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Role of cytochrome P450 reductase in nitrofurantoin-induced redox cycling and cytotoxicity

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Abstract

The one-electron reduction of redox-active chemotherapeutic agents generates highly toxic radical anions and reactive oxygen intermediates (ROI). A major enzyme catalyzing this process is cytochrome P450 reductase. Because many tumor cells highly express this enzyme, redox cycling of chemotherapeutic agents in these cells may confer selective antitumor activity. Nitrofurantoin is a commonly used redox-active antibiotic that possesses antitumor activity. In the present studies we determined whether nitrofurantoin redox cycling is correlated with cytochrome P450 reductase activity and cytotoxicity in a variety of cell lines. Recombinant cytochrome P450 reductase was found to support redox cycling of nitrofurantoin and to generate superoxide anion, hydrogen peroxide, and, in the presence of redox-active iron, hydroxyl radicals. This activity was NADPH dependent and inhibitable by diphenyleneiodonium, indicating a requirement for the flavin cofactors in the reductase. Nitrofurantoin-induced redox cycling was next analyzed in different cell lines varying in cytochrome P450 reductase activity including Chinese hamster ovary cells (CHO-OR) constructed to overexpress the enzyme. Nitrofurantoin-induced hydrogen peroxide production was 16-fold greater in lysates from CHO-OR cells than from control CHO cells. A strong correlation between cytochrome P450 reductase activity and nitrofurantoin-induced redox cycling among the cell lines was found. Unexpectedly, no correlation between nitrofurantoin-induced ROI production and cytotoxicity was observed. These data indicate that nitrofurantoin-induced redox cycling and subsequent generation of ROI are not sufficient to mediate cytotoxicity and that cytochrome P450 reductase is not a determinant of sensitivity to redox-active chemotherapeutic agents.

Keywords

Cytochrome P450 reductase; Hydroxyl radicals; Redox cycling; Antitumor; Cytotoxicity; Chemotherapy

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Introduction

Nitrofurantoin (*N*-(5-nitro-2-furfurylidene)-1-aminohydantoin) is a synthetic nitrofuran-derivative antibiotic commonly used in the treatment of urinary tract infections. Prolonged use of nitrofurantoin is associated with pulmonary fibrosis [1], hepatocellular injury [2], peripheral polyneuropathy [3], and hematological disorders [4]. Nitrofurantoin has also been proposed as a cytotoxic antitumor agent [5,6]. Both the antimicrobial and the cytotoxic effects appear to be related to reduction of the 5-nitro substitution on the molecule (see Fig. 1 for structure). Thus, in susceptible bacteria, nitroreductases convert nitrofurantoin to a highly reactive electrophilic intermediate which attacks ribosomes and inhibits protein synthesis [7]. In humans, pulmonary and hepatic toxicity have been attributed to reactive oxygen intermediates (ROI) generated during redox cycling [8,9]. This involves a one-electron transfer from donors such as NADPH to nitrofurantoin generating a nitro radical anion which, under aerobic conditions, is oxidized back to the parent compound by molecular oxygen (Fig. 1). These reactions produce superoxide, which in turn dismutates to hydrogen peroxide (H₂O₂) and, in the presence of redox-active metals, highly reactive hydroxyl radicals [10]. These ROI damage cellular macromolecules resulting in lipid peroxidation and protein and DNA oxidation [11].

NADPH-cytochrome P450 reductase (EC 1.6.2.4) is a flavoprotein widely distributed in human tissues and tumor cells [12]. It can mediate the transfer of electrons to several enzymes including the superfamily of cytochrome P450s, cytochrome b5, and heme oxygenase, which are essential for the metabolism of xenobiotics and endogenous compounds [13]. Cytochrome P450 reductase has also been identified as a major enzyme catalyzing nitrofurantoin redox cycling [10,14]. In the present studies, we characterized nitrofurantoin redox cycling in a variety of tumor cell lines and recombinant cytochrome P450 reductase. Our results indicate that the ability of nitrofurantoin to redox cycle is directly related to cytochrome P450 reductase activity but not to tumor cytotoxicity. These findings suggest that additional mechanisms mediate the cytotoxic actions of nitrofurantoin in tumor cells.

Materials and methods

Chemicals and reagents

cDNA-expressed rat cytochrome P450 reductase was from microsomal fractions of insect cells (Supersomes; Cat. No. 456514; Lot Nos 5 and 25030; BD Gentest, Woburn, MA). Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) and 2',7'-dichlorodihydrofluorescein diacetate acetyl ester (DCFH-DA) were from Molecular Probes (Eugene, OR). Nitrofurantoin, NADPH, catalase, and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cells and treatments

Mouse cytochrome P450 reductase-overexpressing Chinese hamster ovary cells (CHO-OR), and control cells expressing empty vector (CHO-WT) were kindly provided by Dr. Jun Yan Hong at the University of Medicine and Dentistry of New Jersey (Piscataway, NJ) [15].

Murine lung epithelial cells (MLE 15 cells) were obtained from Dr. Jacob N. Finkelstein at the University of Rochester, NY. All other cell lines were purchased from the American Type Culture Collection (Rockville, MD). CHO-WT, CHO-OR, and PC-3 cells were maintained in Ham's F12K medium. MLE 15, RAW 264.7, C2, S-180, B16, HL-60, HT-29, and HeLa cells were maintained in Dulbecco's modified Eagle's medium. All medium was supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). For CHO cells, the medium was also supplemented with 500 µg/ml hygromycin B (Invitrogen, Carlsbad, CA). Cells were incubated at 37 °C in 5% CO₂ in a humidified incubator. Tissue culture reagents were from Gibco BRL (Grand Island, NY). C2 cells are a continuously grown mouse muscle cell line, while MLE 15 cells are a continuously grown type II lung cell line isolated from SV40 large tumor antigen transgenic mice [16].

To prepare lysates, cells were scraped from culture dishes in phosphate-buffered saline (PBS), washed, and centrifuged (800g, 5 min). Cell pellets were stored at – 70 °C until analysis. Before enzyme assays, cell pellets were resuspended in PBS (~10⁷ cells/0.5 ml) and sonicated on ice using a sonic dismembrator (Artek Systems Inc., Farmingdale, NY). Homogenates were then sequentially centrifuged at 4 °C (3000g and 12,000g, for removal of cellular debris and mitochondrial fractions, respectively). The resulting supernatant fractions were used in enzyme assays. Protein concentrations were quantified using the *Dc* protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

Cell growth inhibition was evaluated as previously described [17]. Briefly, cells were plated at low density (2.5–10 × 10⁴ cells/well) in six-well tissue culture plates and allowed to adhere overnight. The medium was then replaced with growth medium supplemented with increasing concentrations of nitrofurantoin. After 4–5 days, cells were removed from the plates and counted using a Z1 Coulter Particle Counter (Beckman Coulter, Hialeah, FL). Concentrations of nitrofurantoin that caused 50% growth inhibition (IC₅₀) were then determined.

Kinetic analysis of H₂O₂ production

H₂O₂ production was measured using the Amplex red/horseradish peroxidase method [18]. Standard reaction mixes in 100 µl potassium phosphate buffer (50 mM, pH 7.4) contained 0–0.5 mM NADPH, 0–500 µM nitrofurantoin, 25 µM Amplex red, 1 unit/ml horseradish peroxidase, and 1.25 µg/ml cytochrome P450 reductase or 100 µg/ml cell protein. The fluorescent product resorufin was detected using an HTS 7000 Plus Bio Assay Reader (Perkin–Elmer Life Sciences, Shelton, CT) using a 540-nm excitation filter and a 595-nm emission filter. Increases in fluorescence intensity were measured every 2.5 min for 30 min at 37 °C. H₂O₂ production is formed via the one-electron reduction of nitrofurantoin by cytochrome P450 reductase, the interaction of reduced nitrofurantoin with oxygen producing the parent compound and superoxide, and the dismutation of superoxide to hydrogen peroxide. Because the last two steps proceed at very high rates [19], we assumed that the measurement of *K_m* and *V_{max}* reflect the one-electron enzymatic reaction.

Assays for hydroxyl radical and superoxide anion

The generation of 2-hydroxyterephthalate from terephthalate in enzyme assays was used as an indicator of hydroxyl radical production [20]. Standard reaction mixes in 0.2 ml potassium phosphate buffer (50 mM, pH 7.4) contained 6.25 µg/ml cytochrome P450 reductase or 150 µg/ml cell protein from supernatant fractions, 1 mM terephthalate, 0.5 mM NADPH. Reactions were initiated by the addition of Fe³⁺/EDTA (100 µM/110 µM) to the assay mixture. After incubation at 37 °C for 1 h, reactions were stopped by adding an equal volume of ice-cold methanol. 2-Hydroxyterephthalate was quantified by HPLC with fluorescence detection as previously described [20].

Superoxide anion was assayed by the formation of 2-hydroxyethidium from dihydroethidium [21]. Standard reaction mixes described above were used except that Fe³⁺/EDTA was omitted and dihydroethidium (40 µM) was used in place of terephthalate. 2-Hydroxyethidium was detected using a Shimadzu HPLC (Kyoto, Japan) fitted with a Luna C18 column (250 mm × 2.0 mm; Phenomenex, Torrance, CA) and a fluorescence detector with excitation and emission wavelengths set at 510 and 595 nm, respectively. The mobile phase consisted of a linear (10–40%) gradient of acetonitrile in 0.1% trifluoroacetic acid and was run at a flow rate of 0.2 ml/min in 35 min.

Assay for NADPH metabolism

NADPH metabolism was assayed in reaction mixes containing 100 µM NADPH, 7.5 µg/ml cytochrome P450 reductase, with or without 50 µM nitrofurantoin in 1 ml potassium phosphate buffer (50 mM, pH 7.4). Depletion of NADPH in reaction mixes was quantified by decreases in absorbance at 340 nm using a Lambda 20 UV/VIS spectrometer (Perkin–Elmer Life Sciences), scanning at 1 nm/s and recording at 1-nm intervals, repeating the scan in 2.5-min intervals for 25 min.

Cytochrome c reduction assay

A cytochrome *c* reductase (NADPH) assay kit (Sigma–Aldrich) was used to assess enzyme activity. The assay was performed in a 1.1-ml final reaction volume containing 31 µM cytochrome *c* in 300 mM potassium phosphate buffer (pH 7.8), 50 µl cell protein from supernatant fractions, and 0–500 µM nitrofurantoin. The reaction was initiated by the addition of 100 µl NADPH (final concentration, 100 µM). Increases in absorbance at 550 nm were monitored spectrophotometrically. Enzyme activity was expressed as units/mg protein; one unit is defined as 1 nmol cytochrome *c* reduced per min. In this assay, nitrofurantoin did not alter cytochrome P450 reductase-mediated reduction rates of cytochrome *c*.

Measurement of ROI in intact cells

Intracellular ROI were quantified using DCFH-DA in conjunction with flow cytometry as previously described [22]. In brief, cells were suspended in PBS (1 × 10⁶/ml) and incubated with 5 µM DCFH-DA at 37 °C in a shaking water bath for 15 min. Nitrofurantoin was then added. After 3 h, cellular fluorescence was analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA). Data were analyzed by CXP software (Beckman Coulter).

Statistical analysis

Each determination was done in duplicate or triplicate and repeated two or three times. Differences were analyzed for statistical significance using the one-way ANOVA or unpaired *t* test with GraphPad Instat software. $p < 0.05$ was considered significant.

Results

Nitrofurantoin induces redox cycling via cytochrome P450 reductase

Initially, an H_2O_2 -generating system with cDNA-expressed rat cytochrome P450 reductase was used to characterize the ability of nitrofurantoin to redox cycle. Constitutive level of H_2O_2 generation without nitrofurantoin was detected in the reaction mix (Fig. 2A). The addition of nitrofurantoin increased H_2O_2 production in concentration-dependent manner. The K_m and $V_{\max}$ for nitrofurantoin in the reaction with cytochrome P450 reductase were $318.3 \pm 67.9 \mu\text{M}$ and $650.7 \pm 95.8 \text{ nmol } \text{H}_2\text{O}_2/\text{mg protein/min}$, respectively (Table 1). The reaction was dependent on NADPH which is utilized during the enzyme reaction (Fig. 3). Nitrofurantoin was found to increase the $V_{\max}$ for NADPH utilization by cytochrome P450 reductase. With $100 \mu\text{M}$ nitrofurantoin, the $V_{\max}$ for NADPH increased approximately nine fold, with no major changes in its K_m ($p = 0.32$; Table 2). The flavoproteins inhibitor diphenyleneiodonium inhibited nitrofurantoin-induced H_2O_2 generation ($\text{IC}_{50} = 0.2 \mu\text{M}$) (Fig. 4). In contrast, no effects on this reaction were observed with potassium cyanide ($0.1\text{--}30 \mu\text{M}$), an inhibitor of respiratory oxidoreductases, dicoumarol ($0.1\text{--}100 \mu\text{M}$), an NAD(P)H: quinone oxidoreductase 1 (NQO1) inhibitor, or L-NAME ($0.1\text{--}100 \mu\text{M}$), a nitric oxide synthase inhibitor.

Nitrofurantoin also stimulated superoxide anion and hydroxyl radical production supported by cytochrome P450 reductase (Figs. 2B and 2C). As noted for H_2O_2 , constitutive levels of these ROI were detectable in control reactions. Increases in superoxide anion ($p < 0.05$) and hydroxyl radical ($p < 0.01$) generation in the presence of nitrofurantoin were time and concentration dependent (Figs. 2B and 2C). Whereas superoxide dismutase (SOD) inhibited the formation of superoxide ($p < 0.05$) (Fig. 2C), hydroxyl radicals were abolished by catalase ($p < 0.01$) and by DMSO ($p < 0.05$) (Fig. 2B). Production of hydroxyl radicals was dependent on iron, a finding consistent with the ability of nitrofurantoin to generate H_2O_2 during redox cycling, and its subsequent conversion to hydroxyl radicals via the Fenton reaction [23]. As observed with H_2O_2 production, diphenyleneiodonium also inhibited cytochrome-P450-reductase-mediated production of superoxide and hydroxyl radicals (data not shown).

In our next series of experiments, we compared nitrofurantoin redox cycling in CHO cells with stable expression of cytochrome P450 reductase (CHO-OR) and wild-type cells (CHO-WT). CHO-OR cells express 30-fold more cytochrome P450 reductase activity, as measured by cytochrome *c* reduction, than CHO-WT cells [15]. In the absence of nitrofurantoin, constitutive levels of H_2O_2 were generated by both cell types. CHO-OR cells produced approximately 2- to 3-fold more H_2O_2 than CHO-WT cells ($p = 0.01$; Figs. 5A and 5B). Nitrofurantoin caused a concentration-dependent increase in H_2O_2 production in enzyme preparations from both cell types (Figs. 5A and 5B). Significantly greater activity was

detected in CHO-OR cells overexpressing cytochrome P450 reductase. The nitrofurantoin-stimulated $V_{\max}$ for H_2O_2 production in lysates from cytochrome-P450-reductase-overexpressing cells was approximately 16-fold greater than that in wild-type cells (32.9 ± 4.8 nmol H_2O_2 /mg protein/min vs 2.1 ± 0.4 nmol H_2O_2 /mg protein/min, respectively). No major differences in the K_m values for nitrofurantoin in the two cell types were observed ($p = 0.36$; Table 1). In contrast to the kinetic constants for recombinant cytochrome P450 reductase, nitrofurantoin increased both the K_m for NADPH and the $V_{\max}$ for the generation of H_2O_2 in the CHO cells. Thus, in the presence of 100 μM nitrofurantoin, the K_m for NADPH increased about 1.5-fold in CHO-WT cells and more than 9-fold in CHO-OR cells whereas the $V_{\max}$ increased 4-fold in CHO-WT cells and 40-fold in CHO-OR cells (Table 2).

Constitutive production of superoxide anion and hydroxyl radicals was also detected in CHO cells (Figs. 5C and 5D). CHO-OR cells produced greater amounts of hydroxyl radicals than CHO-WT cells ($p < 0.05$). No differences in basal levels of superoxide anion were evident in the two cell types ($p = 0.34$), possibly because of increased SOD in lysates of CHO-OR cells compared to CHO-WT cells (data not shown). Whereas only a relatively small increase in the production of hydroxyl radicals and superoxide anion was noted in CHO-WT cells treated with nitrofurantoin, CHO-OR cells produced significantly greater amounts of these ROI ($p < 0.05$, Figs. 5C and 5D). As observed with recombinant cytochrome P450 reductase, hydroxyl radical formation in these cells was dependent on iron and prevented by catalase and DMSO ($p < 0.05$ for both CHO-WT and CHO-OR cells) (Fig. 5C). Moreover, SOD inhibited superoxide anion production in the cells ($p < 0.05$; Fig. 5D).

Cytochrome P450 reductase and redox cycling of nitrofurantoin in different cell types

We next determined whether nitrofurantoin-induced H_2O_2 generation was correlated with cytochrome P450 reductase activity in various cell types. These results are shown in Table 3. A mouse-lung-derived epithelial cell line, MLE 15, was found to contain the highest NADPH-cytochrome *c* reductase activity and a human prostate cell line, PC-3, exhibited the lowest activity (17.5 ± 1.0 units/mg protein vs 2.5 ± 0.4 units/mg protein, respectively). Table 1 compares nitrofurantoin-induced redox cycling in these cells. The K_m for nitrofurantoin varied from 162.8 ± 13.0 μM in human PC-3 prostate cells to 645.2 ± 88.5 μM in human HT-29 colon tumor cells. The $V_{\max}$ for nitrofurantoin-stimulated H_2O_2 generation varied eight-fold among the cells; the highest activity was detected in HT-29 cells ($V_{\max} = 13.6 \pm 1.4$ nmol H_2O_2 /mg protein/min). As observed with the CHO cells, in all cells except RAW 264.7 macrophages and B16 melanoma, nitrofurantoin increased the K_m and $V_{\max}$ values for NADPH. In the macrophages and melanoma, nitrofurantoin-induced increase in H_2O_2 generation was due to increases in $V_{\max}$ of the enzyme without significant alterations in the K_m (Table 2).

Interestingly, in all of the cell lines except HT-29 cells, redox cycling of nitrofurantoin was found to directly correlate with cytochrome P450 reductase activity (Fig. 6, top). In HT-29 cells, significantly more nitrofurantoin redox activity was detected relative to cytochrome P450 reductase activity. Because cytochrome P450 reductase is a microsomal enzyme [24,25], we next analyzed nitrofurantoin redox cycling in microsomal and cytosolic fractions

of HT-29 cells. For comparison, we also analyzed MLE 15 cells. As expected, in MLE 15 cells, the majority of nitrofurantoin-stimulated H_2O_2 generation appeared in the microsomal fraction of the cells ($p = 0.001$) (Table 4). In contrast, the cytosolic fraction of HT-29 cells contained significantly more nitrofurantoin-stimulated H_2O_2 generation activity than the microsomal fraction ($p = 0.005$). These data indicate that HT-29 cells contain high activity of additional NADPH oxidoreductases in the cytosolic fraction capable of redox cycling nitrofurantoin.

Effects of nitrofurantoin on intracellular ROI production and cell growth inhibition

Because cell lysates were found to redox cycle nitrofurantoin, we next determined whether this was associated with ROI production in intact cells. For these studies we used techniques in flow cytometry in conjunction with the ROI-sensitive probe, DCFH-DA, to evaluate intracellular H_2O_2 production. We found that nitrofurantoin readily induced H_2O_2 in intact CHO cells. In CHO-WT cells, nitrofurantoin increased intracellular H_2O_2 production approximately 7-fold ($p < 0.001$; Fig. 7, left). In contrast, in CHO-OR cells, nitrofurantoin was significantly more effective in increasing intracellular H_2O_2 production. Thus, after nitrofurantoin treatment, a subpopulation of extremely bright CHO-OR cells (approximately 75% of the total number of cells analyzed) increased H_2O_2 production approximately 60-fold ($p = 0.0016$; Fig. 7, right). The remaining cells in a less bright subpopulation also increased H_2O_2 production approximately 5-fold ($p < 0.001$). At the present time, the origin of the subpopulation heterogeneity in CHO-OR cells with respect to H_2O_2 production is not known. However, these data are consistent with the increased ability of CHO-OR cells to redox cycle nitrofurantoin. Interestingly, despite marked differences in nitrofurantoin-induced redox cycling and intracellular H_2O_2 production in CHO-WT and CHO-OR cells, their responses to nitrofurantoin-induced cytotoxicity, as measured by growth inhibition, were similar ($\text{IC}_{50} = 53 \pm 1 \mu\text{M}$ vs $42 \pm 3 \mu\text{M}$ for CHO-WT and CHO-OR cells, respectively) (Fig. 8, left and Table 3). We also found that the different cell types varied approximately 5- to 6-fold in their sensitivity to nitrofurantoin-induced growth inhibition ($\text{IC}_{50} = 14\text{--}80 \mu\text{M}$). Moreover, no correlation was observed between nitrofurantoin redox cycling and growth inhibition (Fig. 6, bottom). For example, although similar cytochrome P450 reductase activity was detected in C2 and S-180 cells (6.1 ± 0.2 units/mg protein vs 7.4 ± 1.0 units/mg protein; $p > 0.05$) and the ability of the two cell types to redox cycle nitrofurantoin was similar ($V_{\text{max}} = 3.5 \pm 0.2$ nmol H_2O_2 /mg protein/min vs 4.4 ± 0.3 nmol H_2O_2 /mg protein/min for C2 and S-180 cells, respectively; $p > 0.05$), S-180 cells were significantly more sensitive to nitrofurantoin-induced cytotoxicity ($p < 0.05$, Fig. 8, right and Table 3). Furthermore, PC-3 cells, which display significantly less nitrofurantoin-induced redox cycling activity than MLE 15 ($p < 0.001$) or HT-29 cells ($p < 0.05$), were 2- to 3-fold more sensitive to nitrofurantoin-induced growth inhibition ($p < 0.001$ comparing PC-3 with MLE 15 cells and $p < 0.01$ comparing PC-3 with HT-29 cells; compare Tables 1 and 3).

Discussion

Several enzymes in mammalian cells that mediate one-electron reduction of nitroaromatic compounds to reactive electrophilic intermediates have been identified including cytochrome P450 reductase [14,26], NADH:lipoamide oxidoreductase [27], NAD(P)H:quinone

oxidoreductase (NQO1) [28], thioredoxin reductase [29], nitric oxide synthase [30], xanthine oxidase [31], and ferredoxin: NADP⁺ reductase [32]. Oxidation of reactive electrophilic intermediates back to the parent compound generates toxic ROI. It is well recognized that nitrofurantoin can undergo a one-electron reduction; however, the enzymatic activity mediating this reaction is not well characterized. Previous studies have demonstrated that microsomal fractions from rat lung and liver can reduce nitrofurantoin, suggesting that this process is mediated by cytochrome P450 reductase [33]. This is supported by the observation that inhibitors of this enzyme suppress reduction of nitrofurantoin in rat hepatocytes [14]. Using purified rat liver cytochrome P450 reductase, it has also been demonstrated that nitrofurantoin undergoes a one-electron reduction to the nitro aromatic anion radical and is reoxidized under aerobic condition [34]. Our data show that redox cycling of nitrofurantoin by a recombinant cytochrome P450 reductase, or lysates from a variety of cell types, generates superoxide anion, hydrogen peroxide, and, in the presence of redox-active iron, hydroxyl radicals. This redox cycling reaction requires NADPH and is inhibited by diphenylenone, demonstrating that it is dependent on the flavin cofactors in the cytochrome P450 reductase.

ROI are known to be cytotoxic and their production during nitrofurantoin redox cycling has the potential to contribute to tissue injury. To examine the relationship between nitrofurantoin-induced ROI production during redox cycling and cytotoxicity, we used cell lines varying in their cytochrome P450 reductase activity. We found that there was a direct correlation between the nitrofurantoin redox cycling and the activity of cytochrome P450 reductase in the cells. This was most evident in CHO cells constructed to overexpress cytochrome P450 reductase in which nitrofurantoin redox cycling was more than 15 times greater than that in wild-type cells. The overexpressing cells also produced approximately six-fold more intracellular ROI following nitrofurantoin treatment. Unexpectedly, no major differences between the two cell types with respect to nitrofurantoin-induced cytotoxicity as measured by growth inhibition were evident. These data suggest that cytotoxicity is independent of nitrofurantoin redox cycling and ROI production. This is supported by similar findings in several cell lines. For example, S-180 cells were two times more sensitive to growth inhibition by nitrofurantoin than C2 cells, despite that the cells displayed similar nitrofurantoin redox cycling activity. Furthermore, although MLE 15 cells displayed approximately four-fold more nitrofurantoin redox cycling activity than PC-3 cells, they were three-fold less sensitive to nitrofurantoin-induced cytotoxicity. These data provide support for the idea that nitrofurantoin redox cycling and subsequent production of ROI does not mediate cytotoxicity. Thus, levels of cytochrome P450 reductase activity in tumor cells may not be predictive for a higher level of sensitivity against nitrofurantoin toxicity.

At the present time, the mechanisms underlying nitrofurantoin toxicity is not known. Among the ROI generated during nitrofurantoin-induced redox cycling, hydroxyl radicals are the most damaging [35,36]. Cytotoxicity may, in part, be related to the ability of nitrofurantoin to generate hydroxyl radicals in each cell line, a process that depends on the availability of redox-active metals such as iron and copper. It is possible that the different cell lines vary in their metal content and that this limits nitrofurantoin-induced cytotoxicity. In this regard, Staubli and Boelsterli [37] and Sturm et al. [38] reported that nitrofurantoin increases the intracellular labile iron pool, a factor that may control redox cycling. Alternatively,

intracellular levels of antioxidants that detoxify and protect against ROI-induced damage may be important in regulating nitrofurantoin-induced cytotoxicity. In this regard, superoxide dismutase, catalase, and antioxidants including alpha-tocopherol, ascorbic acid, and dimethyl sulfoxide have been reported to block nitrofurantoin-induced cytotoxicity in a rat lung explant model [8]. Metabolism of nitrofurantoin to reactive metabolites is another factor that may regulate cytotoxicity. Electrophilic intermediates formed as a result of redox cycling of nitrofurantoin have been reported to bind glutathione and cellular macromolecules [33]. The generation of these metabolites depends on localized oxygen tension which can control bioreduction of nitrofurantoin in target tissues [10]. Reduction of nitrofurantoin to its amine and further derivatization may also generate cytotoxic metabolites [34,39]. Additional studies are needed to characterize metabolism of nitrofurantoin in the various cell types under different conditions and to determine its role in cytotoxicity.

Although redox cycling of nitrofurantoin is directly correlated with cytochrome P450 reductase activity in almost all the cell lines, it is likely that additional oxidoreductases in the cells redox cycle this antibiotic. This is supported by our findings of significant nitrofurantoin redox cycling in the cytosol of HT-29 cells which does not contain cytochrome P450 reductase activity. Low levels of nitrofurantoin redox cycling activity were also evident in cytosolic fractions of MLE 15 cells, suggesting that additional enzymes also redox cycle nitrofurantoin in these cells. In this regard, our laboratory has recently demonstrated paraquat redox cycling by thioredoxin reductase in cytosolic fractions of MLE 15 cells [40]. Studies to identify the NADPH oxidoreductases that are important in redox cycling nitrofurantoin in the cytosolic fraction of cells are in progress. It should be noted that we also cannot exclude the possibility that, in addition to cytochrome P450 reductase, other oxidoreductases mediate redox cycling of nitrofurantoin in the microsomal fraction of cells.

Using recombinant cytochrome P450 reductase, we found that increased nitrofurantoin redox cycling was due to an increase in $V_{\max}$ for NADPH with little change in affinity of the enzyme for the pyridine nucleotide. Moreover, in all cell types except RAW 264.7 and B16 cells, nitrofurantoin redox cycling increased the $V_{\max}$ but appeared to decrease the enzyme affinity for NADPH. The changes in reaction kinetics for NADPH in RAW 264.7 and B16 cells were similar to the recombinant cytochrome P450 reductase. Increases in $V_{\max}$ were not surprising because we are measuring redox cycling in nitrofurantoin-sensitive redox enzymes. However, apparent changes in affinity of NADPH in enzyme assays are most likely due to differences in the kinetic characteristics of various constitutive enzymes in cell lysates that utilize NADPH as a one-electron donor for redox cycling in the absence of nitrofurantoin and the specific characteristics of the enzyme(s) activated by the drug. The mechanisms underlying the distinct reaction kinetics for NADPH in RAW 264.7 and B16 cells are not apparent. It will be of interest to characterize the enzymes in these and the other cell lines that are important in nitrofurantoin redox cycling.

In summary, our data show that nitrofurantoin readily redox cycles with recombinant cytochrome P450 reductase and lysates of many different cell types. In the cells, ROI generated by nitrofurantoin redox cycling directly correlates with cytochrome P450 reductase activity. However, it does not correlate with the cytotoxicity of nitrofurantoin. Thus, in these cells, ROI-induced oxidative stress alone does not mediate the toxicity of

nitrofurantoin. In humans and other animals, nitrofurantoin is known to induce toxicity in many tissues including lung, liver, peripheral nerves and blood. Further studies to determine the role of redox cycling in nitrofurantoin-induced toxicity in these tissues and to identify alternative sites of action of this drug in tumor cells are needed.

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Abbreviations

ROI	reactive oxygen intermediates
CHO	Chinese hamster ovary
MLE	murine lung epithelial
PBS	phosphate-buffered saline
SOD	superoxide dismutase
DMSO	dimethyl sulfoxide

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Fig. 1. Nitrofurantoin redox cycling and ROI generation. The one-electron reduction of nitrofurantoin by enzymes such as cytochrome P450 reductase generates the nitrofurantoin radical

In the presence of oxygen, the radical disproportionates to form superoxide anion and the parent compound. Adapted from Minchin et al. [10].

Fig. 2. Effects of nitrofurantoin (NFT) on ROI production by recombinant cytochrome P450 reductase (P450 OR)

(A) Nitrofurantoin-induced H_2O_2 production. Enzyme reactions were run in absence or presence of increasing concentrations of nitrofurantoin. Accumulation of H_2O_2 in the assay mixes was quantified using the Amplex red/horseradish peroxidase reaction ($n = 3 \pm SE$). (B and C) Nitrofurantoin-induced hydroxyl radical and superoxide production. Reactions were run in the absence or presence of 25 or 100 μM NFT. Hydroxyl radicals in enzyme assays were quantified by the formation of 2-hydroxyterephthalate (2-OH TPT) derived from terephthalate ($n = 2 \pm SD$). In some assays, catalase (400 U/ml) or DMSO (70 mM) was added to prevent hydroxyl radical formation. Note that the generation of hydroxyl radicals was dependent on the presence of redox-active iron. Superoxide in the enzyme assays was quantified as the formation of 2-hydroxyethidium derived from dihydroethidium. In some of the assays, SOD (350 U/ml) was added to inhibit superoxide formation. C shows HPLC tracings of 2-hydroxyethidium formation. One of two representative experiments is shown. All enzyme reaction mixes contained 0.5 mM NADPH. ** $p < 0.01$ compared to rat P450 OR without NFT, # $p < 0.05$ and ## $p < 0.01$ compared to rat P450 OR with 100 μM NFT.

Fig. 3. Nitrofurantoin-stimulated NADPH depletion by recombinant cytochrome P450 reductase
Reaction mixes containing 0.1 mM NADPH were analyzed in 1-ml spectrophotometer cuvettes. Absorbance spectra were recorded at 2.5-min intervals. (Top) Nitrofurantoin-stimulated NADPH depletion; 50 μ M nitrofurantoin was added directly to the cuvette. (Bottom) NADPH metabolism in the enzyme assay in the absence of nitrofurantoin.

Fig. 4. Effects of inhibitors on nitrofurantoin-induced H_2O_2 production via recombinant cytochrome P450 reductase
 H_2O_2 formation was assayed in reaction mixes containing 0.5 mM NADPH, 100 μ M nitrofurantoin, and increasing concentration of diphenyleneiodonium (DPI), potassium cyanide (KCN), dicoumarol, or L-NAME. Control rate is 93.4 ± 11.1 nmol H_2O_2 /mg protein/min; $n = 3 \pm$ SE. ** $p < 0.01$ compared to control.

Fig. 5. Effects of nitrofurantoin on ROI production by CHO-WT and CHO-OR cells (A and B) Nitrofurantoin-induced H_2O_2 production with lysates from CHO-WT cells (A) and CHO-OR cells (B). H_2O_2 production in the assay mixes was quantified in absence or presence of nitrofurantoin every 2.5 min for 30 min ($n = 3 \pm \text{SE}$). Constitutive production of hydrogen peroxide was 8 nmol/30 min/mg protein for CHO-WT cells and 21 nmol/30 min/mg protein for CHO-OR cells. (C and D) Generation of hydroxyl radicals and superoxide anion in reaction mixes stimulated by nitrofurantoin. Reactions were run in the absence or presence of 25 or 100 μM nitrofurantoin (NFT). Hydroxyl radicals in enzyme assays were quantified by the formation of 2-hydroxyterephthalate (2-OH TPT) ($n = 2 \pm \text{SD}$). In some assays, catalase (400 U/ml) or DMSO (70 mM) was added to prevent hydroxyl radical formation. Superoxide in the enzyme assays was quantified by the formation of 2-hydroxyethidium from dihydroethidium. In some of the assays, SOD (350 U/ml) was added to prevent superoxide formation. D shows HPLC tracings of 2-hydroxyethidium formation. One of two representative experiments is shown. * $p < 0.05$ and *** $p < 0.001$ comparing CHO-WT with CHO-OR cells.

Fig. 6. Cytochrome P450 reductase activity, nitrofurantoin (NFT)-induced redox cycling, and cytotoxicity in different cell lines

(Top) Correlation between cellular cytochrome P450 reductase activity and nitrofurantoin-induced H₂O₂ production. Cytochrome P450 reductase activity was measured using a cytochrome *c* reduction assay. The $V_{\max}$ for nitrofurantoin-induced H₂O₂ generation (Table 1) was used to compare nitrofurantoin-induced redox cycling with cellular cytochrome P450 reductase activity. The inset shows the same data with CHO-OR cells omitted. Note the positive correlation between cytochrome P450 reductase activity and nitrofurantoin-induced redox cycling. (Bottom) Lack of correlation between nitrofurantoin-induced redox cycling and cellular cytotoxicity. Redox cycling capacity of the cell lines was plotted against the concentration of nitrofurantoin inhibiting growth by 50% (IC₅₀) in each cell line (Table 3).

(●PC-3, ○CHO-WT, ▼HL-60, △C2, ■S-180, □ HT-29, ◆HeLa, ◇ B16, ▲RAW 264.7, ▽MLE 15, ●CHO-OR).

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Fig. 7. Intracellular ROI production in CHO-WT and CHO-OR cells

DCFH-DA was used to quantify intracellular ROI generation. Cells were incubated with 5 μ M DCFH-DA at 37 $^{\circ}$ C for 3 h in the absence (solid lines) or presence (dashed lines) of 500 μ M nitrofurantoin. Cellular fluorescence was analyzed by flow cytometry. One of three representative experiments is shown. The mean fluorescence of CHO-WT control peak is 1.01 ± 0.04 ($n = 3 \pm \text{SE}$) and 7.25 ± 0.28 after nitrofurantoin treatment. For CHO-OR cells, the mean fluorescence of the control peak is 1.13 ± 0.03 , 5.01 ± 0.21 for subpeak 1, and 66.40 ± 4.49 for subpeak 2 after nitrofurantoin treatment.

Fig. 8. Growth inhibition by nitrofurantoin

Cells were seeded in six-well plates and allowed to adhere overnight. The medium was then replaced with growth medium supplemented with increasing concentrations of nitrofurantoin. Cell growth was assayed after 4–5 days. (Left) Comparison of the growth inhibitory activity of nitrofurantoin in CHO-WT and CHO-OR cells. (Right) Comparison of the growth inhibitory activity of nitrofurantoin in C2 and S-180 cells.

Table 1

Kinetic parameters of nitrofurantoin-stimulated H₂O₂ generation with CYP 450 reductase and microsome-containing fractions from different cell types

Species		Origin	K_m (μ M)*	V_{max} (nmol H ₂ O ₂ /mg protein/min)
	CYP 450 OR	Supersomes	318.3 $\pm$ 67.9	650.7 $\pm$ 95.8
Hamster	CHO-WT	Ovary	150.9 $\pm$ 3.9	2.1 $\pm$ 0.4
	CHO-OR	Ovary	175.9 $\pm$ 21.3	32.9 $\pm$ 4.8
Mouse	MLE 15	Lung	298.1 $\pm$ 27.3	12.1 $\pm$ 1.0
	RAW 264.7	Macrophage	289.5 $\pm$ 42.2	6.7 $\pm$ 0.6
	C2	Muscle	193.8 $\pm$ 7.6	3.5 $\pm$ 0.2
	S-180	Sarcoma	214.4 $\pm$ 18.8	4.4 $\pm$ 0.3
	B16	Melanoma	207.6 $\pm$ 20.3	6.2 $\pm$ 0.4
Human	HL-60	Leukemia	200.0 $\pm$ 26.1	1.8 $\pm$ 0.1
	HT-29	Colon	645.2 $\pm$ 88.5	13.6 $\pm$ 1.4
	HeLa	Cervix	240.3 $\pm$ 16.6	5.7 $\pm$ 0.3
	PC-3	Prostate	162.8 $\pm$ 13.0	3.3 $\pm$ 0.4

* Using increasing concentrations of nitrofurantoin, reaction rates were measured in the linear phase in standard reaction mixes in the presence of NADPH (500 μ M). K_m and V_{max} values were obtained using Michaelis-Menton kinetics. In cell lysates, each point represents the mean of three independent determinations $\pm$ SE. With CYP 450 OR, each point represents the mean of two independent determinations $\pm$ SD.

Table 2

Effects of nitrofurantoin on the kinetic constants for NADPH with recombinant CYP 450 reductase and microsome-containing fractions from different cell types

Species		Treatments	K_m (μM)*	$V_{\max}$ (nmol $\text{H}_2\text{O}_2/\text{mg protein/min}$)
CYP450 OR		-	28.3 ± 5.5	7.8 ± 1.3
		+NFT	19.2 ± 2.3	71.4 ± 13.7
Hamster	CHO-WT	-	28.9 ± 7.9	0.2 ± 0.0
		+NFT	42.2 ± 12.3	0.8 ± 0.2
	CHO-OR	-	30.7 ± 8.9	0.6 ± 0.1
		+NFT	290.3 ± 75.2	24.0 ± 6.2
Mouse	MLE 15	-	19.5 ± 5.2	0.2 ± 0.0
		+NFT	33.7 ± 1.5	2.3 ± 0.2
	RAW 264.7	-	19.7 ± 3.0	0.1 ± 0.0
		+NFT	18.2 ± 2.7	1.7 ± 0.3
	C2	-	13.9 ± 3.3	0.2 ± 0.0
		+NFT	24.9 ± 2.0	1.0 ± 0.0
	S-180	-	6.6 ± 1.0	0.2 ± 0.0
		+NFT	19.5 ± 2.1	1.3 ± 0.2
	B16	-	27.6 ± 5.2	0.4 ± 0.1
		+NFT	28.3 ± 3.5	2.0 ± 0.3
Human	HL-60	-	8.3 ± 1.5	0.1 ± 0.0
		+NFT	13.2 ± 2.6	0.5 ± 0.0
	HT-29	-	ND**	ND
		+NFT	19.6 ± 6.4	2.1 ± 0.2
	HeLa	-	8.2 ± 1.5	0.2 ± 0.0
		+NFT	23.3 ± 1.1	1.7 ± 0.1
	PC-3	-	3.6 ± 0.5	0.1 ± 0.0
		+NFT	58.0 ± 5.8	1.3 ± 0.1

* Using increasing concentrations of NADPH, reaction rates were measured in the linear phase in the absence and presence of nitrofurantoin (NFT; 100 μM). K_m and $V_{\max}$ values were obtained using Michaelis–Menton kinetics. In cell lysates, each point represents the mean of three independent determinations $\pm$ SE. With CYP 450 OR, each point represents the mean of two independent determinations $\pm$ SD.

** The data obtained do not fit Michaelis–Menton kinetics.

Table 3

Cytochrome *c* reductase in microsome-containing fractions from different cell types and ability of nitrofurantoin to inhibit cell growth

Species	Cell type	Origin	Cytochrome <i>c</i> reductase activity (units/mg protein) *	Growth inhibition IC ₅₀ (μM) **
Hamster	CHO-WT	Ovary	2.9 ± 0.6	53 ± 1
	CHO-OR	Ovary	87.1 ± 5.9	42 ± 3
Mouse	MLE 15	Lung	17.5 ± 1.0	55 ± 4
	RAW 264.7	Macrophage	13.5 ± 1.5	51 ± 2
	C2	Muscle	6.1 ± 0.2	80 ± 12
	S-180	Sarcoma	7.4 ± 1.0	41 ± 4
	B16	Melanoma	9.6 ± 0.5	33 ± 4
	HL-60	Leukemia	3.4 ± 0.6	14 ± 1
Human	HT-29	Colon	8.4 ± 1.7	35 ± 1
	HeLa	Cervix	8.9 ± 1.4	41 ± 2
	PC-3	Prostate	2.5 ± 0.4	19 ± 3

* One unit is defined as the reduction of 1 nmol of oxidized cytochrome *c* in the presence of 100 μM NADPH per minute at pH 7.8 at 25 °C. Values for the calculations were taken in the linear phase of the reactions. Each point represents the mean of three independent determinations ± SE.

** IC₅₀, concentration of nitrofurantoin inhibiting cell growth by 50%.

Table 4

Kinetic parameters for nitrofurantoin in different fractions of HT 29 and MLE 15 cells

		K_m (μM) [*]	V_{max} (nmol H ₂ O ₂ /mg protein/min)
Cytosol	HT-29	ND ^{**}	13.8 ± 0.7
	MLE 15	297.5 ± 0.7	3.4 ± 0.8
Microsomal	HT-29	233.2 ± 7.4	6.9 ± 0.2
	MLE 15	228.0 ± 54.3	13.6 ± 0.9

^{*} Using increasing concentrations of nitrofurantoin, reaction rates were measured in the linear phase in standard reaction mixes in the presence of NADPH (500 μM). K_m and V_{max} values were obtained using Michaelis–Menton kinetics. Each point represents the mean of three independent determinations ± SE.

^{**} The data obtained do not fit Michaelis–Menton kinetics.