Likewise, when NBR was overexpressed by 3-fold in the nucleus, the increases in sensitivity under aerobic and hypoxic conditions were 5- and 10-fold greater, respectively, than those seen for mitochondrial/endoplasmic recticulum-localized enzyme overexpressed by 5-fold. Taken together, since aerobic cytotoxicity is less than hypoxic toxicity, the findings reflect the fact that activation of MC by NBR and NPR occurs by an oxygen-sensitive mechanism that results in redox cycling of the MC semiquinone anion radical.

Cell survival assays indicated that the closer the activation of MC to the nuclear DNA target, the greater the extent of cell

![Figure 2](image1.png)

**Fig. 2.** Comparative survival curves for CHO-K1/dhfr− parental and CYTO-NQO1 transfectant cells (A) and CYTO-NQO1 and NLS-NQO1 transfectant cells (B) exposed to 2.5–10 μM MC for 1 h under aerobic (solid symbols) and hypoxic (open symbols) conditions. Parental (●, ○), CYTO-NQO1 (■, □), and NLS-NQO1 (▲, ▲) cells were treated as described under “Experimental Procedures.” Surviving fractions were calculated using the plating efficiencies of aerobic and hypoxic vehicle-treated controls. Points represent the geometric means of 3–7 independent determinations ± S.E.

![Figure 3](image2.png)

**Fig. 3.** Survival curves for CHO-K1/dhfr− parental, CYTO-NQO1 transfectant, and NLS-NQO1 transfectant cells exposed to 10–15 μM MC for 1 h under aerobic (A, solid symbols) and hypoxic (B, open symbols) conditions. Parental (●, ○), CYTO-NQO1 (■, □), and NLS-NQO1 (▲, ▲) cells were treated as described under “Experimental Procedures.” Surviving fractions were calculated using the plating efficiencies of aerobic and hypoxic vehicle-treated controls. Points represent the geometric means of three or four independent determinations ± S.E.
Since it is widely accepted that nuclear DNA is the target of MC (6, 10, 12), the ability of this agent to produce genomic DNA alkylations in cells with nuclear and cytosolic overexpressed NQO1 activity was measured. Using [3H]MC, we found that the degree of sensitivity to MC corresponded to the number of MC-DNA adducts formed under hypoxic and aerobic conditions. Under aerobic conditions, the number of DNA adducts generated from [3H]MC results primarily from the contribution of NBR and NPR to that of the two-electron reducing system(s). The difference between the number of MC-DNA adducts under hypoxic and aerobic conditions for parental, CYTO-NQO1, and NLS-NQO1 cells results in relatively constant increases in cpm/μg DNA under hypoxia of 30, 26, and 32, respectively, over that in air, reflecting the constancy of the added activities of NBR and NPR under hypoxic conditions in transfectants. Furthermore, in agreement with expectations, overexpression of NQO1 activity in the cytosol increased the number of DNA alkylations over that in parental cells, and overexpression in the nucleus caused a further increase in MC-DNA adducts.

In a previous analogous study where the subcellular distribution of NBR was altered, a similar correlation between MC sensitivity and the number of MC-DNA adducts was obtained when comparisons were made between CHO-K1/dhfr - parental, CYTO-NQO1, and NLS-NQO1 cells expressing NBR in either the nucleus or the mitochondrial/endoplasmic reticulum membranes. Despite the fact that 5-fold differences in cell kill were observed between CHO-K1/dhfr - cells expressing NBR in the nucleus and in the mitochondria/endoplasmic reticulum, there was no difference in the number of MC-DNA adducts observed between the two cell types when treated with 10 μM MC under aerobic conditions (29). Since NBR reduces MC by a one-electron pathway to an oxygen-sensitive species, it is probable that under aerobic conditions, the observed enhancement in cell kill produced by MC involved a different mechanism. Thus, MC sensitivity under these conditions probably was not solely a consequence of MC-DNA adduct formation, but in addition reflected increases in damage caused by oxygen radi-
cals produced as by-products of the interaction between the MC semiquinone anion radical and molecular oxygen that have been shown to have toxic effects in EMT6 cells (50, 51).

Taken together, the findings show that activation of MC proximal to its nuclear DNA target through nuclear expression of NQO1 activity or NBR activity results in enhanced cell kill proximal to its nuclear DNA target through nuclear expression of NBR activity. The results also show that activation of MC proximal to its nuclear DNA target through nuclear expression of NBR activity results in enhanced cell kill proximal to its nuclear DNA target through nuclear expression of NBR activity. The results also show that activation of MC proximal to its nuclear DNA target through nuclear expression of NBR activity results in enhanced cell kill proximal to its nuclear DNA target through nuclear expression of NBR activity.

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