Ethonafide-Induced Cytotoxicity Is Mediated by Topoisomerase II Inhibition in Prostate Cancer Cells

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Received November 21, 2006; accepted March 7, 2007

ABSTRACT

Ethonafide is an anthracene-containing derivative of amonafide that belongs to the azonafide series of anticancer agents. The lack of cross-resistance in multidrug-resistant cancer cell lines and the absence of a quinine and hydroquinone moiety make ethonafide a potentially less cardiotoxic replacement for existing anthracene-containing anticancer agents. For this study, we investigated the anticancer activity and mechanism of ethonafide in human prostate cancer cell lines. Ethonafide was cytotoxic against three human prostate cancer cell lines at nanomolar concentrations. Ethonafide was found to be better tolerated and more effective at inhibiting tumor growth compared with mitoxantrone in a human xenograft tumor regression mouse model. Mechanistically, we found that ethonafide inhibited topoisomerase II activity by stabilizing the enzyme-DNA complex, involving both topoisomerase IIα and -β. In addition, ethonafide induced a potent G2 cell cycle arrest in the DU 145 human prostate cancer cell line. By creating stable cell lines with decreased expression of topoisomerase IIα or -β, we found that a decrease in topoisomerase IIα protein expression renders the cell line resistant to ethonafide. The decrease in sensitivity to ethonafide was associated with a decrease in DNA damage and an increase in DNA repair as measured by the neutral comet assay. These data demonstrate that ethonafide is a topoisomerase II poison and that it is topoisomerase IIα-specific in the DU 145 human prostate cancer cell line.
similar to the naphthalene-based DNA intercalator amonafide (NSC-308847, Quinamed), but they differ by the addition of a fourth ring, resulting in a planar, anthracene nucleus. Computer modeling studies indicated that the larger chromophore on azonafide allows for greater DNA distortion and intercalation compared with amonafide (Bear and Remers, 1996). Amanofide displayed poor antitumor efficacy and unpredictable toxicity due to variable metabolism by N-acetyltransferase. The N-acetyl metabolite blocked clearance of the parent compound, leading to excess myelotoxicity in patients with a “fast-acetylator phenotype” (Innocenti et al., 2001). Ethonafide lacks a primary amine, and it is not a substrate for N-acetyltransferase. To maximize drug-DNA interactions, an ethoxy group was added at the 6-position of azonafide, creating ethonafide (Fig. 1) (Sami et al., 1996).

Early studies demonstrated that compared with amonafide, ethonafide was, on average, 80 times more potent in 10 different cancer cell lines (Sami et al., 1996; Remers et al., 1997). In addition, mitoxantrone- and doxorubicin-resistant MCF-7 breast cancer cell lines, and the multidrug-resistant (MDR) WiDr colorectal cancer cell line, were not cross-resistant to ethonafide (Sami et al., 1996). These data suggest that ethonafide is not a substrate for the classic MDR-related transporters. In addition, ethonafide does not contain the quinone and hydroquinone moieties that have been suggested to be responsible for doxorubicin- (Adriamycin; Bedford Laboratories, Bedford, OH) and mitoxantrone (Novantronc; Serono, Inc., Rockland, MA)-induced cardiac toxicity, respectively (Minotti et al., 2004; Aviles et al., 2005). In the human tumor cloning assay, ethonafide was, on average, 10-fold more active in preventing colony formation compared with doxorubicin (Dorr et al., 2001). In a SCID mouse xenograft model, ethonafide was more effective than mitoxantrone in inhibiting tumor cell growth in SCID mice bearing MCF-7 breast or A549 non-small cell lung cancer cells (Dorr et al., 2001). These data suggest that ethonafide may provide a more effective therapeutic option compared with currently used topoisomerase II poisons for patients with existing heart conditions or with chemotherapy-resistant cancers.

Mechanistically, ethonafide has been identified as a DNA intercalator, and it has been identified as a topoisomerase II inhibitor in a cell-free assay using purified topoisomerase IIα (Sami et al., 1996; Mayr et al., 1997). It is unknown whether topoisomerase II inhibition is necessary for ethonafide-mediated cytotoxicity in cancer. In addition, the mechanism of topoisomerase II inhibition by ethonafide was unknown. For this study, we evaluated the cytotoxic activity of ethonafide in normal prostate cancer cells and three human prostate cancer cell lines. We also investigated the nature of topoisomerase II inhibition intracellularly and the importance of topoisomerases IIα and IIβ in ethonafide-mediated toxicity in the DU 145 human prostate cancer cell line.

**Materials and Methods**

**Chemicals and Reagents.** The free base and mesylate salt of ethonafide were provided by AmpliMed Corporation (Tucson, AZ). The free base of ethonafide was used for most of the cell culture studies, and it was solubilized in dimethyl sulfoxide. The mesylate salt of ethonafide is a water-soluble form of the free base, and it was used for the xenograft tumor regression study. All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

**Cell Lines and Tissue Culture.** The DU 145, PC-3, and LNCaP human prostate cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cell lines were cultured in RPMI 1640 medium supplemented with 10% bovine calf serum (HyClone Laboratories, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 2 mM L-glutamine (Hyclone). The PReC normal human prostate epithelial cell line was purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Normal prostate cells were cultured in prostate epithelial cell basal medium supplemented with growth factors (bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin-B, and retinoic acid). The NIH/3T3-based PT67 retroviral packaging cell line (Clontech, Palo Alto, CA) was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine.

All cell lines were maintained under standard cell culture conditions at 37°C and 5% CO2 in a humidified environment. Cell lines were periodically assayed for mycoplasma, and they were found to be negative.

**Cytotoxicity Assay.** Mitochondrial activity of living cells was determined by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a blue formazan based upon a method developed by Mosmann as described previously (Mosmann, 1983; Mayr et al., 1997). Data are expressed as the percentage of growth of control cells calculated from absorbance, corrected for background absorbance. The IC50 value is defined as the drug concentration that inhibits growth to 50% of the vehicle-treated control, and it is calculated from sigmoidal analysis of the dose-response curve using Origin version 6.1 software (OriginLab Corp., Northampton, MA).

**Mice.** Male SCID mice (6–8 weeks old) were purchased from the National Cancer Institute Animal Production Program (Frederick, MD). Mice were housed according to guidelines of the American Association for Laboratory Animal Care under protocols approved by the University of Arizona Institutional Animal Care and Use Committee.

**Xenograft Tumor Regression Study.** Approximately 5 × 10⁶ DU 145 human prostate cancer cells were injected subcutaneously into the right rear flank of SCID mice. Once the tumors reached approximately 60 mm³, the mice were randomized so that the average tumor size in each group (n = 8) was approximately 60 mm³. The following day, mice were injected i.p. with either 0.75 or 1.5 mg/kg of the mesylate salt of ethonafide, 0.45 mg/kg mitoxantrone, or 0.9% sterile saline every 4 days for a total of three doses. The 0.45-mg/kg dose of mitoxantrone was the maximally tolerated dose in SCID mice as established previously in our laboratory (Dorr et al., 2001). Tumor volume and mouse weights were measured two to three times a week.
week. Tumor volumes were determined based on the following equation: \( \text{length} \times \text{width}^2 \). In addition, two calculations were conducted to assess antitumor activity based on National Cancer Institute criteria (Bissery et al., 1991). The tumor growth inhibition (TGI) value was calculated once the median tumor size reached 750 mm\(^3\) as follows: TGI (\%) = (median tumor weight of treated group/median tumor weight of control group) \times 100.

According to National Cancer Institute standards, a TGI \( \leq 42\% \) defines an active compound, and a TGI \( > 10\% \) defines a highly active agent. The next parameter is the tumor growth delay (TGD) value, which is based on the median time in days for tumors in all groups to reach 750 mm\(^3\).

**Topoisomerase II Assay.** The inhibition of topoisomerase II was determined by the Topoisomerase II Assay kit (TopoGEN, Inc., Columbus, OH) according to manufacturer’s instructions. This assay measures the decatenation of kinetoplast DNA (kDNA) by the topoisomerase II enzyme in nuclear extracts isolated from prostate cancer cells. For the kDNA decatenation reaction, 1 \( \mu \)g of nuclear extract was combined with 137 ng of catenated kDNA, double-distilled H\(_2\)O, assay buffer containing 0.5 M Tris-HCl, pH 8.0, 1.20 M NaCl, 100 mM MgCl\(_2\), 5 mM dithiothreitol, and 50 mM ATP, and ethonafide. Reactions were incubated for 30 min at 37°C, and then they were stopped with loading buffer and incubated with 50 \( \mu \)g/ml proteinase K for 15 min. The reactions were separated on a 1% agarose gel at 45 V for 2 to 3 h. Gel was stained with ethidium bromide, and kDNA was visualized under UV light.

**Topoisomerase II-DNA Cross-Linking Assay.** Topoisomerase II-DNA cross-linking was measured according to the method described by Nakamura et al. (2002). In brief, DU 145 cells in the exponential growth phase were treated with ethonafide or 50 \( \mu \)M etoposide and incubated for 1 h along with a vehicle control. Cells were collected and lysed in 500 \( \mu \)l of 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA containing 1% Sarkosyl (Sigma-Aldrich). DNA was isolated by layering lysates on a CsCl gradient and subjecting samples to ultracentrifugation at 148,000g for 20 h at 25°C. The DNA-containing fractions were applied to nitrocellulose membrane, using a dot-blot apparatus, and probed using either a rabbit polyclonal antibody to topoisomerase IIa (Cell Signaling Technology Inc., Danvers, MA) or a mouse monoclonal antibody to topoisomerase IIb (BD Biosciences, Franklin Lakes, NJ), and then they were incubated with the appropriate secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). It is important to note that either 1 or 10 \( \mu \)g of DNA was used when probing for topoisomerase IIa or topoisomerase IIb, respectively. The difference in the amounts of DNA required may be due to the fact that the DU 145 prostate cancer cell line expresses high levels of topoisomerase IIa compared with topoisomerase IIb (van Brussel et al., 1999). Proteins were detected using SuperSignal chemiluminescence substrate (Pierce Chemical, Rockford, IL). The figure presented is representative of at least three experiments.

**Measurement of DNA DSBs.** Ethonafide-induced DNA DSBs were determined by neutral comet single-cell gel electrophoresis (CometAssay kit; Trevigen, Gaithersburg, MD) following a modification of a method described by Oshiro et al. (2001). In brief, DU 145 cells in the exponential growth phase were treated with ethonafide or a vehicle-treated control for 1 h. Cells were collected and resuspended in 1% LMAgarose (Trevigen) and layered carefully onto a slide. Slides were then immersed in ice-cold lysis solution (Trevigen) containing 80 \( \mu \)g/ml proteinase K for 1 h, and then they were incubated at 37°C overnight in the same lysis buffer. Slides were washed with 1 \times Tris borate-EDTA (90 mM Tris-HCl, 90 mM boric acid, and 2 mM EDTA, pH 8.0) for 2 h, followed by electrophoresis at 23 V for 20 min. Slides were then rinsed with double-distilled H\(_2\)O and fixed with 70% ethanol. The agar slides were air-dried, and the DNA was visualized using a PVC 2000 confocal microscope (Nikon, Tokyo, Japan) at an excitation/emission wavelength of 494/521 nm, respectively. At least 50 cells were captured per sample, and the comet moment was calculated using CometScore software (TriTek, Sumerduck, VA). The formula for the comet moment is as follows: \( S_{0-n} = (\text{intensity} \times \text{distance})/\text{total intensity} \) (Kent et al., 1995). The comet moment represents the mean \( \pm \) S.E.M. of three independent experiments with 50 cells analyzed per experiment.

**Immunoblot Analysis.** For detection of proteins, nuclear fractions were obtained according to Dignam et al. (1983). The protein content of the lysates was determined using a BCA colorimetric assay (Pierce Chemical). Protein aliquots of 30 \( \mu \)g/sample were denatured and loaded onto a 10% SDS-polyacrylamide gel; proteins were separated by electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), which was probed with antibodies to topoisomerase IIa (Cell Signaling Technology Inc.), topoisomerase IIb (BD Biosciences), or lamin A/C (Cell Signaling Technology Inc.), and then they were incubated with the appropriate secondary antibody (Jackson ImmunoResearch Laboratories Inc.). Proteins were detected using SuperSignal chemiluminescence substrate (Pierce Chemical).

**Cell Cycle Analysis.** For mitotic index analysis, DU 145 human prostate cancer cells were treated with subtoxic concentrations of ethonafide or the microtubule-inhibiting agent demecolcine for 24 h. Samples were incubated with a phospho-histone H3 (Ser10) monoclonal antibody conjugated to Alexa Fluor 488 (Cell Signaling Technology Inc.). Phosphorylation of the amino terminus of histone H3 occurs in the late stages of G2 and extends through mitosis, making it a suitable marker for mitosis (Hendzel et al., 1997). Samples were then counterstained with propidium iodide (PI). Fluorescence was detected using the BD FACSscan flow cytometer (San Jose, CA) and analyzed using Cell Quest software (BD Biosciences, San Jose, CA). Cells were gated based on controls that were incubated with and without the monoclonal antibody to phospho-histone H3. To measure the percentage of cells in G2 and M phases, cells were incubated with PI and an monoclonal antibody to phospho-histone H3 conjugated to Alexa Fluor 488. Phosphorylation of histone H3 is a mitosis-specific marker, and it can differentiate cells that are in M phase from cells in G2 (Hendzel et al., 1997). Cells expressing the phosphorylated form of histone H3 fluoresced in both the FL1 (Alexa Fluor 488) and FL2 (PI) channels. Cells in the G2 phase do not express phosphorylated histone H3; therefore, they fluoresced in the FL2 channel (PI) only.

**Decrease of Topoisomerase II Expression.** Topoisomerase II expression levels were decreased using RNA interference technology from Open Biosystems (Huntsville, AL). The shRNA retroviral expression constructs that targeted either topoisomerase IIa (clone ID V2HS_94079) or topoisomerase IIb (clone ID V2HS_94089) were then transfected into Escherichia coli stocks. The plasmid DNA was purified using the QIAprep Miniprep kit (QIAGEN, Valencia, CA) and verified with restriction digests. The plasmids were transfected into NIH/3T3-based PT67 retroviral packaging cells (Clontech) using Arrest-In transfection reagent (Open Biosystems). In addition to the two plasmids of interest, PT67 cells were also transfected with a scramble sequence control. Transfected PT67 cells were selected with 2 \( \mu \)g/ml puromycin. The retroviral-containing supernatants from the PT67 cells were then transferred to the DU 145 prostate cancer cell line. The infected DU 145 cancer cells were selected with 0.5 \( \mu \)g/ml puromycin and were grown in the presence of 0.5 \( \mu \)g/ml puromycin to maintain selection pressure. Decreased expression of selected proteins was verified by immunoblot analysis.

**Statistical Analysis.** Results are expressed as mean \( \pm \) S.E.M. The statistical significance of the data was determined by one-way ANOVA followed by a Student-Newman-Keuls multiple comparisons test.

**Results**

Ethonafide-Mediated Anticancer Activity in Human Prostate Cancer Cell Lines in Vivo and in vitro. Ethonafide cytotoxicity in three human prostate cancer cell lines...
was determined by MTT cytotoxicity analysis. The IC$_{50}$ value of ethonafide ranged from 40 to 98 nM after a 3-day exposure in the three prostate cancer cell lines (Table 1). Comparison of the cytotoxicity over time demonstrated that ethonafide was schedule-dependent, as demonstrated by the increasing activity observed with longer exposures. To determine the selectivity of ethonafide for cancer cells versus normal prostate epithelial cells, we examined the activity of ethonafide in PReC cells. These cells are nontransformed prostate epithelial cells that will undergo a finite number of doublings in growth factor-supplemented media. PReC cells exposed to ethonafide for 72 h were significantly less sensitive than any of the human prostate cancer cell lines (Table 1).

To determine the antitumor activity of ethonafide in vivo, a xenograft tumor regression model was established using the DU 145 human prostate cancer cell line. Once tumors reached a volume of 60 mm$^3$, mice were treated every 4 days for a total of three doses with the mesylate salt of ethonafide. To determine the antitumor activity of ethonafide in vivo, a xenograft tumor regression model was established using the DU 145 human prostate cancer cell line. A similar effect was observed using nuclear extracts from the PC-3 human prostate cancer cells. DU 145 human prostate cancer cells were treated with ethonafide for 1 h, DNA was isolated by CsCl density gradient, and then DNA was completely inhibited by ethonafide.

**Table 1**

<table>
<thead>
<tr>
<th>Time</th>
<th>DU 145</th>
<th>PC-3</th>
<th>LNCaP</th>
<th>PReC</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.224 (5.4)</td>
<td>2.2 (52.8)</td>
<td>10 (240)</td>
<td>N.D.</td>
</tr>
<tr>
<td>48</td>
<td>0.668 (3.3)</td>
<td>0.450 (20.6)</td>
<td>0.580 (13.4)</td>
<td>N.D.</td>
</tr>
<tr>
<td>72</td>
<td>0.040 (2.9)</td>
<td>0.086 (6.2)</td>
<td>0.098 (7.1)</td>
<td>0.291 (20.9)</td>
</tr>
<tr>
<td>120</td>
<td>0.016 (1.9)</td>
<td>0.028 (3.4)</td>
<td>0.026 (3.1)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

**Table 2**

<p>| Antitumor activity of ethonafide in the xenograft tumor regression model |
|---------------------------------|-------------|-----------------|</p>
<table>
<thead>
<tr>
<th>TGI</th>
<th>TGD</th>
<th>Wt</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>100</td>
<td>0</td>
<td>20.8</td>
</tr>
<tr>
<td>Ethonafide, 0.75 mg/kg</td>
<td>64</td>
<td>10</td>
<td>18.1</td>
</tr>
<tr>
<td>Ethonafide, 1.5 mg/kg</td>
<td>32</td>
<td>17</td>
<td>17.5</td>
</tr>
<tr>
<td>Mitoxantrone, 0.45 mg/kg</td>
<td>61</td>
<td>10</td>
<td>16.6</td>
</tr>
</tbody>
</table>

The TGI value was calculated once the median tumor size reached 750 mm$^3$. The TGD value is based on the median time (in days) for tumors in all groups to reach 750 mm$^3$. All mice weighed approximately 21 to 22 g before drug treatments.
was probed for DNA-associated topoisomerase IIα and -β protein complexes. As demonstrated in Fig. 3B, increasing concentrations of ethonafide led to increased association of both topoisomerase II isoforms with DNA. Based on pixel density analysis, the stabilization of the complex between topoisomerase II and DNA by 10 μM ethonafide is similar to that of 50 μM etoposide (data not shown). These data suggest that, like etoposide, ethonafide is a topoisomerase II poison. Although both isoforms of topoisomerase II are involved in the drug-stabilized cleavable complex, it is unknown whether both isoforms are essential for ethonafide-mediated cytotoxicity.

**Ethonafide-Induced DNA DSBs.** Topoisomerase II poisons stabilize the cleavable complex, leading to the generation of DNA DSBs. DU 145 cells were incubated with ethonafide for 1 h, and they were analyzed for DNA DSBs using the neutral comet assay. The generation of DNA DSBs, defined by the comet moment, directly correlated with increasing concentrations of ethonafide (Fig. 4, A and B). Furthermore, a strong relationship was observed between the concentrations of ethonafide required to induce DNA DSBs and those required for topoisomerase II inhibition and stabilization of the cleavable complex.

**Cell Cycle Analysis.** Topoisomerase II poisons generally induce a pronounced arrest in the G2 phase of the cell cycle, preventing the cells from progressing to M phase (Skoufias et al., 2004). In contrast, the topoisomerase II catalytic inhibitors can cause an M phase arrest, due to the inhibition of topoisomerase II activity without a large induction of DNA DSBs (Skoufias et al., 2004). To investigate the cell cycle effects of ethonafide, the DU 145 human prostate cancer cell line was incubated with subtoxic concentrations of ethonafide, ranging from 10 to 40 nM, for 24 h. Ethonafide treatment of DU 145 cells led to an increase in G2 from 28 to 70%, and an increase in M phase cells from 3 to 7%, at the 40 nM concentration (Fig. 5). The predominant G2 arrest induced by ethonafide is consistent with data demonstrating that ethonafide stabilizes the topoisomerase II-DNA complex and induces DNA DSBs.

**Isoform Specificity of Ethonafide in the DU 145 Cell Line.** To investigate the in vivo isoform specificity of ethonafide, prostate cancer cell lines with reduced expression of
Topoisomerase IIα (shTIIα) or -β (shTIIβ) were created using shRNA. In addition, a cell line was transfected with a non-specific shRNA scramble sequence, referred to as the scramble control, to account for nonspecific effects of the transfection and selection process. As demonstrated by immunoblot analysis of topoisomerase II protein levels, the α and β isoforms were significantly decreased compared with control levels in the shTIIα and shTIIβ cell lines, respectively (Fig. 6A).

The cytotoxic activity of ethonafide was investigated in the wild-type and transfected cell lines using MTT analysis. Figure 6B demonstrates the dose response of the DU 145 cell lines to ethonafide after a 1-day exposure. Compared with the wild-type, scramble control, and the shTIIβ cell lines, reduced expression of topoisomerase IIα rendered the DU 145 cells approximately 15- and 3.8-fold less sensitive to ethonafide after 1- and 3-day exposures, respectively (Table 3). To ensure that the differential activity of ethonafide in the shRNA-transfected cell lines was not simply due to differences in growth rate, we characterized the growth rates of the wild-type and transfected DU 145 cell lines. The cell doubling times for the wild-type, scramble control, and the shTIIβ cell lines were 31, 31, 41, and 48 h, respectively. It is noteworthy that based on the differences in growth rates, cell densities were adjusted for each cell line to allow for similar final cell counts at the termination of each experiment. The growth rates of the cell lines did not correlate with drug sensitivity, because the slowest growing cell line, shTIIβ, was not significantly resistant to ethonafide.

To examine drug-sensitivity of the shRNA transfected cell lines and to ensure that the shTIIα cell line was not generally resistant to drug-induced cell death, the cytotoxicities of several anticancer agents were examined in the four DU 145 cell lines (Table 3). This included an evaluation of the cytotoxicity of the topoisomerase II poison XK469, which has been shown to have an increased affinity for topoisomerase IIβ in cell-free assays and in cell culture (Gao et al., 1999; Snapka et al., 2001), due to the low potency of this agent, only a 3-day exposure cytotoxicity curve was established. The shTIIβ cell line was approximately 5.5-fold less sensitive to XK469 compared with the control cell lines. The shTIIα cell line was approximately 2.4-fold less sensitive to XK469. All four cell lines were equally sensitive to the alkylating agent melphalan and the microtubule-stabilizing agent docetaxel. These data suggest that the topoisomerase II poison resistance observed in the shTIIα cell line is agent-specific and is not due to a decrease in growth rate or resistance to cell death.

We next hypothesized that the decrease in sensitivity of the shTIIα cell line to ethonafide was, in fact, due to an overall decrease in DNA damage. To further correlate cell death with DNA DSBs and implicate topoisomerase IIα as a direct target of ethonafide, we examined the degree of DNA damage induced by ethonafide in the shTIIα and shTIIβ cell lines. The DU 145 cancer cell lines were incubated with 1 μM ethonafide for 1 h, and the DNA damage was investigated using the neutral comet assay. Comparison of the DNA damage between the cell lines demonstrated that ethonafide induced the least amount of DNA damage in the shTIIα cell line (Fig. 7). Removal of ethonafide followed by a 4-h recovery period demonstrated that the shTIIα cell line removed more DNA DSBs compared with the wild-type, scramble control,
Cytotoxicity of ethonafide and other anticancer agents in the topoisomerase II knockdown cell lines

Data are presented in micromolar and represent the mean ± S.E.M. of the IC_{50} of each agent after a 1- or 3-day exposure. The fold resistance, compared with the average of the wild-type and transfected control cell lines, is shown in parentheses. Due to the low potency at short exposures, the IC_{50} values for XK469 and docetaxel were determined after a 3-day exposure to the respective agents.

<table>
<thead>
<tr>
<th>Exposure Time</th>
<th>Agent</th>
<th>Ethonafide</th>
<th>XK469</th>
<th>Mitoxantrone</th>
<th>Melphalan</th>
<th>Docetaxel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>Wild type</td>
<td>0.174 ± 0.010</td>
<td>N.D.</td>
<td>0.024 ± 0.001</td>
<td>137 ± 59.1</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>Scramble control</td>
<td>0.161 ± 0.039</td>
<td>N.D.</td>
<td>0.034 ± 0.005</td>
<td>129 ± 16.0</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>shTII α</td>
<td>2.57 ± 0.27(15.3)</td>
<td>N.D.</td>
<td>1.09 ± 0.235(37.6)</td>
<td>129 ± 26.7 (0.97)</td>
<td>N.D.</td>
</tr>
<tr>
<td>3 day</td>
<td>Wild type</td>
<td>0.027 ± 0.003</td>
<td>109 ± 11.5</td>
<td>0.0019 ± 3 × 10^{-4}</td>
<td>8.9 ± 1.3</td>
<td>0.0019 ± 2 × 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>Scramble control</td>
<td>0.027 ± 0.002</td>
<td>75.5 ± 6.52</td>
<td>0.0024 ± 1 × 10^{-4}</td>
<td>9.16 ± 0.45</td>
<td>0.0029 ± 8 × 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>shTII α</td>
<td>0.103 ± 0.015 (3.8)</td>
<td>224 ± 26.6* (2.4)</td>
<td>0.0071 ± 0.001 (3.3)</td>
<td>8.5 ± 0.67 (0.94)</td>
<td>0.0025 ± 2 × 10^{-4} (1.0)</td>
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<tr>
<td></td>
<td>shTII β</td>
<td>0.046 ± 0.006 (1.7)</td>
<td>508 ± 21.3 (5.5)</td>
<td>0.0075 ± 4 × 10^{-4} (3.5)</td>
<td>9.89 ± 1.16 (1.1)</td>
<td>0.0039 ± 6 × 10^{-4} (1.6)</td>
</tr>
</tbody>
</table>

N.D., not determined.
* Statistical significance was determined by an ANOVA followed by a Student-Newman-Keuls multiple comparisons test compared with wild-type cell line; p < 0.01.
† Statistical significance was determined by an ANOVA followed by a Student-Newman-Keuls multiple comparisons test compared with wild-type cell line; p < 0.001.

Discussion

Unfortunately, the current treatments for HRPC have not dramatically increased overall patient survival (Tannock et al., 2004). Topoisomerase II remains an important target for anticancer therapies not only due to its critical role in mitosis but also because the enzyme is highly up-regulated in neoplastic tissues, including HRPC, compared with normal tissues (Turley et al., 1997; Hughes et al., 2006). The topoisomerase II poison mitoxantrone is currently approved for use as palliative treatment in HRPC. However, its use is limited due to dose-limiting toxicities and the emergence of drug-resistant tumor (Aviles et al., 2005; Trojan et al., 2005; Michels et al., 2006). To identify new treatments for HRPC, we investigated the mechanism of action of the anthracycline-containing anticancer agent ethonafide. Previous cytotoxicity studies demonstrated that ethonafide retains its cytotoxicity in mitoxantrone and doxorubicin-resistant cancer cell lines (Sami et al., 1996). In addition, ethonafide inhibited tumor growth in xenograft tumor models to a greater degree compared with mitoxantrone (Dorr et al., 2001).

Ethonafide demonstrated significant cytotoxicity in three human prostate cancer cell lines. The greatest activity was seen in the androgen-insensitive DU 145 and PC-3 cancer cell lines, both of which have either a mutated or deleted p53 (Sobel and Sadar, 2005). Interestingly, the most resistant cell line was the androgen-sensitive p53 wild-type LNCaP cell line. It is noteworthy that the LNCaP cell line expresses lower basal levels of topoisomerase IIα compared with the DU 145 and PC-3 cell lines, suggesting that ethonafide activity may correlate with topoisomerase IIα expression (van Brussel et al., 1999). This is in contrast to previous reports that have described the LNCaP cell line as being more sensitive to topoisomerase IIα poisons, including mitoxantrone, compared with the DU 145 and PC-3 cell lines (van Brussel et al., 1999). It is unclear whether p53 status and androgen sensitivity play a role in determining sensitivity to ethonafide. Because p53 negatively regulates the transcription of topoisomerase IIα, these data suggest that ethonafide would be an effective therapy in tumors with inactive or deleted p53 (Sami et al., 1996). In addition, ethonafide was less toxic in the normal prostate epithelial cells compared with the three human prostate cancer cell lines. Therefore, the data suggest that ethonafide is more effective in inducing cell death in less differentiated, transformed, cells compared with normal cells. We also found that in vivo ethonafide was more effective at inhibiting tumor growth and was better tolerated compared with mitoxantrone. Ethonafide may represent a more tolerable and more effective agent against androgen-insensitive human prostate cancer compared with mitoxantrone.

Mechanistically, we found that ethonafide inhibited topoisomerase II activity by stabilizing the topoisomerase-DNA complex. This complex may be the transient cleavable complex, stabilization of which is the defining characteristic of topoisomerase II poisons. The specificity of ethonafide for each topoisomerase II isoform was also investigated. The
down-regulation of both isoforms of topoisomerase II has been shown to render cells resistant to topoisomerase II poisons (Errington et al., 1999; Snapka et al., 2001). In addition to topoisomerase II poisons, Emmons et al. (2006) also demonstrated that the decreased expression of topoisomerase IIβ sensitizes cells to melphalan and that the enzyme seems to play a role in the repair of melphalan-induced crosslinks. Therefore, isoform specificity can also aid in designing combination therapies. For example, agents that are specific for topoisomerase IIα can be used with G2 checkpoint-abrogating agents to force cells into a mitotic cell death (Vogel et al., 2005). Agents specific for topoisomerase IIβ can be combined with DNA-damaging agents to enhance cytotoxicity as observed with the knockdown of topoisomerase IIβ (Emmons et al., 2006).

In our model, stable DU 145 cell lines with decreased expression of topoisomerase IIα (shTIIα) or β (shTIIβ) were created using shRNA. We found that the shTIIα cell line was significantly resistant to ethonafide. Interestingly, the shTIIα cell line was still more sensitive to eto

Omar J. Bandele and Dr. Neil Osheroff at Vanderbilt University for the recombinant topoisomerase IIβ enzyme.

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