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NITRIC OXIDE REVERSES DRUG RESISTANCE BY INHIBITING ATPASE ACTIVITY OF P-GLYCOPROTEIN IN HUMAN MULTI-DRUG RESISTANT CANCER CELLS

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Abstract

Background—Development of resistance to chemotherapy drugs is a significant problem in treating human malignancies in the clinic. Overexpression of drug efflux proteins, including P-170 glycoprotein (P-gp), an ATP-dependent efflux protein, is one of the main mechanisms responsible for multi-drug resistance (MDR). Because our previous studies have shown that nitric oxide (●NO) or its related species inhibit the ATPase activities of topoisomerase II, we hypothesized that ●NO should also inhibit the ATPase activity of P-gp and increase drug accumulation in MDR cells, causing a reversal of drug resistance.

Results—Cytotoxicity and cellular accumulation studies showed that ●NO significantly inhibited the ATPase activity of P-gp in isolated membranes and in NCI/ADR-RES tumor cells, causing an increase in drug accumulation and reversals of adriamycin and taxol resistance in the MDR cells. While ●NO had no effects on topoisomerase II-induced, adriamycin-dependent DNA cleavage complex formation, it significantly inhibited adriamycin-induced DNA double-strand breaks. Electron spin resonance studies showed an increase in adriamycin-dependent hydroxyl radical formation in the presence of an NO-donor.

Conclusions—The reversal of drug resistance is due to inhibition of the ATPase activity by ●NO, resulting in enhancement of the drug accumulation in the MDR cells. Furthermore, DNA damage was not responsible for this reversal of adriamycin resistance. However, formation of adriamycin-dependent toxic free radical species and subsequent cellular damage may be responsible for the increased cytotoxicity of adriamycin by ●NO in NCI/ADR-RES cells.

General Significance—Appropriately designed NO donors would be ideal for the treatment of P-gp-overexpressing tumors in the clinic.

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Keywords

Nitric Oxide; Multi-drug resistance; P-gp protein; Adriamycin; Taxol; Free radical

1. Introduction

The development of multi-drug resistance (MDR) to commonly utilized chemotherapy drugs, e.g., adriamycin (ADR) and taxol (TX), is a significant problem in the treatment of human malignancies in the clinic [1–5]. Studies have identified various mechanisms for MDR, of which the most important is overexpression of P-170 glycoprotein (P-gp) in tumor cells. [1–5]. P-gp is an ATP-dependent efflux protein involved in removing various hydrophobic drugs, including drugs that are structurally very different from one another, from the cancer cells. Although significant progress has been made towards understanding the mechanisms of drug resistance and MDR, significant problems remain in the clinic, as modulation of drug efflux proteins has not been very successful [6].

The roles of nitric oxide ($\bullet\text{NO}$) in the treatment of human cancers is not clear; however, $\bullet\text{NO}$ is reported to induce killing of tumor cells and, thus, has been suggested to play an important role in cancer treatment [7]. $\bullet\text{NO}$ is a short-lived free radical molecule that easily diffuses in tissues. It is an important cellular messenger and plays a role in many important physiological functions, including vasodilatation, apoptosis, and the innate immune response [8]. Recent studies indicate that many of the actions of $\bullet\text{NO}$ result from the generation of its reactive metabolites (NO^+ , N_2O_3 , OONO) and the formation of nitroso groups on reactive sulfhydryl of a protein to form an S-nitrosothiol (SNO) [9]. The latter process, known as S-nitrosylation, results in modulation of protein activity and/or protein stability. $\bullet\text{NO}$ is formed from L-arginine by nitric oxide synthase (NOS) *in vivo*, and three forms of NOS are identified, including neuronal (nNOS), endothelial (eNOS), and a Ca^{2+} -independent inducible isoform (iNOS). High expression of iNOS and increased production of $\bullet\text{NO}$ have been reported in many human tumors, including breast, prostate and colorectal cancers [10–13]. The effects of $\bullet\text{NO}$ are biphasic: At high concentrations it induces DNA damage, apoptosis, and cell death while at low concentrations it induces cell survival and tumor progression [14].

Topoisomerases (topo) are nuclear enzymes responsible for maintaining the topology of DNA and many of DNA's functions in cells. Our recent studies have shown that $\bullet\text{NO}$ -derived species reacted directly with VP-16, a topo II poison, and the products of the reaction were significantly less toxic to tumor cells than the parent drug [15]. Furthermore, we have shown that $\bullet\text{NO}$ or its reactive metabolites induce significant resistance to both topo I and II poisons in human tumor cells [16, 17]. We have shown that both topo I and II in human tumor cells are nitrosylated by $\bullet\text{NO}$ [16, 17]. Nitrosylation of the topo I protein did not affect its activity, nor did it decrease topo I-dependent DNA damage in these tumor cells. However, nitrosylation of topo II decreased both catalytic and relaxation activities *in vitro* and in tumor cells and significantly decreased topo II-induced DNA damage, causing significant resistance to etoposide, amsacrine (m-AMSA) and XK469 in MCF-7 breast

tumor cells [18]. We found that this decrease in topo II activity resulted from the significant inhibition of the ATPase activity of topo II by $\bullet$ NO or $\bullet$ NO-derived reactive species [19].

Because P-gp is an ATP-dependent efflux protein, we hypothesized that $\bullet$ NO would also inhibit its function by inhibiting its ATPase activities, as it does the ATPase activities of topo II, resulting in increased drug accumulation and reversal of MDR. NCI/ADR-RES cells, originally designated as MCF-7/ADR cells, were obtained by continuous treatment of MCF-7 breast cancer cells with increasing concentrations of adriamycin and have been widely used to study the mechanism of adriamycin resistance and MDR [20–22]. However, these cells were found to originate from OVCAR-8 and not from MCF-7 breast cancer cells and, therefore, were redesignated as NCI/ADR-RES [23–25]. These cells overexpress stable P-gp and show significant resistance to ADR, TX and VP-16, among many other known P-gp substrates [20].

In this report, we have used NCI/ADR-RES cells to show that $\bullet$ NO, delivered via a nitric oxide donor, inhibits P-gp activity in NCI/ADR-RES cells and increases adriamycin accumulation, resulting in reversal of adriamycin resistance. Furthermore, $\bullet$ NO also significantly modifies resistance to taxol, a P-gp substrate, in NCI/ADR-RES tumor cells.

2. Materials and Methods

Adriamycin and taxol were gifts of the Drug Synthesis and Chemistry Branch, Developmental Therapeutic Program of NCI, NIH. The P-gp inhibitor PSC-833 (Valspodar) and the nitric oxide donors propylamine propylamine nonoate (PPNO) and diethylenetriamine nonoate (DETNO) were obtained from Cayman Chemicals (Ann Arbor, MI). Stock solutions of PPNO or DETNO were prepared in 0.2 N NaOH and stored at -80°C . Stock solutions of ADR (in H_2O), TX, and PSC-833 (in DMSO) were prepared and stored at -80°C . Fresh drug solutions prepared from stock solutions were used in all experiments. Primary antibody (EPR10364–57) for the detection of P-gp was obtained from Abcam, (Cambridge, MA). For the inhibition of the ATPase activity of P-gp, Solvo's Predeasy ATPase Assay Kit was obtained from Sigma-Aldrich (ST. Louis, MO). DMPO for the trapping of hydroxyl radicals was purchased from Dojindo (Rockville, MD) and used without any further purification.

3. Cell Culture

Human ovarian OVCAR-8 and NCI/ADR-RES cell lines were obtained from the NCI-Frederic Cancer center and were grown in Phenol Red-free RPMI media supplemented with 10% fetal bovine serum and antibiotics. Cells were routinely used for 15–20 passages, after which the cells were discarded, and a new cell culture started from fresh, frozen stock.

3.1. Cytotoxicity and Reversal of Resistance Studies

The cytotoxicity studies were carried out with a cell growth inhibition assay. For the cell count-based growth inhibition studies, 100,000–200,000 cells/well were plated in 2 mL of complete medium onto a 6-well plate (in triplicate) and allowed to attach for 18 h. Cells were treated with various concentrations of ADR or TX for 72 h in complete medium (2

mL). DMSO was included as the vehicle control. Cells were trypsinized, and the numbers of surviving cells were determined by counting the cells in a cell counter (Beckman, Brea, CA). For the reversal of resistance, cells were first treated with PPNO or DETNO (50–100 μ M) for 2 h in medium containing 1% FBS without antibiotics and 200 μ L of PBS (pH 7.0) followed by the addition of various concentrations of drugs and were incubated for 72 h in the complete medium. We used both PPNO and DETNO as our 14 C-NO donors for cytotoxicity studies. PPNO has a short half-life (15 min at 37°C, pH 7.0), while DETNO has a longer half-life (20 h at 37°C, pH 7.0). We found PPNO and DETNO to be more toxic to these ovarian cells than to other tumor cells we have used previously for cytotoxicity studies, e.g., MCF-7 and HT-29 cells at 100 μ M [16, 19]. At 100 μ M, about 25–30% of ovarian cells were found to be dead after 72 h of DETNO or PPNO exposure. Therefore, we chose to use 50 μ M concentrations of these NO-donors, which produced only 10% cell death.

3.2. Inhibition of ATPase Activity

The inhibition of the ATPase activity was carried out using Solvo's SB-MDR1Predeasy-ATP assay kit (Sigma-Aldrich, MO, catalog # SBPE01) according to the manufacturer's instructions. However, inhibitors (Na-Vanadate and cyclosporine A) and PPNO were preincubated with isolated membranes for 30 min before adding ATP to initiate the reaction.

3.3. Accumulation of Adriamycin and Effects of Nitric Oxide

Accumulation of ADR in OVCAR-8 and NCI/ADR-RES cells was carried out as described before by Shen et al. [26] with some modifications. Briefly, about 100,000 cells were seeded in six-well cover slips for 18 h in complete medium. After 18 h, the medium was removed, and the cells were washed once with PBS containing 6 mM glucose (PBS-G; pH 7.2). 2 mL of PBS-G was added, and cells were preincubated with different concentrations of PPNO or PSC-833 (10 μ M) for 1 h. Then ADR (0.1–1.0 μ M) was added, and the cells were incubated for 45 min, and examined using confocal microscopy. Average luminal fluorescence intensity of each cell was quantified using ImageJ software. 20–25 images were taken for each treatment group and controls, and 20 cells per group were analyzed. The means of fluorescence intensity were analyzed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA). The data were subjected to one-way ANOVA and multiple comparisons.

3.4. DNA Damage Studies

SDS-KCl Precipitation Assay—The formation of covalent topo II/DNA complexes in NCI/ADR-RES tumor cells in the presence of ADR and the effects of PPNO on ADR-induced cleavable complex formation were monitored by the SDS-KCl precipitation assay as described previously [27]. Briefly, the DNA of cells growing in the logarithmic phase (1×10^5 cells/mL), seeded in triplicate in six-well plates, was labeled with [methyl- 3 H]-thymidine (1.0 μ ci, 20 Ci/mmol; Perkin-Elmer, Waltham, MA) for 18–24 h. Cells were washed twice with medium, and various concentrations of ADR (dissolved in doubly distilled water) were added and incubated for 1 h. PPNO, when used, was present for 1–2 h before the addition of the drug. Cells were washed with PBS and lysed with 1 mL of prewarmed lysis solution (Topogen, Bueno Vista, Co). After lysis and shearing of DNA, DNA-ADR-topo II-complexes were precipitated with KCl. The precipitate was collected by

centrifugation and washed extensively (4 x) with the washing solution (Topogen) according to the manufacturer's instructions. The radioactivity was counted in a scintillation counter after adding 5 mL of scintillation fluid. PPNO alone had no significant effect on SDS-KCl precipitate formation.

3.5. H2AX Phosphorylation Assay

Detection of ADR-induced double-strand breaks was carried out using the H2AX phosphorylation assay kit (Catalog # 17-344, Millipore-Sigma, Burlington, MA) according to the manufacturer's instructions. Briefly, cell samples were harvested following ADR treatment (1 h), washed, and resuspended in 1X Fixation solution (50 μ l) for 20 minutes on ice. Samples were then washed twice in 1X PBS and resuspended in 1X Permeabilization solution (50 μ l) to which 3.5 μ l of either anti-phospho-Histone H2AX (Ser139) FITC conjugate or negative control mouse IgG-FITC conjugate was added. Samples were incubated on ice for 20 minutes with an occasional gentle mixing. After 20 minutes, 100 μ l of 1X wash solution was added to each sample, pelleted, and resuspended in 150 μ l of 1X PBS. Samples were analyzed using a BD LSR II flow cytometer equipped with FACSDiVa software. Only single cells, devoid of debris, were used in the analysis.

3.6. Western Blot Assay for P-gp

The Western Blot analyses for P-gp were carried out with standard methods, and samples (10–20 μ g of total protein) were electrophoresed under reducing conditions on 3–8% Tris-acetate gels (Novex, Life Technologies, Carlsbad, CA) for 50 minutes at 200 volts. After electrophoresis, proteins were transferred onto nitrocellulose membranes and probed with anti-P-gp and anti-beta actin antibodies. An Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) was used to acquire images.

3.7. Confocal microscopy studies for P-gp Protein in NCI/ADR-RES tumor cells

About 1×10^5 OVCAR-8 and NCI/ADR-RES tumor cells were plated for 18 h at 37°C in culture plates on glass coverslips in the complete medium. The medium was removed and washed with ice-cold PBS. To investigate the presence or absence of P-gp protein, the cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, washed twice for 5 minutes, permeabilized for 5 minutes with 0.5% Triton X-100, and washed twice for 5 minutes. After blocking with 4% fish gelatin in PBS (pH 7.4) for 2 h at room temperature, the cells were incubated with rabbit anti-P-gp antibody (diluted 1:2000 for 2 h, followed by secondary anti-mouse Alexa Fluor 668 antisera (diluted 1:1000) for 1 h. Coverslips containing cells were washed four times and mounted on glass slides using Prolong Gold anti-fade reagent. Cells were stained with DAPI (1 μ g/ml) for 15 min at room temperature and washed with PBS. For the visualization of P-gp protein in cells, fluorescence images were captured on a Zeiss LSM710 (Carl Zeiss Inc, Oberkochen, Germany) using a C-Apochromat 40X/1.20 Water Korr M27 objective. The 633 nm laser line from a HeNe laser at 13% power was used for excitation. Subsequently, a 639–698 nm band pass emission filter was used to collect the images with a pinhole set to yield an optical Z-thickness of 2.4 μ m.

3.8. Electron Spin Resonance Studies

The ESR studies for the detection of hydroxyl radicals were carried out as described previously [22]. Briefly, reaction mixtures were in 1 mL chelex-treated phosphate buffer containing 25 μ M DETPAC (pH 7.4) and glucose (PBS-G), ADR (2–25 μ M), NCI/ADR-RES cells ($1-2 \times 10^6$ /mL), and DMPO (100 mM). Reactions were initiated by adding NADPH (1 mM). Because $\cdot$ NO has been shown to decrease DMPO-OH formation [28], the effect of PPNO on ADR-dependent OH radical formation was examined by incubating cells with PPNO (100 μ M) in PBS-G for 90 min and collecting cells by centrifugation. Cells were washed once with PBS-G and suspended in PBS-G buffer containing 25 μ M DETPAC (pH 7.4) before adding ADR and DMPO. Mixtures were transferred into an ESR flat cell, and the spectra recorded with an ELEXSYS E 500 ESR spectrometer (Bruker Biospin, Billerica, MA) equipped with an ER4122SHQ cavity operating at 9.76 GHz at room temperature. The ESR settings were as follows: scan range, 100 G; modulation frequency, 100 KHz; modulation amplitude, 1.0 G; microwave power, 20 mW; receiver gain, 1×10^4 ; time constant, 327.6 ms and conversion time, 655.36 ms

3.9. Cell Death Studies by Flow Cytometry

Control and treated cells were harvested and resuspended back into their respective media. Cells were analyzed using an LSRII flow cytometer (Benton Dickinson, San Jose, CA) equipped with FACSDiVa software. For each sample, 10,000 cells were analyzed using FACSDiVa software. Changes in cell size were examined using forward-scatter (FSC) light. An increase in FSC indicates an increase in cell size, while a decrease in FSC indicates a decrease in cell size. Changes in cell size were determined using the mean fluorescent intensity (MFI) of the FSC channel.

3.10. Statistical Analysis

All experiments were carried out at least 3 times ($n = 3$) in duplicate. One-way analysis of variance (ANOVA) was used for statistical analysis. The Turkey Multiple comparison's test was used for multiple comparisons. The results are expressed as mean $\pm$ SEM. The differences were considered statistically significant when p values were less than 0.05.

4.0. RESULTS

4.1. Western Blots and Confocal Microscopy for P-gp protein

NCI/ADR-RES cells overexpress P-gp [20, 29]. The Western blot analysis shown in Figure-1 confirms that NCI/ADR-RES cells overexpress P-gp protein while the parental OVCAR-8 cells lack this protein. This was further confirmed in NCI/ADR-RES cells following incubations with the P-gp antibody using confocal microscopy (Figure 1 B).

4.2. Accumulation of Adriamycin in OVCAR-8 and NCI/ADR-RES CELLS

P-gp-overexpressing cells, in general, show significant decreases in drug accumulation compared to non-P-gp-expressing cells [26, 30]. Under similar conditions of drug treatment, NCI/ADR-RES cells accumulated 3 to 5-fold less ADR than the parental OVCAR-8 cells (Figure 2). More interestingly, preincubation of NCI/ADR-RES cells with PPNO

significantly increased ADR concentrations in these cells without affecting the parental cells, suggesting that $\bullet\text{NO}$ inhibited P-gp function in the resistant NCI/ADR-RES cells. This was further confirmed when PSC-833, a competitive modulator of P-gp, similarly inhibited P-gp function and increased ADR accumulation (Figure 2 C).

4.3. Reversal of Drug Resistance by Nitric Oxide In NCI/ADR-RES Cells

Since $\bullet\text{NO}$ and/or related species modulate P-gp transport activity, resulting in an increased drug accumulation, it is possible that this enhanced drug accumulation will also result in reversal of drug resistance in NCI/ADR-RES tumor cells. We examined these possibilities using both ADR and TX by first treating NCI/ADR-RES tumor cells with PPNO (short half-life) and DETNO (long half-life). As shown in Figure 3A, PPNO had no effects on ADR cytotoxicity in OVCAR-8 tumor cells, while it significantly enhanced the cytotoxicity of ADR in the P-gp-containing NCI/ADR-RES cells (Figure 3B). Interestingly, DETNO, under similar conditions (50 μM for 72 h), was significantly better at enhancing ADR cytotoxicity in NCI/ADR-RES cells than PPNO. This may be due to the fact that DETNO has a longer half-life (20 h) than PPNO (15 min). Thus, DETNO releases $\bullet\text{NO}$ (and related active species) continuously and for a longer time than PPNO. A similar observation has also been reported previously, and it has been suggested that a sustained flux of $\bullet\text{NO}$ is more cytotoxic to tumor cells than a short exposure to $\bullet\text{NO}$ [31]. We also examined the effects of $\bullet\text{NO}$ on the cytotoxicity of TX, a substrate of P-gp. As shown in Figure 3C, DETNO again was also extremely effective in reversing cytotoxicity of TX in NCI/ADR-RES cells. DETNO, like PPNO, did not significantly modulate the cytotoxicity of TX in the parental OVCAR-8 tumor cells (not shown).

4.4. Inhibition of ATPase Activity in Isolated Membranes

Our previous studies have shown that $\bullet\text{NO}$ delivered by PPNO inhibited ATPase activity of topo II. We investigated the possibility that PPNO will also inhibit the ATPase activity of P-gp, resulting in the reversal of drug resistance. We used verapamil-activated isolated MDR1 membranes to examine this possibility. Data presented in Figure-4 clearly shows that $\bullet\text{NO}/\bullet\text{NO}$ -related species inhibited the ATPase activity of P-gp. We found that as little as 0.2 μM PPNO was enough to inhibit about 20–30% of the ATPase activity and 20 μM PPNO caused about 50–60% inhibition of the ATPase activity in the isolated membranes. These observations suggest that increases in drug accumulation and resulting reversal of drug resistance in NCI/ADR-Res tumor cells is due to the inhibition of the ATPase activity by $\bullet\text{NO}$.

4.5. DNA Damage Studies

The mechanisms of cytotoxicity of ADR are extremely complicated; however, DNA damage induced by topo II is considered to be one of the mechanisms of action [27, 32]. We evaluated the formation of the DNA-ADR cleavable complexes as a possible mechanism for the reversal of drug resistance using the SDS-KCl precipitation assay in NCI/ADR-ADR tumor cells in the presence of PPNO. While ADR alone at 25 μM significantly (2-fold) enhanced SDS-KCl precipitate formation over the control values (Figure 5A), PPNO had no significant effects on the formation of the topo II-induced cleavable complex formation with ADR in these cells. Furthermore, PPNO did not increase double-strand breaks as detected by

H2AX staining in NCI/ADR-RES cells following treatment with ADR (Figure 5B). In fact, PPNO significantly decreased the ADR-induced double strand breaks (Figure 5B). Taken together, these results would suggest that ADR-induced DNA damage was not responsible for the reversal of MDR in NCI/ADR-RES tumor cells by $\cdot\text{NO}$ or $\cdot\text{NO}$ -related species.

4.6. Free Radical Formation by Adriamycin

Adriamycin is known to undergo redox-cycling following reductive activation to a semiquinone radical in the presence of O_2 , generating significant amounts of toxic species, including hydroxyl radicals, in tumor cells [33–35]. We have previously shown that ADR indeed forms $\cdot\text{OH}$ in NCI/ADR-RES tumor cells, detected as DMPO-OH, although fewer of these radicals were detected compared to the sensitive cells [22, 36]. We examined the formation of OH radicals using DMPO as the spin trap in NCI/ADR-RES cells with and without PPNO. While $\cdot\text{NO}$ has been reported to decrease DMPO-OH adduct formation [28], our results show that there was a 2–3-fold increase (Figure 6B, 6C, and insert) in the formation of DMPO-OH adducts (splitting constant $a^{\text{N}} = a^{\text{H}} = 14.9 \text{ G}$; 1:2:2:1) at lower doses of ADR (2–5 μM) in the presence of PPNO and NADPH. The increased formation of $\cdot\text{OH}$ most likely resulted from enhanced accumulation of ADR in the MDR cells. At higher concentrations of ADR (25–50 μM), however, no significant differences in DMPO-OH adduct formation/trapping were observed (data not shown). We also used DMSO, a specific $\cdot\text{OH}$ scavenger, to determine if ADR-induced DMPO-OH adducts were generated via the formation of free $\cdot\text{OH}$. DMSO reacts with free $\cdot\text{OH}$ to generate a methyl radical ($\cdot\text{CH}_3$) which is then trapped by excess DMPO forming DMPO- CH_3 adducts. Inclusion of DMSO resulted in the formation of DMPO- CH_3 adducts with splitting constants of $a^{\text{N}} = 16.4 \text{ G}$ and $a^{\text{H}} = 23.6 \text{ G}$, indicating that free $\cdot\text{OH}$ are formed by ADR in NCI/ADR-RES cells (Figure 6D).

4.7. Cell Death Studies

Analyses of cell death caused by ADR and TX and the effects of $\cdot\text{NO}$ or $\cdot\text{NO}$ -related species suggest that these drugs induced significant necrosis as indicated by significant swelling (increase in cell size) of NCI/ADR-NCI tumor cells at 72 h (Figure 7). In the presence of DETNO, there was a significant increase in drug-induced necrosis as indicated by an increase in cell swelling of the tumor cells (Figure 7).

5. Discussion

The mechanisms of ADR resistance and MDR are extremely complicated, and various mechanisms have been suggested [37, 38]. In addition to ADR's intercalative and covalent DNA-binding properties [39, 40], it is also a topo II poison [41, 42] and generates hydroxyl radicals *in vitro* and *in vivo*, inducing DNA damage [22, 43]. Mutation of topo II genes and/or a decrease in topo II activity [44] as well as modification of enzymes for drug metabolism and detoxification (phase I and II) play significant roles in ADR resistance in tumor cells [20, 22, 43, 45]. P-gp is an ATP-binding family of drug efflux protein and is involved in removing some chemotherapeutic drugs (ADR, VP-16, Taxol, etc.) from tumor cells, thus decreasing their effectiveness [3–5]. It is also involved in removing various other toxic xenobiotics from normal cells, including brain cells, as a part of the normal defense

mechanism [2]. Overexpression of P-gp has been detected in certain human cancers in the clinic and has been correlated with decreased survival [2, 5]. Thus, it is rationalized that inhibitors of P-gp would be beneficial in the clinic in enhancing/overcoming drug resistance caused by the overexpression of P-gp. Several P-gp inhibitors are now known have entered clinical trials; however, results have not been very encouraging due to their toxicity and/or other side effects [6].

Nitric oxide ($\bullet\text{NO}$), produced intracellularly via NOS catalysis, has been shown to kill tumor cells both *in vitro* and *in vivo* [46]. We have shown that $\bullet\text{NO}/\bullet\text{NO}$ -related species $\bullet\text{NO}/\bullet\text{NO}$ -related species nitrosylates both topo I and II while inhibiting the functions of only topo II [16, 17]. Additional studies showed that this modulation of the topo II function resulted from $\bullet\text{NO}$ -induced inhibition of the ATPase activity of topo II [19]. Because P-gp is an ATP-binding efflux protein, we rationalized that $\bullet\text{NO}$ should also inhibit P-gp activity by inhibiting its ATPase activity, thus inhibiting its function of removing cancer drugs from MDR tumor cells. The consequence of such modulation would result in a sensitization/reversal of drug resistance in tumor cells.

Our studies in isolated membranes containing P-gp showed that $\bullet\text{NO}/\bullet\text{NO}$ -related species significantly inhibited the ATPase activity of P-gp. Furthermore, studies presented in this report demonstrate that $\bullet\text{NO}$, delivered by two different NO-donors, significantly reverse the resistance of ADR and TX, known P-gp substrates in human P-gp-overexpressing MDR tumor cells. In contrast to the MDR cells, these NO-donors were ineffective in modulating the cytotoxicity of ADR or TX in sensitive parental OVCAR-8 ovarian tumor cells, suggesting that $\bullet\text{NO}/\bullet\text{NO}$ -related species inhibited only the activity of the P-gp protein. Our studies also showed that $\bullet\text{NO}/\bullet\text{NO}$ -related species significantly enhanced ADR accumulation in the P-gp-overexpressing MDR cells without affecting its accumulation in the sensitive tumor cells. These observations, taken together, suggest that inhibition of the ATPase activity of P-gp protein in NCI/ADR-RES cells by $\bullet\text{NO}/\bullet\text{NO}$ -related species resulted in increased drug accumulation and the reversal of drug resistance.

The mechanism(s) of reversal of ADR resistance induced by $\bullet\text{NO}/\bullet\text{NO}$ -related species in NCI/ADR-RES cells was also investigated. While ADR is known to cause many biological/pharmacological effects in tumor cells, there are two main mechanisms that have been suggested to play important roles in tumor cell killing [37, 40, 43]. First, ADR induces both single- and double-strand breaks via acting as a topo II poison [27, 41, 42]; and secondly, it generates reactive hydroxyl radicals following reductive activation at or near DNA, inducing DNA damage leading to tumor cell death [33–35, 45]. We have examined both mechanisms in the presence of PPNO as an explanation for the reversal of ADR resistance. We used the SDS-KCl precipitation assay to evaluate topo II-mediated DNA damage; our studies indicated no significant increase in DNA damage in the presence of PPNO at the concentrations of ADR used. This would suggest either that DNA damage did not play any role in the mechanism of tumor cell death in NCI/ADR-RES cells or that PPNO was not involved in the modification of DNA damage induced by topo II with ADR. It is also interesting to note that while ADR (at 25 μM) induced significant amounts of DNA double-strand breaks as detected by an H2AX phosphorylation assay, PPNO had did not increase this ADR-induced double-strand breaks. In fact, PPNO significantly decreased double-

strand breaks induced by ADR. This further confirms our previous findings that $\bullet\text{NO}/\bullet\text{NO}$ -related species inhibit topo II drug-induced DNA double-strand break formation by inhibiting the catalytic activity of topo II [18].

The formation of ADR-dependent $\bullet\text{OH}$, detected using DMPO as a spin trap, was also examined in NCI/ADR-RES cells in the presence of PPNO. Our results confirmed that ADR-induced and NADPH-dependent free radical formation was significantly enhanced in the presence of PPNO. This enhancement of $\bullet\text{OH}$ formation was only observed at lower concentrations of the drug and not at high concentrations of ADR, indicating that at these high concentrations of ADR P-gp protein may not be able to remove/efflux ADR from the cells. Hydroxyl radicals, generated at or near cellular DNA, induces significant DNA damage leading to cell death in the absence of DNA repair. Thus, in NCI/ADR-RES cells, the increased cytotoxicity of ADR caused by PPNO and DETNO may involve enhanced $\bullet\text{OH}$ formation by intracellular ADR, in turn caused by the inhibition of P-gp function by $\bullet\text{NO}/\bullet\text{NO}$ -related species.

It should be noted that $\bullet\text{NO}$ has been reported to cause reversal of *mdr1*-associated drug resistance. Riganti et al. [47] have reported that induction of $\bullet\text{NO}$ production or delivery of $\bullet\text{NO}$ via an NO-donor inhibited the efflux of doxorubicin (adriamycin) in HT-29-dox, a doxorubicin-selected resistant cell line that overexpressed P-gp, resulting in the reversal of doxorubicin resistance. In that study, however, $\bullet\text{NO}$ also increased accumulation of doxorubicin in the sensitive HT-29 cells, suggesting that other mechanisms may also be involved in this. Results shown in our report clearly indicate that this was not the case in sensitive OVCAR-8 tumor cells with PPNO, suggesting that $\bullet\text{NO}/\bullet\text{NO}$ -related species inhibited P-gp only in NCI/ADR-RES cells.

The mechanisms of sensitization/reversal of TX resistance were not studied in this report, but we believe that inhibition of P-gp protein function by $\bullet\text{NO}/\bullet\text{NO}$ -related species would also increase intracellular concentrations of TX in NCI/ADR-RES tumor cells, resulting in an increased drug cytotoxicity. The roles of $\bullet\text{NO}/\bullet\text{NO}$ -related species in specific mechanisms, e.g. effects on stabilization on microtubules by TX, is currently under investigation in our laboratory.

Overcoming drug resistance in the clinic is extremely important for a successful therapy, and we believe that NO-donors offer a promising future direction. The most successful course of action in developing cancer therapies with NO-donors is to develop newer ones that are site-specific to tumor cells and release $\bullet\text{NO}$ following intracellular activation. It is essential to find the right $\bullet\text{NO}$ -delivering compound, which must also be very target-specific to resistant tumor cells in the clinic. Such compounds would be ideal for the treatment of clinically resistant or refractory tumors in the clinic overexpressing ATP-dependent efflux proteins. One such compound, JS-K (O²-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazene-1-ium-1,2-diolate), was found to be an extremely active anticancer agent against human HL60 leukemia cells, human non-small cell lung cancer cells and multiple myeloma cells both *in vitro* and *in vivo* [48, 49]. JS-K was shown to be activated following reactions with glutathione to produce $\bullet\text{NO}$ *in vivo*, catalyzed by glutathione S-transferase in tumor cells [49]. As resistant tumor cells, in general, contain significantly more glutathione and

overexpress glutathione s-transferase (e.g., NCI/ADR-RES cells), compounds like JS-K are expected to be significantly more active in killing these cells and, thus, may be more effective agents in sensitizing/overcoming drug resistance. Work is now in progress to evaluate these possibilities with JS-K and other NO-donors in our laboratories.

6. Conclusions

Our studies described here show that $\bullet\text{NO}$ and/or its related species inhibit P-gp functions by inhibiting its ATPase activity, resulting in the reversal of adriamycin and taxol resistance. This modulation of the resistance is due to $\bullet\text{NO}$ enhancement of drug's accumulation in the MDR cells. Our studies also indicate that DNA damage is not responsible for this reversal of adriamycin resistance. However, a 2–3-fold increase in adriamycin-dependent $\bullet\text{OH}$ formation and the resulting free radical-dependent cellular damage may be responsible for $\bullet\text{NO}$'s increasing the cytotoxicity of adriamycin in NCI/ADR-RES cells. Our studies further suggest that appropriately designed nitric oxide donors may be useful in the clinic for treating human tumors that overexpress P-gp protein or in other diseases where pharmacoresistance results from the presence of ATP-dependent efflux proteins.

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ABBREVIATIONS

P-gp	P-glycoprotein 170
MDR	multi-drug resistance
ADR	Adriamycin
TX	taxol
$\bullet\text{NO}$	nitric oxide
topo	topoisomerase
PPNO	propylamine nonoate
DETNO	diethylenetriamine nonoate
PSC 833	Valspodar
PBS-G	phosphate saline buffer containing glucose
ESR	electron spin resonance

DMPO	5'-dimethyl pyrroline-N-oxide
•OH	hydroxyl radical

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Highlights

- Nitric oxide inhibits Pgp protein functions in human MDR tumor cells.
- Nitric oxide increases cellular accumulation of adriamycin in MDR tumor cells.
- Nitric oxide reverses resistance to adriamycin and taxol in MDR tumor cells.
- The reversal of drug resistance is not due to adriamycin-induced DNA damage.
- Reversal of adriamycin resistance may be, in part, due to increased $\cdot\text{OH}$ formation.

Figure 1: Western blot analysis (A) for P-gp proteins in OVCAR-8 (lane-1) and NCI/ADR-RES (lane-2) tumor cells and confocal microscopy studies for P-gp proteins in OVCAR-8 and NCI/ADR-RES tumor cells (B).

Figure 2:

The relative accumulation of ADR in OVCAR-8 and NCI/ADR-RES tumor cells (**panel A**) and effects of PPNO and PSC 388 on ADR accumulation in OVCAR-8 (**panel B**) and in NCI/ADR-RES tumor cells (**panel C**). Values represent three separate experiments. *** p values < 0.0001 compared with control (no treatment). ### p values < 0.0001 from ADR alone.

Figure 3:

Cytotoxicity of ADR (■-■) in OVCAR-8 tumor cells (panel A) in the presence of PPNO (●-●, 50 μ M) and DETNO (▲-▲, 50 μ M) and in NCI/ADR-RES tumor cells following 72 h of drug treatment (**Panel B**). Cells were counted as described in the methods section. **Panel C** shows the effects of PPNO and DETNO on the reversal of Taxol resistance under similar conditions. Values represent three separate experiments carried out in triplicate. ***, ** and * p values 0.001, 0.005, and 0.05, respectively, compared with concentration-matched samples. ###, ##, and # values 0.001, 0.005 and 0.05, respectively, from concentration-matched samples.

Figure 4:

Inhibition of the ATPase activity of P-gp in isolated MDR1 membranes by PPNO. The inhibition studies were carried out according to the manufacturer's instructions. Inhibitors (Na Vanadate and cyclosporine A), and various concentrations of PPNO were incubated with verapamil-activate membranes for 30 min prior to the addition of ATP. ** p values 0.005 compared to 0.02, 0.2 and 2.0 μM PPNO, respectively.

Figure 5:

Panel A: Formation of cleavage complexes in NCI/ADR-RES cancer cells by ADR alone (□) and in the presence of PPNO (100 μM; ■). Cells were preincubated with PPNO for 1–2 h and then incubated with ADR (5.0 and 25.0 μM) for 1 h in complete medium (2 mL). * p values ≤ 0.05 compared with control. **Panel B:** Flow cytometric analysis for ADR-induced double-strand breaks using the γ -H2AX phosphorylation assay following treatment with ADR (5 and 25 μM) alone (□) and in the presence of PPNO (100 μM; ■). Cells were preincubated with PPNO for 1 h before adding ADR and then incubated for 1 h in complete medium (2 mL). The SDS-KCl precipitation assay and the γ -H2AX analysis were carried out as described in the methods section. **, p value ≤ 0.005 compared to adriamycin alone and *, p value ≤ 0.05 compared to control.

Figure 6:

Spin trapping of hydroxyl radicals formed from adriamycin ($2.5 \mu\text{M}$) by ESR in the presence of DMPO (100 mM) and NADPH (1 mM) in NCI/ADR-RES tumor cells in the presence (C) or in the absence (B) of PPNO ($100 \mu\text{M}$) and (A) no adriamycin. The cells were incubated with PPNO in PBS-G buffer at 37°C for 90 min, collected by centrifugation, washed once with PBS-G buffer, and suspended in PBS-G buffer containing $25 \mu\text{M}$ DETPAC and adding DMPO and ADR ($5 \mu\text{M}$). The reactions were initiated by adding NADPH (1 mM) as described in the methods section and the resulting spectra were recorded. The DMPO-OH

adduct had the following hyperfine coupling constants: $a^N = a^H = 14.9$ G. Spectra (**D**) was obtained in the presence of DMSO (0.5 M) and 10 μ M ADR showing the presence of both DMPO-OH (o) and DMPO-CH₃ (c) adducts with $a^N = 16.3$ G and $a^H = 23.6$ G. The receiver gain for **A** and **B** was 3×10^4 , and 1×10^4 for **C** and **D**.

Insert: Quantification of DMPO-OH adducts formed in the presence (**C**) and absence (**B**) of PPNO using low field line of the DMPO-OH adduct spectrum.

Figure 7:

Effects of DETNO (50 μ M) on ADR- and Taxol-induced cell death of NCI/ADR-RES tumor cells. NCI/ADR-RES cells were treated with 25 μ M ADR or 0.5 μ M taxol with and without DETNO for 72 h in the complete media. Cells were collected and analyzed by flow cytometry as described in the methods section. **Panel A**, in absence of DETNO; **Panel B**, in the presence of DETNO.