RESEARCH

Phenoxodiol Treatment Alters the Subsequent Response of ENOX2 (tNOX) and Growth of HeLa Cells to Paclitaxel and Cisplatin

D. James Morré · Nicole McClain · L.-Y. Wu · Graham Kelly · Dorothy M. Morré

Abstract Phenoxodiol is an experimental anticancer drug under development as a chemosensitizer intended to reverse multidrug resistance mechanisms in ovarian and prostate cancer cells to most standard cytotoxics. The putative molecular target of phenoxodiol is a cell-surface, tumor-specific NADH oxidase, ENOX2 (tNOX), with phenoxodiol having no apparent effect on the constitutive form of this enzyme ENOX1 (CNOX). Using ENOX2 as the target, this study was conducted to explore the temporal relationship between phenoxodiol and paclitaxel or cisplatin in achieving chemosensitization in HeLa cells which are relatively resistant to both paclitaxel and cisplatin. Sequential addition of phenoxodiol and paclitaxel or phenoxodiol and cisplatin showed greater inhibition of HeLa cell ENOX1 activity and growth compared to adding the drugs simultaneously or individually. In parallel, a similar chemosensitizing response of phenoxodiol for cisplatin was observed. ENOX1 was not affected and *trans*-platinum had no effect. With spent media from phenoxodiol-treated cells sensitivity was enhanced to both paclitaxel and cisplatin if the cells were first pretreated with phenoxodiol. Similar results were obtained with ENOX2-enriched preparations stripped from the surfaces of phenoxodiol-treated cells. In keeping with a speculative prion model, it seems as though the ENOX2 "remembers" the phenoxodiol and "teaches" other ENOX2 molecules to respond to paclitaxel and cisplatin as if phenoxodiol were still present.

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Introduction

Phenoxodiol [2H-1-benzopyran-7-0,1,3-(4-hydroxyphenyl)] is an investigational anticancer drug currently being studied in Phase II and Phase III clinical trials for its ability to reverse chemoresistance to platinum-based and taxane-based chemotherapies in platinum- and taxane refractory, late-stage ovarian cancer. Phenoxodiol is cytotoxic and cytostatic across a broad range of cancer cell types [1]. The mechanism of action is thought to be associated with the ability of phenoxodiol to prevent phosphorylation and activation of a number of different pro-survival signaling proteins including sphingosine kinase [2] and Akt, with inhibition of phosphorylation of antiapoptotic factors (e.g., XIAP, Flip_{short}) being key to induction of apoptosis via the Fas death receptor pathway [3]. Phenoxodiol is also a potent sensitizer of most standard chemotoxic agents including taxanes (paclitaxel, docetaxel), platinums (cisplatin, carboplatin), gemcitabine, topotecan, and etoposide, as well as reversing resistance in tumor cells to these same drugs.

The primary molecular target for phenoxodiol has been unknown, although we recently provided evidence that it is a cancer-specific and growth-related, cell-surface hydroquinone [NAD(P)H] oxidase with protein disulfide–thiol interchange activity, referred to as tumor-specific NADH oxidase tNOX (ENOX2) [4]. Phenoxodiol inhibits the

tumor-specific ENOX2 without any effect on the constitutive NADH oxidase ENOX1 (CNOX) [4], providing an explanation for the drug's apparent high specificity in vitro and lack of significant toxicity in vivo [3].

The clinical development of phenoxodiol as a chemosensitizer of drugs such as taxanes in tumors that have become refractory to such drugs is based on the assumption that phenoxodiol initiates a biochemical change within the cell that either reverses or overrides chemoresistance. The build-up of NADH in the cytoplasm of tumor cells as a consequence of inhibition of plasma membrane electron transport [4], and the potential effects of that buildup on a wide range of biochemical processes is a rational explanation [2]. Consequently, the treatment regimen for the use of phenoxodiol in combination therapy was based traditionally on the delivery of both drugs simultaneously. That is, phenoxodiol was maintained in plasma at continuously steady-state levels, providing a 'preconditioning' effect on the tumor cell at the time that the second drug such as paclitaxel or carboplatin was administered [5].

The basis for this rationale came into question, however, with a clinical observation of patients in a Phase II study. In that study, patients with late-stage ovarian cancer that had relapsed following multiple therapies including taxanes were treated with phenoxodiol as a single-agent therapy until they showed disease progression. Following this, a number of patients were re-treated with paclitaxel as a form of salvage therapy at times varying from 5 to 25 days following the cessation of phenoxodiol therapy. Surprisingly, a high proportion of these patients subsequently showed both complete or partial responses to a considerably greater extent than might have been expected on the basis of recovery of taxane sensitivity with time [5]. Equally, it was difficult to rationalize that this represented a chemosensitizing effect of phenoxodiol given that the median time of phenoxodiol therapy was approximately 20 times the half-life of the drug.

The purpose of this study was to determine if the clinical observation of persistent chemosensitization by phenoxodiol even if the drug was no longer present could be replicated in vitro, and to determine if an interaction between phenoxodiol and ENOX2 (tNOX) might provide a rational explanation for this effect.

Materials and Methods

Materials

Phenoxodiol was from Marshall Edwards, Inc, 140 Wicks Road, North Ryde, Australia. All other chemicals were from Sigma or from suppliers indicated.

Growth of Cells

HeLa (ATCC CCL-2) (human cervical adenocarcinoma cells) were cultured in minimal essential medium (Eagle), with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/l sodium bicarbonate, 0.2 mM nonessential amino acids, 1.0 mM sodium pyruvate, and supplemented with 10% bovine calf serum (heat-inactivated) plus 50 μg/l gentamycin sulfate. Cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C. Media were renewed every 2–3 days.

Preparation of ENOX2-Enriched Spent Media

HeLa cells were grown for 24 h in the presence of 1 μM phenoxodiol. The media were removed, the cells were washed, and fresh media lacking phenoxodiol were added. After three additional days of growth during which the phenoxodiol-exposed ENOX2 molecules were shed into the media, the media were collected for addition to fresh cells, and control media were collected in parallel but from cells not exposed to phenoxodiol. The spent media were added to the fresh HeLa cells in the following week after 48 h storage at −40°C. After an additional 48 h, the cells were treated with 0.025 μM paclitaxel or 2.5–10 μM cisplatin and growth was measured 48 h later. The experiments were in duplicate with good agreement.

Spectrophotometric Assay of NADH Oxidase

NADH oxidase (ENOX) activities were determined from the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-MES (pH 7.2), 1 mM KCN to inhibit mitochondrial oxidase activity, and 150 μM NADH, 1 μM GSH, 0.03% H_2O_2 at 37°C with temperature control (±0.5°C) and stirring [6]. Activities were measured using paired Hitachi U3210 spectrophotometers. Assays were initiated by the addition of enzyme source. Assays were for 1 min and were repeated on the same sample every 1.5 min for the times indicated. A millimolar extinction coefficient of 6.22 was used to determine specific activity.

Since NADH is an impermeant substrate, only the cell surface activity was measured with external NADH as substrate. The inhibitor capsaicin (1 μM) was added near the end of the assay to distinguish the drug-resistant ENOX1 form of the activity from the tumor-specific drug-inhibited ENOX2 form of the activity.

Protein disulfide–thiol interchange was determined spectrophotometrically from the activation of scrambled and inactive RNase [7] or from the cleavage of an artificial dithiodipyridine substrate [8]. Hydroquinone oxidase activity was estimated according to Kishi et al. [9] with

reduced coenzyme Q_{10} (Tishcon, Westbury, NY) as the substrate.

Proteins were estimated by the bicinchoninic acid method [10]. Bovine serum albumin was the standard.

Growth Measurements

HeLa cell growth was determined using a 96-well plate assay in which cells were fixed by glutaraldehyde and stained with 1% aqueous crystal violet. The absorbance was determined at 580 nm using an automated plate reader.

Chemosensitization

To assay for chemosensitization, HeLa cells were first exposed to phenoxodiol for the times indicated after which the phenoxodiol was removed by repeated washing. Paclitaxel or cisplatin were then added and subsequent growth was compared to that of the cells where either the phenoxodiol was not removed or the cells were not treated with phenoxodiol.

Transmission of Chemosensitizing Effect of Phenoxodiol (Prion-Like Mechanism)

Prions are infectious agents implicated in mammalian neurodegenerative diseases referred to generally as transmissible spongiform encephalopathies. Infectivity is associated with aberrant conformations of host-encoded proteins induced by the prion itself in an autocatalytic process [11]. The self-sustaining autocatalytic propagation of the infective state appears to involve a model whereby these proteins structurally encode and stably store information and which, through 'learning and teaching', are able to sustain and propagate their altered structural states imparted by external stimuli. In experiments to test the above prion model as a basis for phenoxodiol-induced chemoresistance, spent media containing ENOX molecules shed from untreated HeLa cells or HeLa cells pretreated with phenoxodiol were collected and added back to cells not previously exposed to phenoxodiol. Alternatively, ENOX preparations released from the surface of phenoxodiol-treated cells, extensively washed to remove traces of phenoxodiol, or phenoxodiol-untreated cells were tested in parallel.

Equilibrium Binding

Paclitaxel-2(2-Benzoyl-Ring-UL-^{14}C) (52.3 mCi/mmol) was obtained from Sigma (St. Louis, MO) and diluted with ethanol. For binding studies, a multicell rotating Teflon cell equilibrium dialyzer (Spectrum Equilibrium Dialyzer, Spectrum Medical Instruments, Los Angeles, CA) with a

dialyzing volume of 1 ml and a 47 mm diameter membrane (12–14 kDa molecular mass cutoff) was used as described [12]. The dialysis membranes were prepared by soaking in water for 30 min followed by 30% ethanol for 30 min and several changes of distilled water. Both sides of the chamber were supplied with 0.5 ml final volume of 50 mM Tris–HCl. To one side of the chamber the pactitaxel in ethanol and the fraction to be evaluated were added. An equivalent amount of ethanol was added to the opposite side of the chamber and an equilibrium was established by rotating the cells at about 100 rpm at 25°C overnight or longer as indicated. A 100 μl sample was withdrawn from each chamber and radioactivity was determined by liquid scintillation spectrometry. Radioactivity was determined at the 99% confidence level. Determinations were in triplicate to increase the confidence of specific high-affinity binding to approximately ±5%. Experiments were in duplicate.

Two-Dimensional Gel Electrophoresis and Western Blots

The presence of both ENOX1 and ENOX2 in spent media of HeLa cells was verified by two-dimensional (2D) gel electrophoresis and Western blots with ENOX1-specific [13] and ENOX2-specific [14] antibodies.

The ENOX2 proteins were first enriched and concentrated from the culture media by binding to nickel-agarose and then eluting. After release of the proteins, the samples were electrophoresed in the first dimension by using a commercial flatbed electrophoresis system (Ettan IPGphor 3, Amersham-Pharmacia Biotech) with IPG dry strips (Amersham) as described [14]. A linear pH range of 3–10 on 7 cm IPG strips was used. The IPG strips were rehydrated with the samples overnight at room temperature. The strips were focused at 50 mA per strip and at constant voltage of 300 V for 15 min, 600 V for 30 min, and 1,000 V for 1 h. The samples were then electrophoresed at an increasing voltage to 4,000 V for 2 h. Finally, the strips were focused at a constant 4,000 V for 28,000 volt-hours. After isoelectric focusing, the IPG strips were re-equilibrated for 30 min in 2.5% (w/v) SDS, 6 M urea, 30% (v/v) glycerol, and 100 mM Tris–HCl (pH 8.8). The strips were placed onto linear SDS-PAGE gels (10% [w/v] polyacrylamide) and electrophoresed at a constant 250 V for 80 min. The samples were then transferred to nitrocellulose membranes by electroblotting using the Bio-Rad Trans-Blot Electrophoretic Transfer Cell. The membranes were blocked using milk protein (5% low-fat dry milk) at room temperature for 1 h. Detection was with an ENOX2-specific recombinant single-chain variable region antibody (scFv) carrying an S-tag (overnight at 4°C) followed by alkaline phosphatase-linked anti-S (Novogen cat. #69598-3) and, after washing, detection with Western Blue

nitrotetrazolium (NBT) substrate (Promega, Madison, WI, cat. # S3841) for 5–10 min at 4°C.

Results

Effect on ENOX1 (CNOX) and ENOX2 (tNOX)

HeLa cells contain both the constitutive ECTO-NOX form ENOX1 (CNOX) and a cancer-related ECTO-NOX form ENOX2 (tNOX). In this study, ENOX1 activity proved to be resistant to phenoxodiol, cisplatin, and paclitaxel at concentrations as high as 100 μM. In contrast, ENOX2 activity of HeLa cells was inhibited by cisplatin with an EC_{50} of approximately 0.1 μM, and was somewhat more resistant to paclitaxel with an EC_{50} of >1 μM. When assayed under reducing conditions, the EC_{50} for inhibition of ENOX2 by phenoxodiol was 200 nM (Table 1).

Following a phenoxodiol washout, inhibition by phenoxodiol was no longer evident (i.e., the drug is reversibly bound). However, addition of either paclitaxel or cisplatin to the phenoxodiol pretreated and washed cells resulted in an enhanced inhibition with the EC_{50} to cisplatin and paclitaxel being reduced to <0.05 μM and that for paclitaxel to <0.5 μM (Table 1). ENOX1 activity remained unresponsive to both drugs.

The two drugs added together or cisplatin or paclitaxel added after phenoxodiol in series with the phenoxodiol still present did not show chemosensitization and they frequently were antagonistic (Table 2).

Temporal Effect of Phenoxodiol and Drugs on Cell Survival

The above findings were reproduced in growth experiments where sequential application of phenoxodiol followed by paclitaxel gave augmented growth inhibition whereas phenoxodiol pretreatment followed by simultaneous addition of paclitaxel was antagonistic (Table 2). Results with cisplatin were similar (Table 3).

Table 1 Response of ENOX2 activity of HeLa cells under reducing conditions (in the presence of 100 μM GSH) to phenoxodiol and cisplatin alone or in combination

Drug	EC_{50} (nM)
Phenoxodiol	200
Paclitaxel	>1000
Cisplatin	100
Trans-platinum	>1000
Pretreat with phenoxodiol, wash to remove drug, and then add cisplatin	<50
Pretreat with phenoxodiol, wash to remove drug, and then add paclitaxel	<500

In the 96-well plate assay of Table 4 comparing two different concentrations of phenoxodiol, phenoxodiol and cisplatin added together were antagonistic. However, phenoxodiol treatment followed by washout and then cisplatin resulted in greater inhibition than with cisplatin alone.

Transmissability of Chemosensitizing Effect of Phenoxodiol

Spent media free of phenoxodiol, prepared as described in section "Methods", was added to fresh HeLa cells and the cells were treated with or without 0.025 μM paclitaxel after 48 h (Fig. 1). Growth measured 48 h later was inhibited by 45%. In contrast, when added to cells grown in media from control cells, growth was inhibited by only 20%. Paclitaxel (0.025 μM) added to the fresh media inhibited the growth by about 5% (Fig. 1).

A smaller but still statistically significant response to cisplatin was seen as a result of incubating HeLa cells in spent media from cells previously exposed to phenoxodiol (Fig. 2). In contrast to data of Fig. 1 for paclitaxel, the effect was clearly evidenced only in the slope of the dose–response curves. The addition of spent media from the previously phenoxodiol-treated cells to HeLa cells not previously exposed to phenoxodiol reduced the EC_{50} of cisplatin inhibition from 10 to 7.5 μM (Fig. 2).
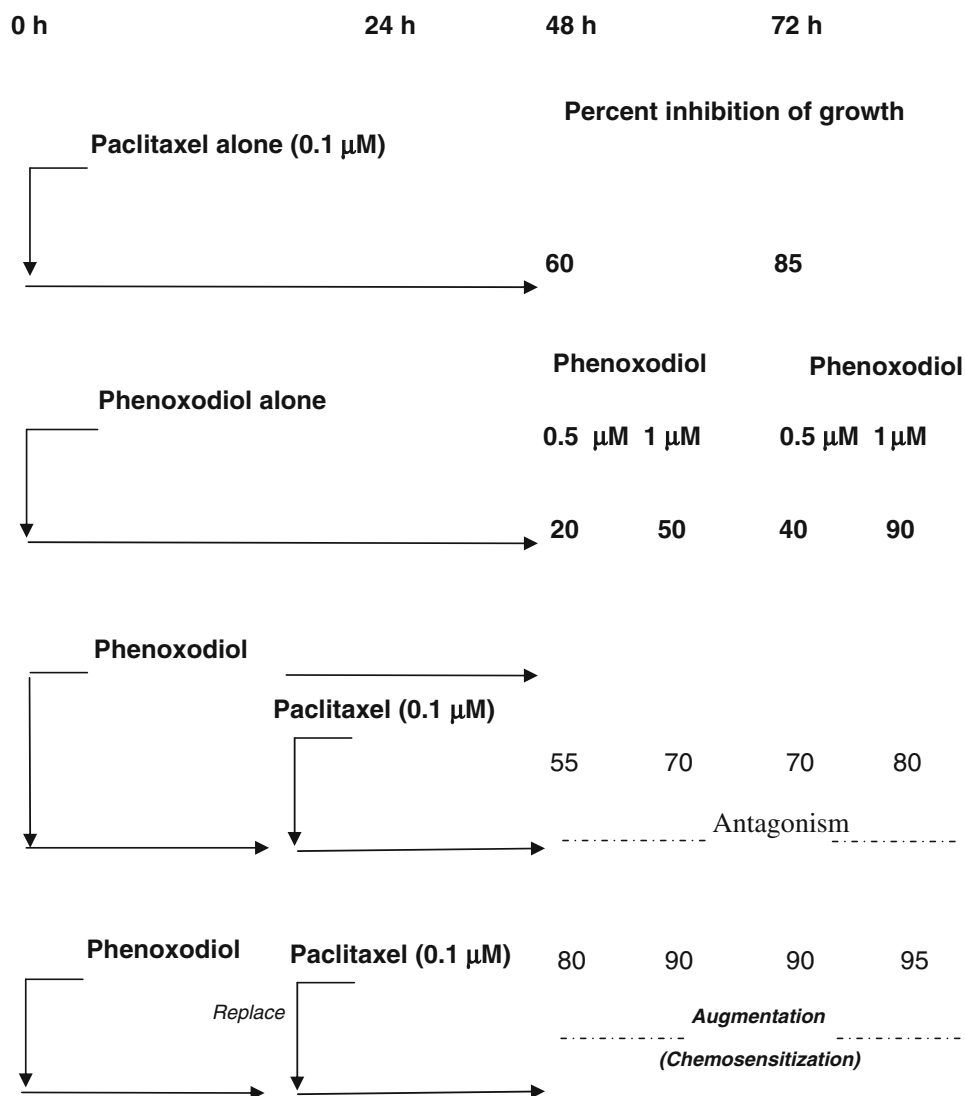
The shed forms of both ENOX1 (Fig. 3a) and three isoforms of differing isoelectric point of the 80 kDa cervical cancer-specific shed form of ENOX2 (Fig. 3b) were present in the spent media. Also present was the 34 kDa fully processed ENOX2 isoform as verified by antibody localization on one-dimensional SDS-PAGE (not shown).

These results were subsequently extended to ENOX2-enriched preparations released from the surface of phenoxodiol-pretreated HeLa cells by the method of del Castillo et al. [15] (abbreviated Cellex) and then added back to cells not having been treated with phenoxodiol. The differences, while small, were significant, and do indicate some form of transmissible response to the phenoxodiol affecting both the response to paclitaxel (Fig. 4) and to cisplatin (Fig. 5).

The ENOX2 preparation released from HeLa cells (control Cellex) was, of itself, inhibitory to the growth of HeLa cells (Figs. 4 and 5). Addition of 0.04 μM paclitaxel resulted in a further inhibition of about 12% (Fig. 4). The ENOX2 preparation released from HeLa cells treated with phenoxodiol and then extensively washed and recultured for 48 h (phenoxodiol Cellex) elicited an even greater inhibition and addition of 0.04 μM paclitaxel inhibited the growth by a further 20%.

With 1 μM cisplatin, there was only a small effect of the addition of control Cellex on inhibition (Fig. 4). However, with phenoxodiol Cellex, there was an enhanced inhibition by 1 μM *cis*-platinum of 10% (Fig. 5).

Table 2 Response of cultured HeLa cells to paclitaxel under different conditions of phenoxodiol treatment

0 h	24 h	48 h	72 h
		Percent inhibition of growth	

Paclitaxel alone (0.1 μM)

60   85

		Phenoxodiol	**Phenoxodiol**

Phenoxodiol alone

		0.5 μM	1 μM	0.5 μM	1 μM
		20	50	40	90

Phenoxodiol

Paclitaxel (0.1 μM)

55   70   70   80

Antagonism

Phenoxodiol   **Paclitaxel (0.1 μM)**   80   90   90   95

Replace

Augmentation

(Chemosensitization)

Simultaneous phenoxodiol + paclitaxel was antagonistic whereas phenoxodiol followed by paclitaxel was synergistic

Paclitaxel Binding

We were unable to demonstrate binding of paclitaxel either to recombinant ENOX2, to the HeLa cell surface, to isolated HeLa plasma membranes, to isolated HeLa microsomes, or to ENOX2 released from the surface of HeLa cells by low pH according to del Castillo et al. [15] (Cellex). However, if isolated microsomes enriched in HeLa plasma membranes were incubated with phenoxodiol for longer periods of time, the membranes gradually began to show specific paclitaxel binding in a time-dependent manner (Table 5). After 2 days of incubation, binding with a kDa of 20 μmol was observed. After 4 days of incubation, binding with a kDa of 1.9 ± 0.1 μmol was observed.

During this time, the relative number of binding sites (n) increased from 0.04 to 0.07 μmol per 150 μl of microsomes (75 μg protein).

Discussion

Chemosensitization is a relatively new concept in cancer management, but one having exceptional promise to restore drug sensitivity in drug-resistant patients. Our work has focused on the ENOX proteins with particular emphasis on ENOX2 which is the drug target for the experimental isoflavene chemosensitizer phenoxodiol. The principal significance of the work rests in the development of a

Table 3 Response of cultured HeLa cells to cisplatin under different conditions of phenoxodiol treatment

0 h	24 h	48 h	72 h

Cisplatin alone (1 μM)

Percent inhibition of growth

40   75

Phenoxodiol alone

		Phenoxodiol		Phenoxodiol	
		0.5 μM	1 μM	0.5 μM	1 μM
		20	50	40	90

Phenoxodiol

Cisplatin (1 μM)

35   75   60   90

Antagonism

Phenoxodiol   **Cisplatin (1 μM)**   45   85   85   100

Replace

Augmentation

(Chemosensitization)

Simultaneous phenoxodiol + cisplatin was antagonistic whereas phenoxodiol followed by cisplatin was synergistic

model to explain phenoxodiol chemosensitization of drug-resistant cancers to taxanes and cisplatins.

ENOX2 is a cancer-specific form of a family of growth-related and time-keeping proteins of the cell surface [16] with both hydroquinone or NADH oxidation and protein disulfide–thiol interchange activities (ENOX proteins) [17] that responds to several known or suspected quinone-site inhibitors, all possessing anticancer activity [18]. ENOX2 inhibitors include, in addition to phenoxodiol [4], (−)-epigallocatechin-3-gallate [19], capsaicin [20], adriamycin [21], and the antitumor sulfonylureas [22]. All inhibit both the NADH oxidation and protein disulfide–thiol interchange activities of ENOX2 with no effect on either activity of ENOX1 [18, 23] or growth of noncancer cells as

the basis for their cancer specificity. The synthetic isoflavenes inhibit both NADH and hydroquinone oxidation [4] as well as the protein disulfide–thiol interchange. However, inhibition of the protein disulfide–thiol interchange portion of the cycle occurs incrementally over a span of about 60 min to suggest that the isoflavenes interact in a unique manner with ENOX2 in its conformational state that carries out protein disulfide–thiol interchange [4]. This is the portion of the ENOX cycle that drives cell enlargement such that an eventual cessation of growth is the result [4] followed several hours later by apoptosis [3].

Normally both ENOX1 and ENOX2 activities of HeLa cells with NADH as a substrate do not respond to paclitaxel (Table 1). However, a response to paclitaxel was seen, if

Table 4 Response of cultured HeLa cells to phenoxodiol alone, cisplatin alone, and the two in combination after 48 and 72 h

Treatment	% Inhibition of growth	
	48 h	72 h
Phenoxodiol alone (μM)		
0.5	22 ± 5	21 ± 6
1	40 ± 5	45 ± 9
Cisplatin alone (μM)		
1	40 ± 6	60 ± 6
Phenoxodiol + cisplatin (μM)		
0.5 + 1	26 ± 4	43 ± 7
1 + 1	32 ± 7	50 ± 4
Phenoxodiol, remove after 48 h and replace with cisplatin (μM)		
0.5 + 1	60 ± 4**	71 ± 2**
1 + 1	74 ± 11**	88 ± 6**

** Significantly different from cisplatin alone $P < 0.001$

Fig. 2 Growth of HeLa cells in culture and effect of media from untreated (control) or phenoxodiol-treated cells on subsequent response to cisplatin. The cells were grown and treated with phenoxodiol as in Fig. 1. Following phenoxodiol treatment, the media were removed and the cells were washed twice with fresh media and fresh media was added to the wells. After 3 days of growth, the media were collected, stored frozen for 48 h, and then added to fresh HeLa cells. After 48 h of growth, 2.5, 5, or 10 μM cisplatin were added and growth was measured after an additional 48 h of growth. Control media was prepared exactly in parallel from cells not exposed to phenoxodiol. The *solid symbols* were media from untreated HeLa cells and the *open symbols* were media from cells pretreated with 1 μM phenoxodiol. The EC_{50} for cisplatin was shifted from 10 μM for the untreated cells to about 7.5 μM for the media from the phenoxodiol-treated cells

ENOX2 was reversibly inhibited by the addition of 10 μM phenoxodiol, and the activity returned to its original level after phenoxodiol was removed. With simultaneous phenoxodiol and paclitaxel addition, paclitaxel inhibition was diminished. Augmentation of cisplatin or paclitaxel inhibition of ENOX2 activity by phenoxodiol pretreatment required that the phenoxodiol be removed prior to cisplatin or paclitaxel addition. With sequential phenoxodiol–paclitaxel treatment, ENOX2 activity was inhibited completely by paclitaxel. Phenoxodiol treatment followed by addition of paclitaxel to the phenoxodiol-containing suspension also resulted in paclitaxel inhibition but only to a level approximately 50% of that where the phenoxodiol was washed out prior to paclitaxel addition. Again ENOX1 activity was unaffected. These findings show chemosensitization of ENOX2 by sequential addition of phenoxodiol and paclitaxel to a much greater degree than when the two drugs were presented simultaneously. These findings parallel results from growth experiments where sequential application of phenoxodiol followed by paclitaxel gave augmented growth inhibition whereas phenoxodiol pretreatment followed by simultaneous addition of paclitaxel was antagonistic.

A similar pattern of chemosensitization was seen with cisplatin whereas *trans*-platinum was inactive. Cisplatin

Fig. 1 Growth of HeLa cells in culture and effect of media from untreated (control) or phenoxodiol-treated cells on subsequent response to 0.025 μM paclitaxel (taxol). The phenoxodiol concentration was 1 μM. HeLa cells plated in 96-well assay format were treated with 1 μM phenoxodiol for 24 h. The media were removed and the cells were washed twice with fresh media and fresh media was added to the wells. After 3 days of growth, the media were collected, stored frozen for 48 h, and then added to fresh HeLa cells. After 48 h of growth, 0.025 μM paclitaxel was added and growth was measured after an additional 48 h of growth. Control media was prepared exactly in parallel from cells not exposed to phenoxodiol

the cells were first treated with 10 μM phenoxodiol for 72 min, and then washed free of phenoxodiol. Under these conditions, addition of 1 μM paclitaxel inhibited ENOX2 activity but was without effect on ENOX1 activity. Thus, phenoxodiol treatment induced paclitaxel sensitivity in a previously paclitaxel-resistant ENOX2 conformer.
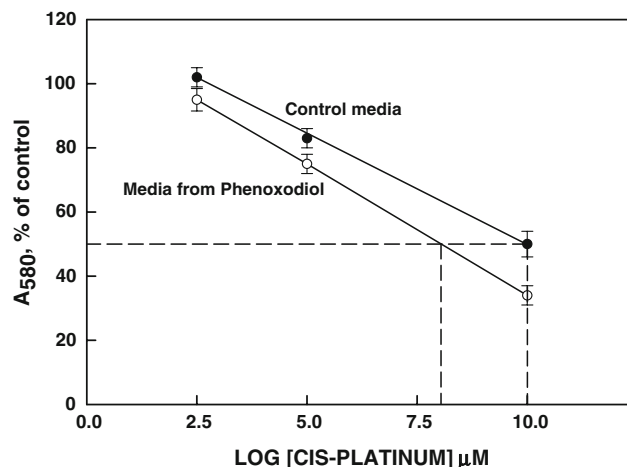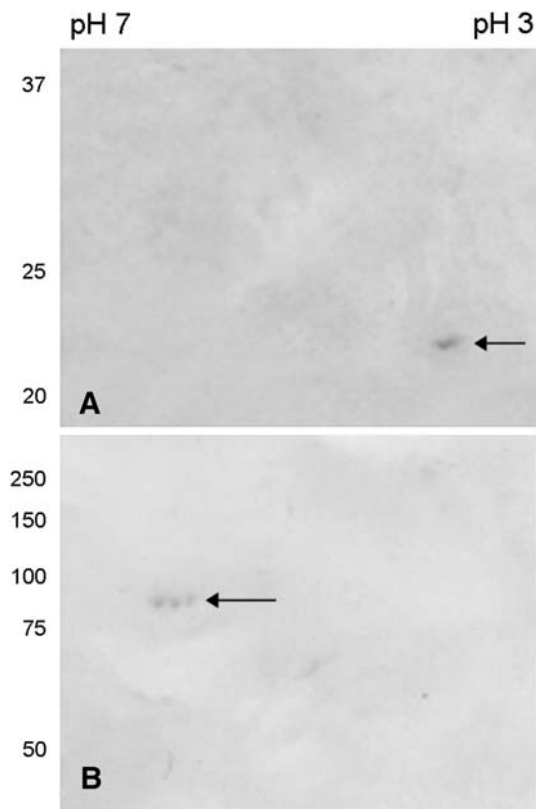
Fig. 3 Western blots of 2D gel electrophoretic separations of spent media [14] from cultured HeLa cells showing the presence of the 29 kDa fragment of ENOX1 normally shed into sera [13] (**a**, *arrow*) and three isoforms of the 80-kDa shed serum isoform of ENOX2 characteristic of cervical cancer [14] (**b**, *arrow*). Detection used human ENOX1-specific antisera (**a**, [13]) and a pan-ENOX2-specific recombinant antibody (**b**, [14])

Fig. 4 Response of HeLa cells in culture to a ENOX2-enriched fraction stripped from the HeLa cell surface by treatment at low pH by the method of del Castillo et al. [15] (Cellex) and response to 0.04 μM paclitaxel (taxol). The ENOX2-enriched fraction was, of itself, inhibitory to the growth of HeLa cells and elicited a stronger response to paclitaxel. HeLa cells were treated for 24 h with 1 μM phenoxodiol and washed to remove the phenoxodiol. The surface ENOX protein were removed by the low pH treatment and added to fresh cells in the amounts indicated. After 48 h of growth, 0.04 μM paclitaxel was added and growth was measured after an additional 48 h of growth

alone even in the absence of phenoxodiol inhibited the ENOX2 activity of HeLa cells. However, sequential phenoxodiol followed by cisplatin treatment resulted in greater inhibition than seen with cisplatin alone (Table 3).

The above findings with both paclitaxel and cisplatin establish a pattern of restoration of drug sensitivity to ENOX2 and growth inhibition as a result of phenoxodiol pretreatment without precedence in the cancer therapeutic literature. To explain the mechanism of ENOX2, chemosensitization may be the result of learning and teaching based on the prion model. Simply put, for platinum or paclitaxel resistance, the ENOX2 becomes modified in a way so that it does not respond to platinum or paclitaxel. The resistant ENOX2 proteins, having learned, then modify ("teach") incoming naïve ENOX2 molecules to be paclitaxel or platinum resistant. To overcome the resistance, the ENOX2 population requires relearning to become paclitaxel or platinum susceptible. Phenoxodiol seems to possess that capability.

The only effect on ENOX2 structure that we have been able to observe is a statistically significant shift ($P < 0.02$)
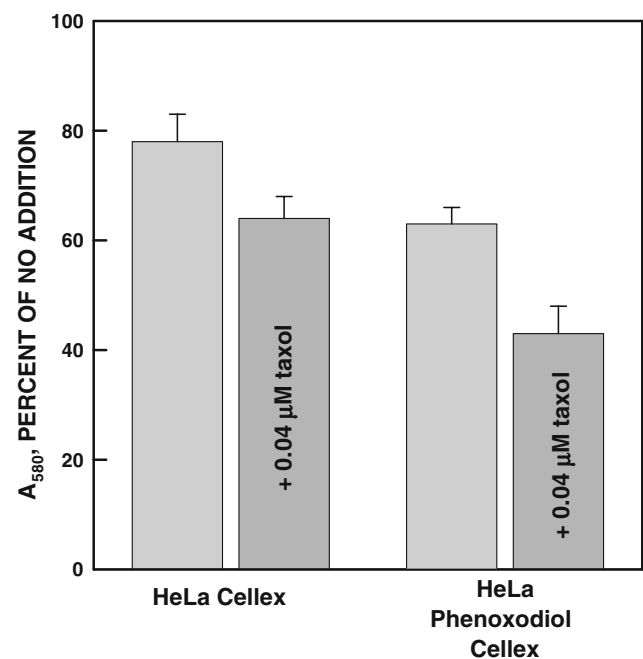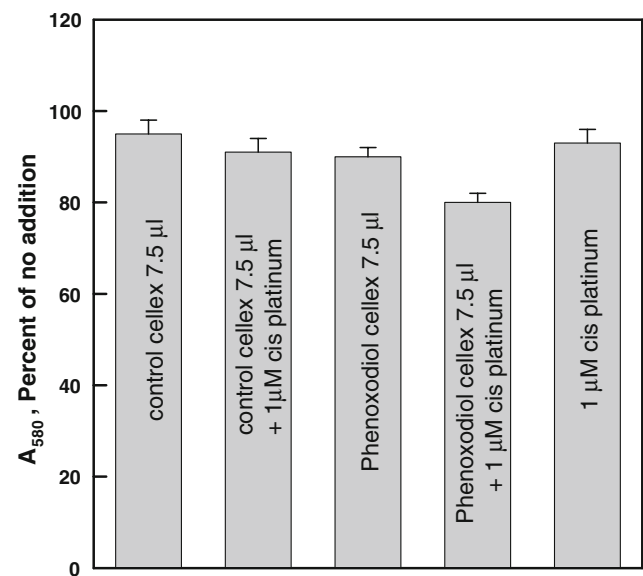

Fig. 5 As in Fig. 3 except response to 1 μM cisplatin. The response to phenoxodiol treatment followed by cisplatin (cis platinum) was significantly different from cisplatin alone ($P < 0.03$)

Table 5 Binding of paclitaxel to plasma membranes from HeLa cells

Days of incubation	kDa (μM)	N (μmol)[a]
0	>100	–
2	20 ± 5	0.4
4	19 ± 0.1	0.7

[a] Per 150 μl (75μg protein) of plasma membrane-enriched microsomes

in the isoelectric point of about 0.2 pH units upon ENOX2 addition. The ENOX1 protein does not respond to phenoxodiol; hence, responses may be attributed to ENOX2 which is the confirmed phenoxodiol target protein [4].

Prions in mammals reproduce by recruiting normal cellular isoforms of the prion protein and causing their conversion into the disease-causing isoform [24, 25] likely in some sort of template-assisted process. This process of learning and teaching offers a mechanism for autopropagation of conformational changes in response to an external signal such as phenoxodiol which is then functionally retained in succeeding generations long after the stimulus is removed. This type of memory-related process has been suggested as a more general mechanism extending beyond disease-causing prions [11, 26].

To test the prion model for phenoxodiol-induced chemosensitization, we have used tNOX-containing extracts from phenoxodiol-treated cells that, when added back to untreated cells, imparted a chemosensitization response. Thus, some degree of chemosensitization seems to have been achieved by merely adding back ENOX2 molecules that have been exposed to phenoxodiol but to which phenoxodiol is no longer bound. Such an observation is consistent with a prion-like process of learning and teaching. In support of this model is the observation that phenoxodiol chemosensitization is retained upon removal of the phenoxodiol and does not appear to be attenuated as the cells continue to grow and divide.

A similar situation has been reported for plants where an ENOX activity is activated by the synthetic plant growth auxin regulator, 2,4-dichlorophenoxyacetic acid (2,4-D) [27]. The 2,4-D-induced component of the ENOX activity is unregulated, resistant to proteinase K, and leads to death of the plants. After a period of time, nearly all of the ENOX activity of 2,4-D-treated stem tissues, including most of the ENOX1 activity, becomes unregulated and proteinase K resistant. To explain these observations, recruitment of ENOX1 proteins by the 2,4-D-activated ENOX (dNOX) proteins was postulated, followed by a conversion of the ENOX1 proteins into a likeness of the dNOX protein much in the manner as the creation of infective prion proteins from noninfective prion proteins by an interaction of one with the other [27]. 2,4-D-activated ENOX is postulated to recruit normal ENOX1 proteins and

cause them to undergo a permanent activation through a conformational alteration. The converted molecules are then considered to participate in the conversion of other ENOX1 proteins. The net result is a propagation of the 2,4-D response and a net loss of unconverted ENOX1 proteins. Eventually, an ENOX population is established of proteins largely refolded to resemble the 2,4-D-activated ENOX and an overall entrainment to produce predominantly a single 2,4-D-activated ENOX activity and growth response. Once the process is initiated by 2,4-D, the 2,4-D is no longer required for the subsequent propagation of the response leading to death of the plant.

We have previously reported that ENOX2 has many properties in common with prions (resistance to proteases, heat, and chemical degradation; unusual solubility characteristics; β-sheet character; and ability to form amyloid rods) [28]. ENOX2 also has the ability to impart proteinase K resistance to proteins that are normally susceptible to proteinase K digestion. This property, that of converting a normal form of a protein into a likeness of itself, is one of the defining characteristics of the group of proteins designated as prions [24, 25].

The basis for chemosensitization of tumor cells by phenoxodiol to cisplatin and paclitaxel [29] has been without explanation. The correlation throughout our work between the resistance of ENOX2 to paclitaxel and/or cisplatin and resistance of cell growth to paclitaxel and/or cisplatin is strongly suggestive of cause and effect. Similarly, the ability of phenoxodiol to impart sensitivity both to cisplatin and paclitaxel may be that of a prion-like mechanism of conformer formation discussed above. However, the effect of phenoxodiol in inducing paclitaxel sensitivity of ENOX2 at the HeLa cell surface has been more difficult to explain than the growth response. Somehow phenoxodiol must be altering the ENOX2 protein to render it paclitaxel sensitive. The simplest explanation would be that phenoxodiol treatment results in paclitaxel binding by ENOX2. This explanation is supported by our findings where microsome fractions enriched in plasma membranes acquire an ability to specifically bind paclitaxel when incubated for relatively long periods of time in the presence of phenoxodiol.

References

1. Brown, D. M., Kelly, G. E., & Husband, A. J. (2005). Flavanoid compounds in maintenance of prostate health and prevention and treatment of cancer. *Molecular Biotechnology, 30*, 253–270. doi: 10.1385/MB:30:3:253.
2. De Luca, T., Morré, D. M., Zhao, H., & Morré, D. J. (2005). NAD$^+$/NADH and/or CoQ/CoQH$_2$ ratios from plasma membrane electron transport may determine ceramide and sphingosine-1-

phosphate levels accompanying G$_1$ arrest and apoptosis. *BioFactors (Oxford, England), 25*, 43–60.

3. Kamsteeg, J., Rutherford, T., Sapi, E., Hanczaruk, B., Shahabi, S., Flick, M., et al. (2003). Phenoxodiol—an isoflavone analog—induces apoptosis in chemoresistant ovarian cancer cells. *Oncogene, 22*, 2611–2620. doi:10.1038/sj.onc.1206422.

4. Morré, D. J., Chueh, P.-J., Yagiz, K., Balicki, A., Kim, C., & Morré, D. M. (2007). ECTO-NOX target for the anticancer isoflavene phenoxodiol. *Oncology Research, 16*, 299–312. doi:10.1159/000102153.

5. Goss, G., Quinn, M., Rutherford, T., & Kelly, G. A. (2005). A randomized Phase II study of phenoxodiol with platinum or taxane chemotherapy in chemoresistant epithelial ovarian cancer, fallopian tube cancer and primary peritoneal cancer. *European Journal of Cancer* (Suppl. 3), 261.

6. Morré, D. J., & Morré, D. M. (2003). Spectroscopic analyses of oscillation in ECTO-NOX-catalyzed oxidation of NADH. *Nonlinearity in Biology Toxicology and Medicine, 1*, 345–362. doi:10.1080/15401420390249916.

7. Lyles, M. M., & Gilbert, H. F. (1991). Catalysis of the oxidative folding of ribonuclease A by protein disulfide isomerase dependence of the rate on the composition of the redox buffer. *Biochemistry, 3*, 613–619. doi:10.1021/bi00217a004.

8. Morré, D. J., Gomez-Rey, M. L., Schramke, C., Em, O., Lawler, J., Hobeck, J., et al. (1999). Use of dipyridyl-dithio substrates to measure directly the protein disulfide-thiol interchange activity of the auxin stimulated NADH, protein disulfide reductase of soybean plasma membranes. *Molecular and Cellular Biochemistry, 200*, 7–13. doi:10.1023/A:1006916116297.

9. Kishi, T., Morré, D. M., & Morré, D. J. (1999). The plasma membrane NADH oxidase of HeLa cells has hydroquinone oxidase activity. *Biochimica et Biophysica Acta, 1412*, 66–77. doi:10.1016/S0005-2728(99)00049-3.

10. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mailia, A. K., Gartner, F. H., Provenzano, M. D., et al. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry, 150*, 76–85. doi:10.1016/0003-2697(85)90442-7.

11. Tompa, P., & Friedrich, P. (1998). Prion proteins as memory molecules: A hypothesis. *Neuroscience, 86*, 1037–1043. doi:10.1016/S0306-4522(98)00148-1.

12. Morré, D. J., Morré, D. M., Stevenson, J., MacKellar, W., & McClure, D. (1995). HeLa plasma membranes bind the antitumor sulfonylurea LY181984 with high affinity. *Biochimica et Biophysica Acta, 1244*, 133–140.

13. Sedlak, D., Morré, D. M., & Morré, D. J. (2001). A drug-unresponsive and protease-resistant CNOX protein from human sera. *Archives of Biochemistry and Biophysics, 386*, 106–116. doi:10.1006/abbi.2000.2180.

14. Hostetler, B., Weston, N., Kim, C., Morré, D. M., & Morré, D. J. (2008). Cancer site-specific isoforms of ENOX2 (tNOX), a cancer-specific cell surface oxidase. *Clinical Proteomics*. doi:10.1007/s12014-008-9016-x.

15. del Castillo-Olivares, A., Yantiri, F., Chueh, P.-J., Wang, S., Sweeting, M., Sedlak, D., et al. (1998). A drug-responsive and protease-resistant 34 kD NADH oxidase from the surface of HeLa cells. *Archives of Biochemistry and Biophysics, 358*, 125–140. doi:10.1006/abbi.1998.0823.

16. Morré, D. J. (1995). NADH oxidase activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea N-(4-methylphenylsulfonyl)-N'-(4-chlorophenyl)urea (LY181984) at an external site. *Biochimica et Biophysica Acta, 1240*, 201–208. doi:10.1016/0005-2736(95)00164-7.

17. Morré, D. J., Chueh, P.-J., Lawler, J., & Morré, D. M. (1998). The sulfonylurea-inhibited NADH oxidase activity of HeLa cell plasma membranes has properties of a protein disulfide-thiol oxidoreductase with protein disulfide-thiol interchange activity. *Journal of Bioenergetics and Biomembranes, 30*, 477–487. doi:10.1023/A:1020594214379.

18. Morré, D. J. (1998). NADH oxidase: A multifunctional ecto-protein of the eukaryotic cell surface. In H. Asard, A. Bérczi, & R. Caubergs (Eds.), *Plasma membrane redox systems and their role in biological stress and disease* (pp. 121–156). Dordrecht, The Netherlands: Kluwer Academic Publishers.

19. Morré, D. J., Bridge, A., Wu, L.-Y., & Morré, D. M. (2000). Preferential inhibition by (−)-epigallocatechin-3-gallate of the cell surface NADH oxidase and growth of transformed cells in culture. *Biochemical Pharmacology, 60*, 937–946. doi:10.1016/S0006-2952(00)00426-3.

20. Morré, D. J., Chueh, P.-J., & Morré, D. M. (1995). Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. *Proceedings of the National Academy of Sciences of the United States of America, 92*, 1831–1835. doi:10.1073/pnas.92.6.1831.

21. Morré, D. J., Kim, C., Paulik, M., Morré, D. M., & Faulk, W. P. (1997). Is the drug-responsive NADH oxidase of the cancer cell plasma membrane a molecular target for adriamycin? *Journal of Bioenergetics and Biomembranes, 29*, 269–280. doi:10.1023/A:1022414228013.

22. Morré, D. J., Wu, L.-Y., & Morré, D. M. (1995). The antitumor sulfonylurea N-(4-methylphenylsulfonyl)-N'-(chlorophenyl)urea (LY181984) inhibits NADH oxidase activity of HeLa plasma membrane. *Biochimica et Biophysica Acta, 1240*, 11–17. doi:10.1016/0005-2736(95)00164-7.

23. Morré, D. J., Sedlak, D., Tang, X., Chueh, P.-J., Geng, T., & Morré, D. M. (2001). Surface NADH oxidase of HeLa cells lacks intrinsic membrane binding motifs. *Archives of Biochemistry and Biophysics, 392*, 251–256. doi:10.1006/abbi.2001.2436.

24. Griffith, J. S. (1967). Self-replication and scrapie. *Nature, 315*, 1043–1044. doi:10.1038/2151043a0.

25. Prusiner, S. B. (1994). Biology and genetics of prion diseases. *Annual Review of Microbiology, 48*, 655–686. doi:10.1146/annurev.mi.48.100194.003255.

26. Robertson, E. D., & Sweatt, J. D. (1998). A biochemical blueprint for long-term memory. *Learning & Memory (Cold Spring Harbor, N.Y.), 6*, 381–388.

27. Morré, D. J., Morré, D. M., & Ternes, P. (2003). Auxin-activated NADH oxidase activity of soybean plasma membranes is distinct from the constitutive plasma membrane NADH oxidase and exhibits prion-like properties. *In Vitro Cellular & Developmental Biology. Plant, 39*, 368–376. doi:10.1079/IVP2003417.

28. Kelker, M., Kim, C., Chueh, P.-J., Guimont, R., Morré, D. M., & Morré, D. J. (2001). Cancer isoforms of a tumor-associated cell surface NADH oxidase (tNOX) has properties of a prion. *Biochemistry, 40*, 7351–7354. doi:10.1021/bi010596i.

29. Morré, D. J., Dick, S., Bosneaga, E., Balicki, A., Wu, L.-Y., McClain, N., et al. (2008). tNOX (ENOX1) target for chemosensitization—low-dose responses in the hormetic concentration range. *American Journal of Pharmacology and Toxicology, 3*, 16–26.