Effect of Nitric Oxide on the Anticancer Activity of the Topoisomerase-Active Drugs Etoposide and Adriamycin in Human Melanoma Cells

Birandra K. Sinha, Ashutosh Kumar, Suchandra Bhattacharjee, Michael G. Espey, and Ronald P. Mason

Laboratory of Toxicology and Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina (B.K.S., A.K., S.B., R.P.M.); and National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland (M.G.E.)

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ABSTRACT

Nitric oxide (NO) was originally identified as an innate cytotoxicin. However, in tumors it can enhance resistance to chemotherapy and exacerbate cancer progression. Our previous studies indicated that NO/NO-derived species react with etoposide (VP-16) in vitro and form products that show significantly reduced activity toward HL60 cells and lipopolysaccharide (LPS)-induced macrophages. Here, we further confirm the hypothesis that NO generation contributes to VP-16 resistance by examining interactions, leading to increased DNA damage and apoptosis. Furthermore, we found that coculturing A375 melanoma cells with LPS-induced macrophage RAW cells also significantly reduced VP-16 cytotoxicity and DNA damage in A375 cells. We also examined the interactions of NO with another topoisomerase active drug, Adriamycin, in A375 cells. In contrast, to VP-16, NO caused no significant modulation of cytotoxicity or Adriamycin-dependent apoptosis, suggesting that NO does not interact with Adriamycin. Our studies support the hypothesis that NO oxidative chemistry can detoxify VP-16 through direct nitrogen oxide radical attack. Our results provide insights into the pharmacology and anticancer mechanisms of VP-16 that may ultimately contribute to increased resistance, treatment failure, and induction of secondary leukemia in VP-16-treated patients.

Introduction

Topoisomerases constitute an important class of nuclear enzymes responsible for maintaining the topology and function of DNA. Inhibition and/or interference with topoisomerase functions lead to cell death. A large number of clinically active anticancer drugs (e.g., etoposide, adriamycin, and camptothecin) interact with and poison topoisomerases, causing significant DNA damage and cell death. Etoposide (VP-16), a topoisomerase II (topo II) poison, is active against lung and testicular cancers and lymphoma (Henwood and Brogden, 1990). Adriamycin (Adr), also a Topo II poison (Liu, 1989), is active against lung and testicular cancers, bladder, breast, lung, and multiple myelomas (Sinha, 1995).

VP-16 is rapidly metabolized by cytochrome P-450 (P450) and peroxidases to o-dihydroxy and o-quinone derivatives of VP-16 (Haim et al., 1986, 1987a,b; VanMaanen et al., 1987, 1989; Kagan et al., 2001), and the formation of these metabolites requires the generation of a VP-16 radical (VP-16·) formed from the oxidation of the 4′-OH group of VP-16 (Haim et al., 1987a,b). VP-16 metabolites bind to cellular components (Haim et al., 1986, 1987b) and cause DNA strand cleavage through a Topo II-mediated mechanism (Sinha et al., 1990; Gantchev and Hunting, 1998). VP-16 causes oxidative stress both in vitro and in vivo by oxidizing glutathione and forming the glutathione thiol radical (Katki et al., 1987). In addition, VP-16-o-quinone has been reported to form glutathione adducts in HL60 cells (Fan et al., 2006).

Adriamycin has been reported to intercalate into DNA and binds covalently to DNA and proteins via generation of reactive species through reductive bioactivation (Sinha, 1980; Sinha et al., 1984). Reductive activation also generates free radicals via the formation of semiquinone radical and its reaction with molecular oxygen (Kalyanaraman et al., 1980; Myers et al., 1987; Sinha et al., 1987). Although the covalent binding to DNA and formation of free radicals are implicated in the mechanisms of Adr cytotoxicity (Myers et al., 1987; Sinha et al., 1987) and cardiotoxicity (Doroshow et al., 1990; VanMaanen et al., 1987; VanMaanen et al., 1989), the oxidative chemistry and production of radicals have not been systematically studied.

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ABBREVIATIONS: Adr, adriamycin; P450, cytochrome P450; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; iNOS, inducible nitric-oxide synthase; L-NIL, 6-(1-iminoethyl)-L-lysine dihydrochloride; LPS, lipopolysaccharide; MTT, microculture tetrazolium toxicity; NO, nitric oxide; NO2, nitrogen dioxide; PBS, phosphate-buffered saline; VP-16, etoposide; VP-16, VP-16-phenoxyl radical; Topo II, topoisomerase II.
1983; Rajagopalan et al., 1988), poisoning of Topo II is considered to play an important role in tumor cell death.

NO and/or its products (NO\textsubscript{2}, ONOO\textsuperscript{−}) have been shown to play important roles in cancer biology, including the innate immune response, neovascularization, cancer metastasis, and cell death (Jenkins et al., 1995; Wink et al., 1998; Xu et al., 2002; Chen et al., 2008). Exposure to NO (via NO donors) modifies activities of certain anticancer drugs, including Adr (Cook et al., 1997; Wink et al., 1997; Evig et al., 2004). NO and/or its products react with a wide variety of substrates, including amines, thiols, and phenols (Janzén et al., 1993; Cudic and Ducrocq, 2000; Yenes and Messeguer 1999). NO-derived species rapidly react with phenols to form phenoxyl radicals, quinones, and nitrated phenols. The formation of nitrotyrosine in cellular proteins has been associated with modifications of protein activities (Curtis et al., 1996; Chatterjee et al., 2009). Furthermore, NO has been reported to react with the tyrosyl radical of ribonucleotide reductase, resulting in the inhibition of DNA synthesis and ultimately cell death (Lepoivre et al., 1994).

VP-16 contains a phenolic OH group in the 4′-position, and both the cytotoxicity and the binding of VP-16 to Topo II depend on the presence of this moiety (Loike and Horwitz, 1976; Long et al., 1984; Sinha et al., 1990). We have recently shown that the phenolic OH of VP-16 reacts with NO-derived species to generate VP-16, o-quinone, and other products, thereby modulating the cytotoxicity of VP-16 toward cancer cells (Sinha et al., 2013). In this report, we have further examined the interactions of VP-16 and Adr with endogenously formed NO via iNOS catalysis in A375 human melanoma cells. Our results support the conclusion that the reaction of NO with VP-16 diminishes its cytotoxic activity toward cancer cells. In contrast, endogenously formed NO in A375 cells had no significant effects on Adr cytotoxicity, indicating that Adr did not interact with NO or NO-derived species. This work extends our in vitro work to the cellular systems and confirms the same processes observed for VP-16 in vitro also operates in tumor cells.

**Materials and Methods**

VP-16 and Adriamycin were the gift of the Drug Synthesis and Chemistry Branch, Development Therapeutic Program of the National Cancer Institute, National Institutes of Health. Human topoisomerase and SDS/KCl precipitation assay kits were obtained from Topogen (Columbus, OH). The nitric oxide synthase (inducible, iNOS) inhibitor N\textsuperscript{ω}-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL) was obtained from Cayman Chemicals (Ann Arbor, MI). Caspase-3 activity was measured by an Abcam assay kit (Cambridge, MA). A primary antibody for the analysis of iNOS was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A nitric oxide assay kit was purchased from Thermo Fisher Scientific (Waltham, MA).

**Cytotoxicity Studies.** A375 melanoma cell lines and macrophage RAW 264.7 cells [American Type Culture Collection (ATCC), Manassas, VA] were grown in phenol red–free RPMI 1640 media supplemented with 10% fetal bovine serum and antibiotics. A375 cells and RAW 264.7 were used for 15 passages, after which the cells were discarded and a new cell culture was started from fresh-frozen stock. The intracellular reaction between VP-16 and endogenous NO was examined in A375 cells by preincubating cells either in the presence or absence of an iNOS inhibitor (L-NIL) for 4 hours before the addition of VP-16. A375 cells (2000/well) were plated in phenol red–free medium and cultured for 96 hours. Dimethylsulfoxide was included as the vehicle control, and the cytotoxicity was measured by the microculture tetrazolium toxicity (MTT) assay, as described elsewhere (Alley et al., 1988).

Coculture experiments for cytotoxicity with A375 cells and induced RAW cells were performed in six-well plates separated by 0.4-μM membrane filters (Costar, Corning, NY). Macrophage RAW cells were induced as described previously elsewhere (Sinha et al., 2013). For the cytotoxicity studies, 200,000–250,000/well of A375 cells were plated in 3 mL of complete medium into a six-well plate (in duplicates) and were allowed to attach for 4–6 hours. Membranes were inserted, and various concentrations of VP-16 were added onto the top of membranes containing cells induced with lipopolysaccharide (LPS) (1 × 10\textsuperscript{4}) and incubated for 48 hours. Membranes and RAW cells were removed, A375 cells were washed once with ice-cold phosphate-buffered saline (PBS) and trypsinized, and the cytotoxicity was determined by counting the cells. Nitrite concentrations were determined using the Greiss reagent, as described previously elsewhere (Sinha et al., 2013).

**Western Blot Assay.** Samples (10 μg of total protein) were electrophoresed under reducing conditions through 4%–12% Bis-Tris NuPage acrylamide gels (Invitrogen, Carlsbad, CA). After electrophoresis, proteins were transferred onto a nitrocellulose membrane and probed with anti-iNOS and anti-β actin antibodies. An Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) was used to acquire images. Rabbit polyclonal anti-iNOS antibody was used to quantify iNOS proteins in samples using a standard enzyme-linked immunosorbent assay (ELISA) (Rangelova et al., 2008).

**Caspase-3 Assay.** Caspase-3 activity in the A375 cell lysate was measured by a colorimetric assay kit (Abcam) using the manufacturer's instructions. In brief, 1 million cells were treated with VP-16 (25 and 50 μM) in the presence or absence of L-NIL (250–400 μM) for 24 hours. Cells were collected by centrifugation and lysed with the lysis buffer (Abcam) by incubating for 10 minutes on ice. Cells were centrifuged at 10,000g for 5 minutes, supernatant was collected, and the protein concentration was determined by the BCA assay. Equal amounts of the protein (200–400 μg) were incubated with 10 mM DTT (dithiothreitol) containing the reaction buffer, followed by the addition of caspase-3 substrate (DEVD-Pna, 4 mM), and the reaction mixture was incubated at 37°C for 2–4 hours. The sample was analyzed for absorbance at 405 nm in a multiwell plate reader, and results were expressed as substrate utilization per milligram protein.

**SDS-KCl Precipitation Assay.** The formation of covalent Topo II/DNA complexes with VP-16 in the presence or absence of L-NIL was quantitated by the SDS-KCl precipitation assay, as described elsewhere (Liu et al., 1983). In brief, DNA of A375 cells growing in the logarithmic phase (2 × 10\textsuperscript{6} /mL) was labeled with [methyl-\textsuperscript{3}H]thymidine (10 μCi, 2 Ci/mmol, PerkinElmer Life and Analytical Sciences, Waltham, MA) for 18–24 hours. Cells were collected and washed twice with the medium, diluted in fresh medium, and seeded into a six-well plate at a density of 2 × 10\textsuperscript{5} cells/mL. L-NIL was preincubated with the cells for 18 hours during the labeling phase. VP-16, dissolved in dimethylsulfoxide, was added and incubated for 1 hour. Cells were washed with PBS (2 times) and lysed with 1 mL of prewarmed lysis solution (Topogen). After lysis and shearing of DNA, DNA–Topo II–VP-16 complexes were precipitated with KCl. The precipitate was collected by centrifugation and washed extensively (4 times) 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.

The effects of endogenously generated NO from iNOS of LPS-induced macrophage RAW cells on SDS-KCl precipitation in A375 cells were investigated as described in the cytotoxicity studies, except that the drug exposure was for 90 minutes and the samples were processed for the SDS-KCl precipitation assay as described previously. In some experiments, VP-16 (10 μM) was preincubated with LPS-induced RAW cells (0.5 × 10\textsuperscript{5} cells/mL, 30 minutes), the mixtures were added to six-well plates fitted with membrane filters (on top), and they were allowed to interact with the \textsuperscript{3}H-labeled A375 cells (in bottom, 200–250,000 cells/well) for 90 minutes. The medium was removed, and the cells were washed once with PBS; A375 cells...
were lysed with prewarmed lysis buffer, and the samples were processed as described previously.

**Metabolism of VP-16 in A375 Cells.** The metabolic studies with VP-16 in A375 cells in the presence and absence of L-NIL were performed similarly to those described previously elsewhere (Haim et al., 1987a,b). In brief, 1 × 10⁶/ml of cells in 5 ml of phenol red-free medium were seeded in a 100 × 15 mm Petri dish (Falcon; Becton Dickinson, Franklin Lakes, NJ) and were allowed to attach for 4 hours. L-NIL (400 μM) was added to the cells and incubated for 18 hours before VP-16 (100 μM) was added at various time points. At the end of the incubation, the cells were washed with ice-cold PBS, gently scraped in 5 ml of ice-cold PBS, and collected by centrifugation (2000g, 5 minutes). The cells were suspended in 1 ml of ice-cold PBS, lysed with sonication (4°C), and the resulting mixture extracted with chloroform (4 × 1 ml). The combined organic layers were removed under argon, and the residue was dissolved in 200 μl of methanol and analyzed by high-performance liquid chromatography (HPLC) as described previously elsewhere (Haim et al., 1987a,b), except that a C18 column was used and MeOH-water (60:40) was the mobile phase. Under these conditions, VP-16 and VP-16-quinoine had retention times of 2.1 minutes and 2.7 minutes, respectively.

**Results**

Using purified Topo II enzyme, we had previously reported that products formed from reactions of NO and/or NO-derived species with VP-16 were significantly less active than the parent drug in inducing DNA cleavage and were not cytotoxic to HL60 cells (Sinha et al., 2013). In this report, we examined whether endogenous formation of NO catalyzed by iNOS in cancer cells could affect the cytotoxicity of VP-16 and Adr. To assess this, we used a human melanoma A375 tumor cell line, which has been shown to express iNOS and to produce levels of NO that are relatively low (Tang and Grimm, 2004; Chanvorachote et al., 2006; Sikora et al., 2010). The presence of iNOS was confirmed in A375 cells with a primary antibody for iNOS (Fig. 1A), and our results are similar to those described previously elsewhere (Sikora et al., 2010). Furthermore, treatment of A375 cells with 10 ng/ml LPS for 18 hours significantly induced this protein, further confirming the presence of the inducible form of NOS in A375 cells (Fig. 1B). It is interesting to note that under these experimental conditions the formation of NO/NO₂ was not detected by the Griess reaction (data not shown). This observation is similar to those previously published by Sikora et al., (2010) and Chin and Deen (2010), as the amount of NO synthesis in A375 cells is well below (100–150 nM) the detection limits of the Griess reaction.

Examination of caspase-3 activity, a marker for apoptosis, in A375 cells showed that VP-16 alone (25 and 50 μM) induced 2.5- and 3.5-fold increases in caspase-3 activity, respectively, after 18 hours (Fig. 2A). Inhibition of iNOS with L-NIL (400 μM) further enhanced caspase-3 activity 4.5- and 6.0-fold in the presence of 25 μM and 50 μM VP-16, respectively. Consistent with these data, application of L-NIL (400 μM) significantly enhanced VP-16 cytotoxicity in A375 cells (2.5–3.5-fold; Fig. 2B). The relative IC₅₀ (concentration required to inhibit cell growth by 50%) is presented in Table 1. Under these conditions, L-NIL alone had no significant effect on caspase-3 activity or A375 cell viability.

Because LPS significantly induced iNOS (Fig. 1), we examined the cytotoxicity of VP-16 after induction of A375 cells with LPS. Consistent with the increase in the expression of iNOS, VP-16 cytotoxicity was further decreased in the induced A375 cells (Fig. 3A; Table 1). More interestingly, the presence of L-NIL significantly sensitized A375 cells to VP-16 (Fig. 3A; Table 1), which suggests that increased NO formation from iNOS was responsible for the decrease in VP-16 cytotoxicity in A375 cells. To further examine the roles of exogenously generated NO on VP-16 cytotoxicity in A375 cells, we performed coculture studies with LPS-induced macrophage RAW cells. As shown in Fig. 3B and Table 1, exogenously formed NO from iNOS in RAW cells further decreased VP-16 cytotoxicity (>15-fold), suggesting that NO generated from the induced RAW cells reacted with VP-16 and formed nontoxic species. Additionally, the data presented in Table 2 show that considerable amounts of NO were generated from the induced RAW cells, and the presence of VP-16 (in coculture cytotoxicity studies) significantly decreased NO formation, indicating a reaction between VP-16 and NO. Furthermore, there were no significant differences in nitrite formation between the top and bottom layers (compartments) of the six-well plates, suggesting free diffusion of NO across the membrane. In contrast, the cytotoxicity of VP-16 was not significantly affected (data not shown) in the presence of noninduced RAW cells, indicating that NO/NOSpecies generated in the induced RAW cells from iNOS catalysis were responsible for this decrease in VP-16 cytotoxicity.

Adriamycin is a Topo II-active drug that induces protein-associated DNA damage leading to cell death, and thus it is similar to VP-16 in its mode of action. We examined the effects

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**Fig. 1.** (A) The Western blots for iNOS and effects of LPS (10 ng/ml) in A375 cells. (B) Quantitation of the iNOS protein in A375 cells using rabbit polyclonal anti-iNOS antibody in the standard ELISA assay as described in Materials and Methods. For the Western blots, A375 cells were seeded at 1 × 10⁶ in a six-well plate. LPS (10 ng/ml) was added and exposed for 14 to 16 hours. Cells were collected, lysed, and examined for iNOS protein by Western blots. For the Western blots, 10 μg of proteins were loaded, and actin was used to assess equal loading of the proteins. Data are the mean of three separate experiments performed in duplicate. **P < 0.005 compared with the untreated control.**
of endogenously generated NO in Adr-induced apoptosis and cytotoxicity in A375 cells. Under similar conditions, the presence of L-NIL had no significant effects on caspase-3 activity induced by Adr (Fig. 4). Furthermore, the presence of L-NIL had no effects on the cytotoxicity of this drug (Fig. 4) in A375 cells. These observations would indicate that Adr does not react with NO/NO-related species.

Effects of L-NIL on VP-16–Induced Cleavable Complex in A375 Cells. To determine whether iNOS catalysis influences the formation of a VP-16/Topo II complex, we evaluated the formation of the cleavage complex by VP-16 in the presence of L-NIL using an SDS/KCl precipitation assay (Liu et al., 1983). As expected, treatment of A375 cells with VP-16 (10 μM) significantly increased (6- to 7-fold) the formation of the SDS/KCl precipitate (Fig. 5) over the untreated controls. The presence of L-NIL further increased this complex formation (10-fold), equivalent to the effects of VP-16 alone at 5 times the concentration (50 μM; Fig. 5). This result suggests that inhibition of NO formation in A375 cells by L-NIL increased the DNA damage induced by VP-16 in a Topo II–dependent manner.

To further confirm that NO or NO-derived species were responsible for this increase in VP-16–mediated Topo II–dependent DNA damage, we performed coculture experiments with induced macrophage RAW cells. Coincubation of A375 cells with induced RAW cells significantly decreased VP-16–dependent SDS-KCl precipitate formation (Fig. 5B). Similar effects were noted when the incubations were performed either simultaneously or with preincubated VP-16 (Fig. 5B). Taken together, these observations strongly suggest that NO and associated nitrogen oxides can react with VP-16, thereby making it less effective at inducing apoptosis and DNA damage and resulting in increased VP-16 resistance toward cancer cells.

Metabolism of VP-16 in A375 Melanoma Cells. To examine the effects of endogenously produced NO on VP-16 metabolism in A375 cells, VP-16 was incubated with A375 cells for various times in the presence or absence of L-NIL. The HPLC analysis (Fig. 6) clearly indicated that VP-16 is rapidly metabolized in A375 cells, and a significant amount of presence of L-NIL using an SDS/KCl precipitation assay (Liu et al., 1983). As expected, treatment of A375 cells with VP-16 (10 μM) significantly increased (6- to 7-fold) the formation of the SDS/KCl precipitate (Fig. 5) over the untreated controls. The presence of L-NIL further increased this complex formation (10-fold), equivalent to the effects of VP-16 alone at 5 times the concentration (50 μM; Fig. 5). This result suggests that inhibition of NO formation in A375 cells by L-NIL increased the DNA damage induced by VP-16 in a Topo II–dependent manner.

To further confirm that NO or NO-derived species were responsible for this decrease in VP-16–mediated Topo II–dependent DNA damage, we performed coculture experiments with induced macrophage RAW cells. Coincubation of A375 cells with induced RAW cells significantly decreased VP-16–dependent SDS-KCl precipitate formation (Fig. 5B). Similar effects were noted when the incubations were performed either simultaneously or with preincubated VP-16 (Fig. 5B). Taken together, these observations strongly suggest that NO and associated nitrogen oxides can react with VP-16, thereby making it less effective at inducing apoptosis and DNA damage and resulting in increased VP-16 resistance toward cancer cells.

Metabolism of VP-16 in A375 Melanoma Cells. To examine the effects of endogenously produced NO on VP-16 metabolism in A375 cells, VP-16 was incubated with A375 cells for various times in the presence or absence of L-NIL. The HPLC analysis (Fig. 6) clearly indicated that VP-16 is rapidly metabolized in A375 cells, and a significant amount of
The presence of L-NIL completely inhibited the quinone formation of NO species with VP-16. Our present studies clearly show that inhibition of formation of NO (and NO-related species) by L-NIL increased caspase-3–dependent apoptosis by VP-16 (2-fold), with a concomitant increase (2.5- to 3.5-fold) in VP-16 cytotoxicity to A375 tumor cells (Fig. 2, A and B; Table 1). Induction of iNOS in A375 cells with LPS further decreased VP-16 cytotoxicity to A375 tumor cells (Fig. 2, A and B; Table 1). These studies taken together strongly suggest that inhibition of formation of NO (and NO-related species) by L-NIL increased caspase-3–dependent apoptosis by VP-16 (2-fold), with a concomitant increase (2.5- to 3.5-fold) in VP-16 cytotoxicity to A375 tumor cells (Fig. 2, A and B; Table 1). Induction of iNOS in A375 cells with LPS further decreased VP-16 cytotoxicity to A375 cells. Interestingly, inhibition of NO/NO-related species by L-NIL resulted in sensitization of cells to VP-16-dependent DNA damage (Sinha et al., 2013).

Discussion

The impact of NOS activity on tumor biology is diverse and dependent on a myriad of host factors (Ambs and Glynn, 2011). Likewise, NO and its nitrogen oxide products can affect cancer therapy either positively or negatively (Hickok and Thomas, 2010). The present study extends our work in vitro to tumor cells and shows significant oxidative chemistry of NO with VP-16.

In our previous study we showed that the reaction of NO/NO species with VP-16 initiates through the obligate intermediary of the 4′-phenoxy radical, with subsequent formation of o-quinone and short-lived VP-16 nitrogen oxide species (Sinha et al., 2013). NO reacts with phenolic compounds (Janzen et al., 1993; Yenes and Messeguer, 1999; Cudic and Ducrocq, 2000), and the initiator in these reactions is believed to be the autoxidation of NO (Liu et al., 1998; Espey et al., 2001) with the formation of the NO2 radical, which can attack the electron-rich phenolic OH group to generate the phenoxy radical (Hogg et al., 1996; Goss et al., 1999).

Interaction of VP-16 with Topo II has been shown to cause both single- and double-strand DNA breaks (Long et al., 1984; Glisson et al., 1986; Sinha et al., 1988), which in the absence of DNA repair results in cell death. Indeed, a significant decrease in VP-16–dependent DNA damage and cell death has been observed in cell lines where the activity of Topo II had been compromised (Glisson et al., 1986; Sinha et al., 1988). Our previous studies clearly showed that products of VP-16 exposed to NO (in the presence of O2) were no longer cytotoxic to HL60 cells and lacked sufficient ability to induce Topo II–dependent DNA damage (Sinha et al., 2013).

To further address the relevance of this NO chemistry observed in vitro, iNOS-expressing human melanoma A375 cells were used to directly elucidate the role of NO in the cytotoxicity of VP-16. Our present studies clearly show that inhibition of formation of NO (and NO-related species) by L-NIL increased caspase-3–dependent apoptosis by VP-16 (2-fold), with a concomitant increase (2.5- to 3.5-fold) in VP-16 cytotoxicity to A375 tumor cells (Fig. 2, A and B; Table 1). Induction of iNOS in A375 cells with LPS further decreased VP-16 cytotoxicity to A375 cells. Interestingly, inhibition of NO/NO-related species by L-NIL resulted in sensitization of cells to VP-16. These studies taken together strongly suggest that NO/NO-related species react with VP-16 and generate products that are significantly less cytotoxic to A375 cells.

Coculture studies performed with LPS-activated macrophage RAW cells (which generate large quantities of NO/NO-related species) show a further (>15-fold) decrease in VP-16 cytotoxicity to A375 cells. This is a very significant finding, as...
this decrease in VP-16 cytotoxicity in the presence of exogenously generated NO from macrophage cells is quite large compared with that found with endogenously generated NO from iNOS catalysis in A375 cells (2.5- to 3.5-fold). Our findings would suggest that even in those cancer cells expressing low levels of iNOS, infiltrating macrophages would further increase VP-16 resistance by increasing detoxification of VP-16 via NO-dependent chemistry. In tumors, the presence of macrophages is well established, and they play significant roles in tumor progression, neovascularization, and poor survival (Jenkins et al., 1995; Lewis and Pollard, 2006; Pollard, 2008; Ambs and Glynn, 2011).

Nitric oxide has been reported to stabilize Bcl2, resulting in decreased cytotoxicity of cis-platinum in A375 cells (Tang and Grimm, 2004; Chanvorachote et al., 2006), thus raising the possibility of a general apoptotic resistance mechanism for...
NO independent of direct VP-16 reactions. However, our current study shows that endogenously/exogenously formed NO (or NO-derived species) reacts with VP-16 to generate less active metabolites of VP-16, resulting in a decrease in both Topo II–mediated DNA damage and cytotoxicity. In contrast, L-NIL had no significant effects on Adr-induced apoptosis or cytotoxicity in A375 cells, which would indicate that Bel2 did not play a role in VP-16 activity.

Inhibition of cellular NO formation by L-NIL caused an increase in intracellular unmodified VP-16, resulting in increased DNA damage and cytotoxicity. Our observations with Adr further confirm that L-NIL did not affect apoptosis nor did it significantly affect its cytotoxicity. Similar observations were also noted with Camptothecin, a Topo I–active drug, where the presence of L-NIL had no significant effect on caspase-3 activity or its cytotoxicity in A375 cells (unpublished observations), lending support to the conclusion that L-NIL did not modulate VP-16 pharmacology by nonspecific effects on caspase-3 or Topo II activities. We conclude, therefore, that iNOS catalysis within A375 tumor cells was sufficient to directly detoxify active VP-16 via NO/NO2-dependent oxidative reactions.

NO readily partitions into a hydrophobic medium, such as the interior of membranes (Liu et al., 1998). This, in combination with a relatively high rate of NO generation from iNOS catalysis, facilitates NO autoxidation and formation of NO2 (Hogg et al., 1996; Goss et al., 1999; Moller et al., 2007). The organic solubility of VP-16 may likewise localize and enhance interactions with reactive nitrogen oxides. The occurrence of both augmented iNOS catalysis (Amb and Glynn, 2011) and alterations in cancer cell plasma membrane architecture (Zhuang et al., 2002; Moller et al., 2007; Miersch et al., 2008) will significantly influence iNOS-dependent detoxification of VP-16 and resistance toward cancer cells.

While in human patients VP-16 is exclusively metabolized by Cyp3A4 in the liver to its dihydroxy derivative, it is possible that VP-16 could also be metabolized by Cyp3A4 in A375 melanoma cells to the catechol derivative and o-quinone. The roles of Cyp3A4 in A375 cells were not investigated in our present study, but inhibition of o-quinone formation by L-NIL would suggest that NO was involved in its formation in A375 cells. The formation of the o-quinone was rapid and decreased significantly with time, suggesting binding to proteins and DNA. The formation of VP-16-o-quinone by NO-dependent pathways is very significant in light of the role of the quinone in the induction of acute myeloid leukemia in patients treated with VP-16. It has been reported that VP-16 metabolites increase Topo II–dependent cleavage near leukemia-associated MLL translocation breakpoints (Lovett et al., 2001). Furthermore, Vlasova et al. (2011) have shown that the VP-16 radical formed from 1-electron oxidation can redox cycle, leading to enhanced Topo II–mediated strand breaks and MLL gene translocation. Our previous studies have shown that VP-16 is readily oxidized by NO chemistry to its phenoxyl radical, an obligatory intermediate in the formation of the VP-16-o-quinone (Sinha et al., 2013).

We conclude from our findings that NO oxidative chemistry occurs in A375 cancer cells and can detoxify VP-16. Our results provide insight into the pharmacology and anticancer activities of VP-16 in tumors that may ultimately contribute to increased resistance and treatment failure. VP-16 is currently used for the treatment of a variety of cancers (e.g., lung, melanoma, and breast) that are known to express iNOS (Chen et al., 2008; Grimm et al., 2008). It is tempting to suggest that the use of VP-16 and related anticancer drugs capable of reacting with NO/NO2 may be ill-advised for patients harboring cancers with intensive iNOS activity. Furthermore, in tumors that do not express significant amounts of iNOS, infiltrating macrophages may further detoxify VP-16 and render tumor cells resistant to VP-16, as shown in our studies with melanoma cells.

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Authorship Contributions
Participated in research design: Sinha, Kumar, Bhattacharjee, Mason.
Conducted experiments: Sinha, Kumar, Bhattacharjee
Contributed to new reagents or analytic tools: Kumar, Bhattacharjee.
Performed data analysis: Sinha, Kumar, Bhattacharjee, Espey.
Wrote or contributed to writing of the manuscript: Sinha, Mason.

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