

5-Methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione, a mechanism-based inhibitor of NAD(P)H:quinone oxidoreductase 1, exhibits activity against human pancreatic cancer *in vitro* and *in vivo*

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Abstract

The enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1) has been found to be up-regulated in pancreatic cancer as well as many other solid tumors. A recent study showed that inhibition of NQO1 in pancreatic cancer cells using the nonselective inhibitor dicumarol suppressed the malignant phenotype. The authors suggested that inhibition of cell growth might result from an increase in intracellular superoxide production due to inhibition of NQO1. We have recently shown that NQO1 can directly scavenge superoxide and this effect may become physiologically relevant in cells containing high NQO1 levels. We therefore tested the hypothesis that 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione (ES936), a specific mechanism-based inhibitor of NQO1, would be an effective agent for the treatment of pancreatic tumors. The human pancreatic tumor cell lines BxPC-3 and MIA PaCa-2 contain high levels of NQO1 activity and protein as verified by immunoblot and immunocytochemical staining of human pancreatic tumor cells. ES936 treatment inhibited NQO1 activity by >98% in MIA PaCa-2 and BxPC-3 cells. In addition, ES936 treatment induced growth inhibition [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay] in MIA PaCa-

2 and BxPC-3 cells with an IC₅₀ of 108 and 365 nmol/L, respectively. Treatment of MIA PaCa-2 cells with ES936 also inhibited the ability of these cells to form colonies and grow in soft agar in a dose-dependent manner. Treatment of mice carrying MIA PaCa-2 xenograft tumors with ES936 resulted in a significant difference in growth rates in ES936-treated and DMSO-treated (control) tumors. Our data did not show an increase in either intracellular superoxide production or oxygen consumption after treatment of cells with ES936, contrary to the effects seen with dicumarol. In summary, mechanism-based inhibitors of NQO1, such as ES936, may be useful therapeutic agents for the treatment of pancreatic cancer, although the underlying mechanism seems to be independent of superoxide generation. [Mol Cancer Ther 2006;5(7):1702–9]

Introduction

Pancreatic cancer has the worst prognosis of all cancers with a 5-year survival rate of <3%, accounting for the fourth largest cause of cancer deaths in the United States (1, 2). It is among the most rapidly fatal cancers with a 1-year survival rate of <20% and the incidence of the disease is increasing (1, 3). The most common treatment option is surgery and/or radiation but, because of the problems with early detection of this disease, most patients have advanced or nonresectable disease at presentation (1, 3). There are few therapeutic options in this disease and new chemotherapeutic approaches to treatment are desperately needed.

NAD(P)H:quinone oxidoreductase 1 (NQO1; DT-diaphorase) is an obligate two-electron reductase that reduces quinones to their corresponding hydroquinones. A number of possible cellular functions of NQO1 have recently been summarized (4). NQO1 is up-regulated in most human solid tumors (5–7) and pancreatic tumors are no exception. NQO1 has been reported to be expressed at higher levels in pancreatic tumors than in normal tissue (2, 8) and our own immunohistochemical studies have confirmed the elevated expression of NQO1 in pancreatic tumors with little to no NQO1 protein detectable in adjacent uninvolved tissue (9). Cullen et al. (10) recently showed that dicumarol, an inhibitor of NQO1, led to inhibition of cell growth, inhibition of the *in vitro* malignant phenotype, and increased intracellular levels of superoxide in pancreatic cancer cells. These authors also showed that dicumarol-induced cell growth and superoxide levels could both be abrogated by transfection of pancreatic cancer cells with superoxide dismutase (10). From these data, an innovative mechanism was proposed,

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one that involved NQO1 inhibition, increased levels of superoxide, and inhibition of cell growth and the *in vitro* malignant phenotype (10). Our own work has shown that NQO1 can directly scavenge superoxide (11), and in cells containing high levels of NQO1, such as pancreatic cancers, scavenging of superoxide by NQO1 may represent an alternative pathway for superoxide detoxification (10, 12). These data provided a potential mechanistic basis for the effects of NQO1 inhibitors in pancreatic cancer.

However, dicumarol is known to have many ancillary effects other than inhibition of NQO1, and its growth inhibitory effects may be unrelated to modulation of NQO1 activity. We have previously cautioned against overreliance on dicumarol as a specific inhibitor of NQO1, listing a dozen enzymes that have been reported to be inhibited by dicumarol mainly in the dehydrogenase and reductase categories (13). A variety of other proteins have also been shown to be inhibited by dicumarol, including a variety of stress-activated protein kinases (14), nuclear factor κ B (14), glutathione S-transferase (15), glutathione peroxidase (15), and UDP-glucuronosyltransferase (16). Dicumarol has also been reported to uncouple mitochondrial oxidative phosphorylation (17). The nonspecificity of dicumarol led us to develop specific inhibitors of NQO1 that can be employed to reliably test the hypothesis that NQO1 inhibitors may be useful agents in the therapy of pancreatic tumors.

We have developed inhibitors of NQO1 and have characterized one compound as a potent and specific mechanism-based inhibitor of NQO1. This compound is an indolequinone, 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione (ES936), which is active at inhibiting NQO1 both *in vitro* and *in vivo* (18–20). Mechanism-based (suicide) inhibitors of NQO1 are characterized by enzymatic reduction to generate an unstable hydroquinone that undergoes chemical rearrangement, leading to covalent binding of the inhibitor into the active site of NQO1. Mechanism-based inhibition of NQO1 has been characterized using biochemical methods, mass spectrometry, and X-ray crystallography (19). Concentrations of ES936 required to induce growth inhibition (IC_{50}) and inhibition of NQO1 activity in human cancer cell lines are in the nanomolar range. Consistent with a mechanism-based mode of action, the reduction of ES936 by NQO1 in cellular systems results in NQO1 inhibition without affecting NQO1 protein levels.⁴ ES936 can therefore be reliably employed to test the hypothesis that NQO1 inhibitors may be useful agents in the therapy of pancreatic tumors. Due to the high levels of NQO1 in pancreatic cancer relative to uninvolved pancreatic tissue, this approach has the potential to afford considerable selectivity against pancreatic tumors.

Materials and Methods

Chemicals

NADH, 2,6-dichlorophenol-indophenol, dicumarol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT), β -lapachone, and 2,3-dimethoxy-1,4-naphthoquinone were obtained from Sigma Chemical (St. Louis MO). Dihydroethidium was obtained from Invitrogen (formerly Molecular Probes, Eugene OR). ES936 was synthesized by C.J. Moody as previously described (21).

Cell Lines

MIA PaCa-2 human pancreatic carcinoma cells and BxPC-3 human pancreatic adenocarcinoma cells were obtained from American Type Culture Collection (Manassas VA). MIA PaCa-2 cells were grown in DMEM adjusted to contain 4 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10% (v/v) fetal bovine serum, 2.5% (v/v) horse serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin. BxPC-3 cells were grown in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate, and adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Both cell lines were maintained in a humidified incubator containing 5% carbon dioxide at 37°C.

Immunodetection of NQO1 in Human Pancreatic Cancer Cells

NQO1 protein expression was detected by fluorescence microscopy in BxPC-3 and MIA PaCa-2 cells by immunocytochemistry using anti-NQO1 mouse monoclonal antibodies (clone A180) and Texas red-conjugated goat anti-mouse immunoglobulin G secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Briefly, cells were grown on glass coverslips in complete medium, fixed in 4% (v/v) formaldehyde, permeabilized with cold methanol (–20°C), and then blocked in 5% (w/v) bovine serum albumin in PBS for 1 hour at room temperature. Hybridoma cell supernatant containing mouse monoclonal antibodies (immunoglobulin G1) from clone A180 was diluted 1:5 in 10 mmol/L Tris-HCl (pH 8) containing 150 mmol/L NaCl and 0.4% (v/v) Tween 20 (TBST). Cover slips were incubated with the antibody solution for 1 hour at room temperature. Texas red-conjugated goat anti-mouse immunoglobulin G secondary antibodies were diluted 1:5,000 in TBST, then added for 30 minutes at room temperature. Immunoblot analysis of NQO1 was done in BxPC-3 and MIA PaCa-2 sonicates using anti-NQO1 mouse monoclonal antibodies (clone A180) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibodies (Jackson ImmunoResearch Laboratories) with chemiluminescence detection as previously described (7).

NQO1 Activity Measurements

Cell culture medium was aspirated and cells were washed twice with PBS, scraped into 25 mmol/L Tris-HCl (pH 7.4) containing 250 mmol/L sucrose and 5 μ mol/L FAD, followed by probe sonication on ice. Sonicates were then centrifuged at 13,000 rpm for 10 minutes at 4°C and the supernatant was recovered for NQO1 activity assays and protein concentration determinations (22). NQO1 activity measurements were done as described by Ernster (23) and modified by Benson et al. (24) using

⁴ Unpublished data.

2,6-dichlorophenol-indophenol as a substrate. Reactions (1 mL) contained 25 mmol/L Tris-HCl (pH 7.4), 0.7 mg/mL bovine serum albumin, 0.2 mmol/L NADH, and 40 μ mol/L 2,6-dichlorophenol-indophenol. Reactions were started by the addition of a small volume (2–5 μ L) of cell supernatant. Reactions were done in the absence and presence of 20 μ mol/L dicumarol. NQO1 activity is defined as the dicumarol-inhibitable reduction of 2,6-dichlorophenol-indophenol measured at 600 nm at 30°C.

Growth Inhibition Assays

Growth inhibition in MIA PaCa-2 and BxPC-3 cell lines following treatment with ES936 was measured using the MTT assay. For these studies, cells were seeded at 1×10^3 to 2×10^3 per well (96-well plates in triplicate) and allowed to attach for 16 hours. Cells were treated with ES936 for 72 hours in complete medium. Growth inhibition was determined by measuring cellular reduction of MTT to the formazan product extracted from cells by the addition of DMSO. Absorbance of the extract was determined at 550 nm using a microplate reader.

Clonogenic Assays

The inhibition of the colony-forming ability of MIA PaCa-2 cells was assayed by seeding 5,000 cells in complete medium into 100-mm tissue culture plates and cells were allowed to grow. Cells were then exposed to a concentration range of ES936 in complete medium. After 1 week at 37°C, the medium was removed, cells were rinsed in PBS, fixed in 3% (v/v) acetic acid, 10% (v/v) methanol for 2 minutes, then stained with 0.2% (w/v) crystal violet, 10% (v/v) formalin in PBS, and colonies were counted manually. Colonies were defined as ≥ 50 cells.

Growth in Soft Agar

For these experiments, 2×10^3 to 5×10^3 cells were suspended in 3 mL of complete medium containing 0.3% (w/v) low-melt agarose (Invitrogen, Carlsbad, CA) in the presence and absence of ES936. This mixture was then plated into 60-mm tissue culture plates containing 0.5% (w/v) agar in complete medium. Cells were allowed to grow for 10 to 14 days and colonies (≥ 50 cells) were counted. Plating efficiency was defined as [(number of colonies) / (number of cells plated)] $\times 100$.

ES936 Antitumor Activity in Human Pancreatic Xenograft Tumors

All experiments were approved by the University of Colorado Health Sciences Center Animal Care and Use Committee and were carried out according to approved protocols. Female athymic nude mice (Ncr *nu/nu*; National Cancer Institute, Frederick, MD) were received at 5 to 6 weeks of age and were allowed to acclimate for 2 weeks in sterile microisolator cages with constant temperature and humidity. Mice had free access to food and water. MIA PaCa-2 cells in log-phase growth were harvested on the day of use. Cells were suspended in 75:25 unsupplemented medium/Matrigel and 0.1 mL (2×10^7 cells) was injected s.c. into the right flank of each animal. After inoculation of tumor cells, mice were monitored daily, weighed twice weekly, and digital caliper measurements were begun when tumors became

visible. When tumors had grown to ~ 200 mm (~ 14 days after implantation), tumor-bearing mice were randomized into control and drug treatment groups. For these studies, ES936 was administered at the highest dose permitted (5 mg/kg) due to solubility limitations in DMSO. ES936 (dissolved in sterile DMSO) was injected i.p. daily for 10 days at a dose of 5 mg/kg. No obvious toxicities were observed in the control (DMSO) or ES936-treated animals and there was no difference in body weight between control and ES936-treated animals. Tumor volume was calculated by the formula $(L \times W^2) / 2$, where L is the longer measurement of the tumor and W is the smaller tumor measurement. T/C (ratios of tumor volumes of treated and control tumors as an indicator of drug efficacy) percent growth inhibition of tumors and tumor doubling times were calculated as previously described (25).

Detection of Intracellular Superoxide

Intracellular superoxide production was measured using the oxidation of dihydroethidium (26). For these experiments, MIA PaCa-2 cells were harvested by trypsinization, washed in PBS, counted, and 1×10^6 cells were treated with vehicle (DMSO) or NQO1 inhibitors in complete medium for 4 hours at 37°C. After drug treatments, 10 μ mol/L dihydroethidium was added and cells were incubated for an additional 30 minutes; after which, the cells were centrifuged and resuspended in 1 mL of PBS. Conversion of dihydroethidium to a fluorescent oxidized product was measured by flow cytometry (FACScan, Becton-Dickinson).

Oxygen Uptake Studies

MIA PaCa-2 cells were harvested by trypsinization, washed in PBS, counted, and equal numbers of cells were resuspended in complete medium with or without drugs for the indicated time at 37°C. Oxygen consumption (Biological Oxygen Monitor, Yellow Springs Instrument Company, Yellow Springs, OH) was measured

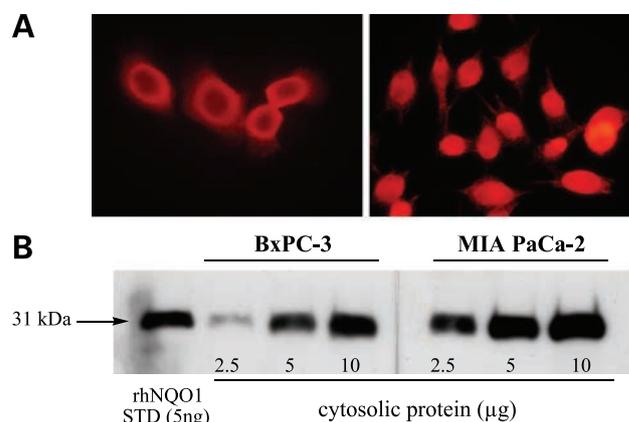


Figure 1. Immunodetection of NQO1 in human pancreatic cancer cells. **A**, immunocytochemical staining of NQO1 in BxPC-3 (*left*) and MIA PaCa-2 (*right*) using Texas red-conjugated secondary antibodies. **B**, immunoblot analysis of NQO1 in BxPC-3 and MIA PaCa-2 cells.

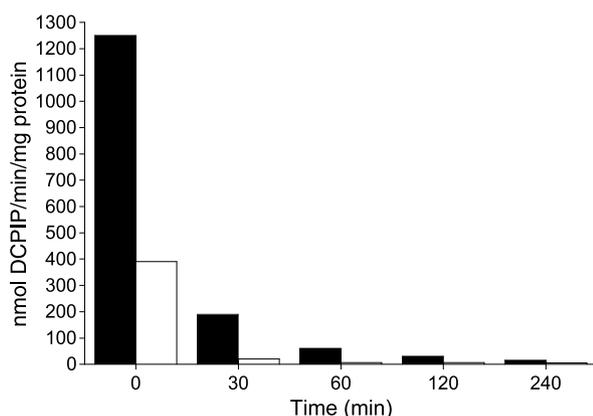


Figure 2. Inhibition of NQO1 catalytic activity in human pancreatic cancer cells by ES936. NQO1 catalytic activity (nmol 2,6-dichlorophenol-indophenol/min/mg protein) was measured in pancreatic cancer cell lines after treatment with ES936 (100 nmol/L) for the indicated time points. *Solid columns*, MIA PaCa-2 cells; *open columns*, BxPC-3 cells.

in complete medium with 5×10^6 cells in suspension at 37°C. The oxygen utilization was measured from 0 to 20 minutes and the rate of oxygen consumption was calculated from 5 to 15 minutes. Calculations of dissolved oxygen were corrected for temperature (37°C) and pressure (29.9 mm Hg).

Results

Immunocytochemical staining and immunoblot analysis of NQO1 in BxPC-3 and MIA PaCa-2 cell lines (Fig. 1) confirmed previous reports of high levels of NQO1 expression in these cell lines (10). We have shown that ES936 is an effective and potent inhibitor (>98% inhibition) of NQO1 in MIA PaCa-2 and BxPC-3 cells (Fig. 2). We have examined inhibition of NQO1 by ES936 in a number of different cell types (data not shown) and the kinetics of NQO1 inhibition depends on the amount of enzyme in cells (20). MIA PaCa-2 cells contain considerably greater amounts of NQO1 than BxPC-3 cells (see immunoblot, Fig. 1B) and thus require a longer incubation period at equimolar concentrations of ES936 to effectively inhibit NQO1.

The effect of ES936 on the viability of human pancreatic cancer cells was assayed using a variety of methods. Growth inhibition (MTT) was measured in BxPC-3 (Fig. 3A) and MIA PaCa-2 (Fig. 3B) cell lines following treatment with ES936. The ability to form colonies (clonogenic assay, Fig. 3C) and inhibition of growth in soft agar (anchorage-dependent growth, Fig. 3D) were also measured in MIA PaCa-2 cells following treatment with ES936. Results from these studies clearly showed that ES936 can induce growth inhibition, inhibit colony formation, and reverse the malignant phenotype of pancreatic tumor cells at concentrations of ES936 that result in near complete inhibition of NQO1. Finally, studies were then done using MIA PaCa-2 xenograft tumors implanted into nude mice (Fig. 4). For

these studies, ES936 was dosed at 5 mg/kg (i.p.) daily for 10 days, and then treatment was stopped. Tumor volumes were calculated from control and ES936-treated mice during drug treatment and for 10 days following cessation of therapy (Fig. 4A). The difference in growth rates between ES936-treated and DMSO-treated control tumors was significant (Fig. 4B, *left*). In addition, inhibition of tumor growth rates by ES936 was significantly greater during treatment (days 1–10; Fig. 4B, *left*) when compared with the same tumors after cessation of ES936 therapy (days 11–20; Fig. 4B, *right*). The maximal T/C value based on tumor volume analysis was 47%.

It has previously been reported that treatment of human pancreatic cell lines with the NQO1 inhibitor dicumarol resulted in increased production of intracellular superoxide (10). In similar experiments, we compared intracellular production of superoxide in MIA PaCa-2 cells treated with either dicumarol or ES936 (Fig. 5). For these experiments, cells were treated with the NQO1 inhibitor dicumarol or ES936 for 4 hours, followed by the addition of dihydroethidium. The redox-cycling quinone 2,3-dimethoxy-1,4-naphthoquinone was included separately as a positive control. Results from these experiments confirmed previous reports (10) that dicumarol treatment significantly increased the intracellular production of superoxide in MIA PaCa-2 cells. In our studies, however, no increased intracellular production of superoxide was detected following treatment with ES936 (Fig. 5) under conditions that resulted in >98% inhibition of NQO1. Oxygen consumption studies (Clark electrode) using intact MIA PaCa-2 cells revealed that treatment with dicumarol resulted in an immediate >3-fold increase in oxygen consumption in MIA PaCa-2 cells (Table 1; Fig. 6A). In the same experiments, no increase in oxygen consumption was observed in MIA PaCa-2 cells treated with ES936. After 1 hour of treatment, a significantly higher rate of oxygen consumption was measured in dicumarol-treated cells compared with untreated controls (Table 1; Fig. 6B). There was no increase in the rate of oxygen consumption in cells treated with ES936 at 30 minutes or 1 hour (Table 1; Fig. 6B). In these experiments, the rate of oxygen consumption in untreated controls was inhibited by >90% after the addition of 1 mmol/L KCN (Fig. 6A), confirming that, in these experiments, O₂ consumption was due primarily to cellular respiration.

Discussion

We have previously examined the inhibitory effects of ES936 on NQO1 in breast (20), colon (18, 20), and bone marrow endothelial cells.⁴ Data presented in this manuscript clearly show that the mechanism-based inhibitor ES936 will effectively inhibit NQO1 activity and inhibit cell growth in human pancreatic tumor cells. Cullen et al. (10) showed that dicumarol, a reversible competitive inhibitor of NQO1, induced growth inhibition in human pancreatic

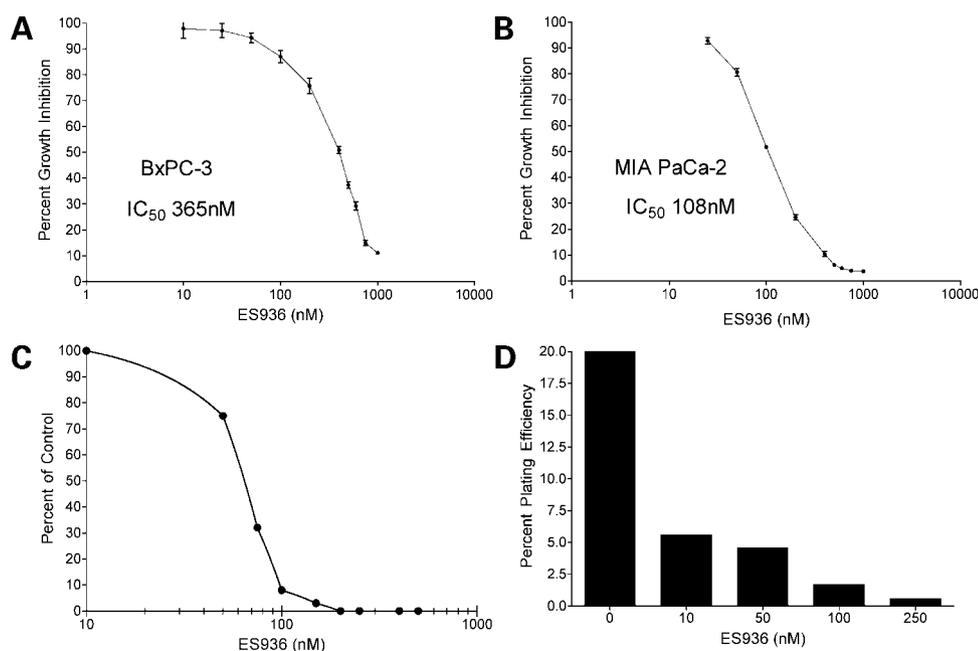


Figure 3. Effect of ES936 on growth inhibition, colony formation, and anchorage-dependent growth in human pancreatic cancer cells. Growth inhibition (MTT) was measured in BxPC-3 cells (A) and MIA PaCa-2 cells (B) following 72-h treatment with ES936. Colony forming ability (C) and anchorage-dependent growth (D) were measured in MIA PaCa-2 cells following continuous treatment with ES936.

cancer cells. Dicumarol is known to have many ancillary effects other than inhibition of NQO1 and its growth inhibitory effects may be unrelated to modulation of NQO1 activity (13–17).

In view of the relative nonspecificity of dicumarol as an inhibitor of NQO1, the development of ES936 as a mechanism-based inhibitor of NQO1 provided an excellent tool to test the hypothesis that NQO1 inhibitors might be useful agents in the therapy of pancreatic cancer. We found that ES936 was effective in inhibiting the growth of pancreatic tumor cells *in vitro* (MTT assay) in both MIA PaCa-2 and BxPC-3 pancreatic cancer cell lines. Additional experiments with MIA PaCa-2 cells showed that ES936 inhibited colony formation (clonogenic assay) and was able to inhibit the *in vitro* malignant phenotype (growth in soft agar) at low nanomolar concentrations. The *in vitro* data were reinforced by *in vivo* experiments in the MIA PaCa-2 xenograft system. Despite the use of a non-optimized dosing protocol, we showed that ES936 (5 mg/kg/d) was able to slow the growth of pancreatic cancer cells when compared with controls. When treatment with ES936 was stopped, the growth rate of tumors reverted to control levels, indicating that ES936 required continuous exposure to exert its inhibitory effects.

Cullen et al. (10) showed that dicumarol increased superoxide production in pancreatic tumors and our own data showed that high levels of NQO1 may scavenge superoxide (11). An attractive mechanism was proposed whereby treatment with NQO1 inhibitors results in elevated levels of superoxide, which induced toxicity and growth inhibition in pancreatic cancer cells (10, 12). In our studies, we also detected increased levels of superoxide in MIA PaCa-2 cells treated with dicumarol but we were unable to detect elevated levels of superoxide after

treatment with ES936. Our inability to detect an increase in intracellular superoxide production following ES936 treatment may be due to the sensitivity of the assay; however, such low levels of superoxide production would not be expected to induce toxicity via oxidative stress. Alternatively, superoxide is an important signaling molecule and Cullen et al. (27) have shown that pancreatic cancer cells are markedly sensitive to redox changes; thus, it remains a possibility that low fluxes of superoxide may be involved in activating growth inhibitory pathways in pancreatic tumor cells.

The ability of dicumarol to increase superoxide production in pancreatic cancer cells may be unrelated to inhibition of NQO1. Cullen et al. (10) showed that the transfection of pancreatic cancer cell lines with manganese superoxide dismutase greatly inhibited the production of superoxide and cytotoxicity induced by dicumarol. Because manganese superoxide dismutase is localized primarily in mitochondria, dicumarol could be targeting the mitochondria directly. Interestingly, dicumarol has been used as a mitochondrial uncoupling agent for almost 50 years (17). Studies in isolated mitochondria (28) and cells (29) have shown that treatment with dicumarol resulted in increased rates of respiration. Our studies showed that treatment of MIA PaCa-2 cells with dicumarol resulted in an immediate 3-fold increase in oxygen consumption and >2-fold increase in intracellular superoxide production compared with untreated controls. The disruption of mitochondrial homeostasis by a wide variety of compounds, including uncoupling agents, has been shown to lead to increased levels of intracellular superoxide (30, 31). However, treatment of pancreatic cells with ES936 at a concentration that resulted in essentially complete inhibition of cellular NQO1 did not affect the rate of oxygen consumption or

intracellular superoxide production, suggesting a different mechanism of action. One interpretation of these data is that dicumarol may be acting primarily as a mitochondrial uncoupling agent in MIA PaCa-2 cells while ES936 is targeting another pathway through NQO1.

The role of NQO1 in pancreatic cancer may not be related to superoxide detoxification but instead may reflect an alternative mechanism where NQO1 could regulate the function or stability of important signaling molecules through protein-protein interactions. We have previously shown that NQO1 can bind with p53 (32) and it has been reported that NQO1 can modulate the stability of p53 (33). Because the NAD(P)H binding site and quinone acceptor site overlap in NQO1, the adduction of ES936 into the active site will prevent further reduction of the FAD

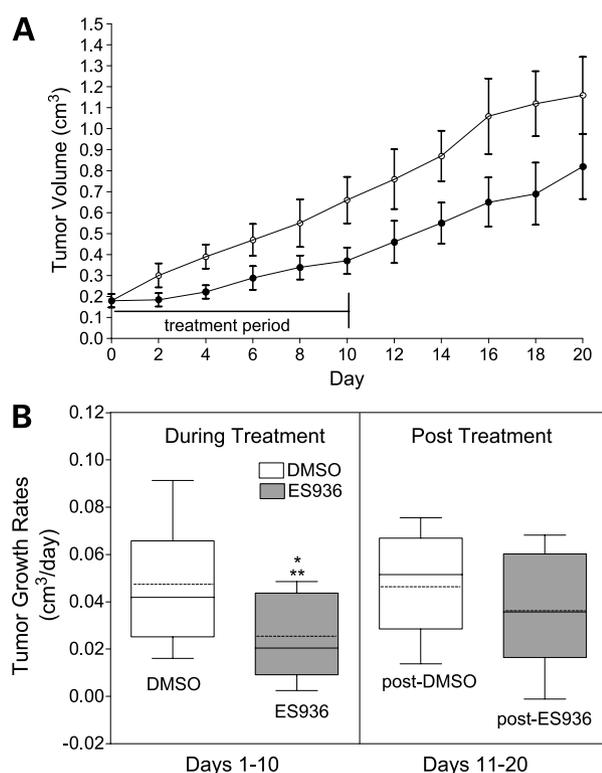


Figure 4. Inhibition of pancreatic xenograft tumor growth in nude mice following treatment with ES936. MIA PaCa-2 xenograft tumors were grown on the flanks of nude mice and then treated with ES936 (5 mg/kg i.p.) daily for 10 d. **A**, tumor volumes during and for 10 d after cessation of therapy. **B**, tumor growth rates were measured daily during ES936 treatment (days 1–10; left) and for 10 d following ES936 treatment (days 11–20; right). Tumor measurements were done as described in Materials and Methods. Values are presented as a box plot ($n = 10$ animals) with mean and median values represented by the dotted and solid middle lines. Top and bottom solid lines of the box plot represent 75% and 25%, respectively; error bars represent 95% and 5% of the data. Growth rates were calculated using linear regression analysis of tumor size versus days over the designated time period. *, $P < 0.02$, significantly different compared with control mice over days 1 to 10; **, $P < 0.004$, significantly different compared with ES936-treated group on days 11 to 20 (paired t test). Maximal T/C from a conventional tumor analysis was 47%.

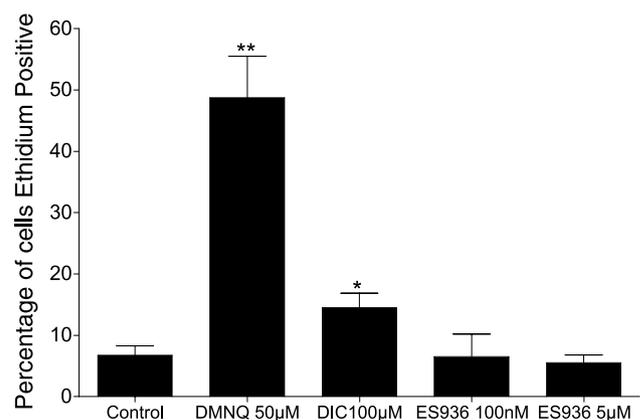


Figure 5. Effect of NQO1 inhibitors on intracellular superoxide production in human pancreatic cancer cells. Intracellular superoxide was measured in MIA PaCa-2 cells by following the oxidation of dihydroethidium after 4-h treatment with NQO1 inhibitors (dicumarol or ES936). The oxidation of dihydroethidium to an ethidium-like fluorescent product was measured using flow cytometry. The redox-cycling quinone 2,3-dimethoxy-1,4-naphthoquinone was used as a positive control for the generation of intracellular superoxide. *Columns*, mean of four separate determinations; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$, significantly different from untreated control (one-way ANOVA using Dunnett's multiple comparison test).

cofactor by NAD(P)H and the inactivated enzyme will reside permanently in the oxidized state. Molecular modeling studies have shown that the oxidized and reduced forms of NQO1 have different conformations (34). It is possible then that inactivation of NQO1 by ES936 inhibits transformation of NQO1 into a reduced conformation, preventing NQO1 from binding important signaling molecules. We are currently studying the effect of reduced and oxidized NQO1 in protein-protein interactions using immunoprecipitation and immunocytochemistry.

Taken together with previous work, our data suggest that inhibitors of NQO1 may be effective agents in the therapy of pancreatic cancer. Given the absence of a common mechanism of action involving superoxide production in

Table 1. Effect of NQO1 inhibitors on the rate of respiration in MIA PaCa-2 cells

Treatment	Time	O ₂ consumption*
Untreated	0	3.1 ± 0.2
Dicumarol 100 µmol/L	0	10.1 ± 0.3 [†]
ES936 100 nmol/L	0	3.5 ± 0.3
Untreated	1 h	2.6 ± 0.5
Dicumarol 100 µmol/L	1 h	4.3 ± 0.5 [‡]
ES936 100 nmol/L	30 min	2.19 ± 0.8
ES936 100 nmol/L	1 h	2.9 ± 0.6

*nmol O₂/min/million cells; mean ± SD; $n = 3$ separate determinations.

[†] $P < 0.01$ compared with untreated, one-way ANOVA using Dunnett's multiple comparison test.

[‡] $P < 0.05$ compared with untreated (1 hour), one-way ANOVA using Dunnett's multiple comparison test.

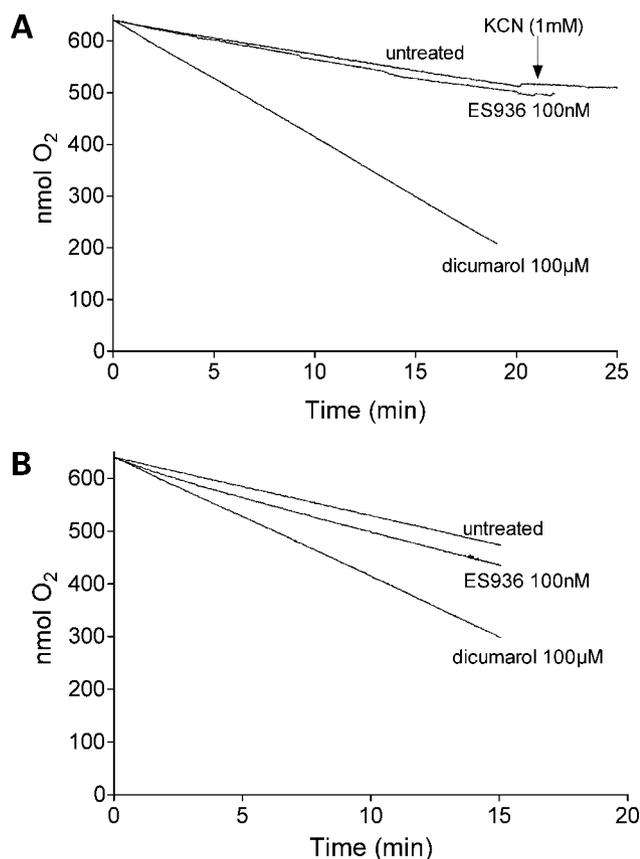


Figure 6. The rate of oxygen consumption in human pancreatic cancer cells following treatment with NQO1 inhibitors. Polarographic studies showing rate of oxygen consumption in MIA PaCa-2 cells in suspension in complete medium using a Clark electrode immediately (**A**) or 1 h (**B**) after the addition of NQO1 inhibitors.

experiments using dicumarol and ES936, however, the evidence linking NQO1 inhibition to pancreatic tumor growth inhibition remains correlative at best. It is still plausible that the effects of both dicumarol and ES936 in pancreatic cancer cells are unrelated to NQO1 inhibition. Caution should be used until a wide range of ES936 analogues with varying abilities to inhibit NQO1 are examined for their effect on intracellular superoxide production and their ability to inhibit the growth of pancreatic tumor cells.

In summary, we show that ES936 is an effective inhibitor of NQO1 in pancreatic tumor cells and is effective against pancreatic tumors both *in vitro* and *in vivo* although its mechanism of action seems to be independent of superoxide generation.

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5-Methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione, a mechanism-based inhibitor of NAD(P)H:quinone oxidoreductase 1, exhibits activity against human pancreatic cancer *in vitro* and *in vivo*

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