Etoposide Quinone Is a Covalent Poison of Human Topoisomerase IIβ

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ABSTRACT: Etoposide is a topoisomerase II poison that is utilized to treat a broad spectrum of human cancers. Despite its wide clinical use, 2−3% of patients treated with etoposide eventually develop treatment-related acute myeloid leukemias (t-AMLs) characterized by rearrangements of the MLL gene. The molecular basis underlying the development of these t-AMLs is not well understood; however, previous studies have implicated etoposide metabolites (i.e., etoposide quinone) and topoisomerase IIβ in the leukemogenic process. Although interactions between etoposide quinone and topoisomerase IIβ have been characterized, the effects of the drug metabolite on the activity of human topoisomerase IIβ have not been reported. Thus, we examined the ability of etoposide quinone to poison human topoisomerase IIβ. The quinone induced ∼4 times more enzyme-mediated DNA cleavage than did the parent drug. Furthermore, the potency of etoposide quinone was ∼2 times greater against topoisomerase IIβ than it was against topoisomerase IIα, and the drug reacted ∼2−4 times faster with the β isoform. Etoposide quinone induced a higher ratio of double- to single-stranded breaks than etoposide, and its activity was less dependent on ATP. Whereas etoposide acts as an interfacial topoisomerase II poison, etoposide quinone displayed all of the hallmarks of a covalent poison: the activity of the metabolite was abolished by reducing agents, and the compound inactivated topoisomerase IIβ when it was incubated with the enzyme prior to the addition of DNA. These results are consistent with the hypothesis that etoposide quinone contributes to etoposide-related leukemogenesis through an interaction with topoisomerase IIβ.

Etoposide is an integral component of chemotherapeutic regimens that are used to treat hematological malignancies, somatic tumors, germ cell tumors, and other human cancers.1−6 The drug targets type II topoisomerases, enzymes that generate transient double-stranded breaks in the double helix.5,7−11 These enzymes regulate DNA supercoiling and remove knots and tangles from the genome. Etoposide kills cells by inhibiting the ability of type II topoisomerases to ligate DNA, which leads to the accumulation of double-stranded breaks in the genome.1−6 These breaks induce DNA recombination−repair processes and have the potential to activate apoptosis.3,5,8,12 However, if cells survive drug treatment, they may carry stable chromosomal translocations or other rearrangements.3,8,12−17

Despite the wide use of etoposide, there is a well-established correlation between chemotherapeutic regimens that include the drug and the development of therapy-related acute myeloid leukemias (t-AMLs) that feature rearrangements in the MLL (mixed lineage leukemia) gene at chromosomal band 11q23.12,14,17−24 Initially, as many as 12% of patients treated with etoposide developed t-AMLs.19−22,25 Once high-risk schedules were identified and eliminated, that number subsequently dropped to ∼2−3%.19−22,26

Several studies suggest that therapy-related leukemic translocation breakpoints in MLL are derived directly from chromosomal breaks generated by type II topoisomerases.3,12−15,17,27 However, the molecular events that link the initiating DNA cleavage event and the resulting translocation are not well defined. Recent work indicates that topoisomerase II-mediated DNA strand breaks are processed and eventually resected by an alternative nonhomologous end joining pathway.12,28−32 Furthermore, there is evidence that the induction of t-AMLs following etoposide treatment is influenced by the ability of cells to metabolize the drug and the ability of these metabolites to interact with one of the two topoisomerase II isoforms.12,33,34

Etoposide can be metabolized by a number of cellular pathways.35−38 In a cytochrome P450-mediated pathway (Figure 1), one of the two methoxy groups on the E-ring of etoposide is converted to a hydroxyl moiety by oxidation by CYP3A4. The resulting etoposide catechol can be further oxidized to a quinone metabolite by the actions of myeloperoxidase and other oxidases (Figure 1).36−41 The high concentration of myeloperoxidase in hematopoietic cells (∼3% of the cell by weight) is consistent with a role for etoposide quinone in the generation of leukemic chromosomal translocations.27,33,42,43 A further epidemiological study has linked a polymorphism in the 5′-promoter region of CYP3A4 (i.e., CYP3A4-V) with a lower risk of t-AMLs that involve MLL
gene translocations. This polymorphism is believed to decrease the rate of cytochrome P450-mediated production of etoposide catabolism, lending further credence to the postulated role for etoposide metabolites in the leukemogenic process.

Human cells encode two isoforms of topoisomerase II, α and β. These isoforms share extensive amino acid sequence identity (~70%), but have distinct patterns of expression and separate nuclear functions. Topoisomerase IIα is essential for the survival of proliferating cells, and its expression is proliferation-dependent. In contrast, topoisomerase IIβ is dispensable at the cellular level, and its presence cannot compensate for the loss of topoisomerase IIα in human cells. The cellular concentration of topoisomerase IIβ is independent of proliferation status, and the enzyme appears to play an important role in transcription.

All clinically used topoisomerase II-targeted anticancer drugs affect the activities of both enzyme isoforms. However, the degree to which topoisomerase IIα and IIβ are targeted by any given drug and the relative contributions of either isoform to the curative effects of drugs are not well understood. The above notwithstanding, in vivo and cellular studies suggest that topoisomerase IIβ is the enzyme primarily responsible for generating the breaks in MLL that initiate t-AMLs. First, in a skin carcinogenesis model, the incidence of secondary malignancies was greatly diminished in a skin-specific top2b-knockout mouse. Second, in a murine cell model, etoposide-induced DNA sequence rearrangements and double-strand breaks were dependent on the presence of topoisomerase IIβ. Third, in a human cellular system, the majority of MLL breaks generated by etoposide, as well as the genotoxic effects of the drug, appeared to be mediated primarily by topoisomerase IIβ.

Despite the proposed roles of etoposide metabolites and topoisomerase IIβ in the induction of t-AMLs, the effects of etoposide quinone on this isoform have not yet been described. Therefore, we characterized the ability of etoposide quinone to alter enzyme-mediated DNA cleavage and ligation. Results indicate that the quinone induces ~4 times more DNA cleavage than etoposide and appears to function by a different mechanism than that of the parent drug. These findings support a role for etoposide metabolites and topoisomerase IIβ in etoposide-associated t-AMLs.

### Experimental Procedures

**Enzymes and Materials.** Human topoisomerase IIβ was expressed in *Saccharomyces cerevisiae* JEL1Δtop1 cells and purified as described previously. The enzyme was stored at −70 °C as a 1.5 mg/mL (4 μM) stock in 50 mM Tris-HCl (pH 7.7), 0.1 mM EDTA, 750 mM KCl, 5% glycerol, and 8 μM DTT (carried from the enzyme preparation). Negatively supercoiled pBR322 DNA was prepared using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Etoposide was obtained from Sigma-Aldrich. Drugs were prepared as 20 mM solutions in 100% DMSO and stored at −70 °C.

**Synthesis of Etoposide Quinone.** Etoposide quinone was synthesized and purified according to previously published procedures with slight modifications. The purity was determined to be >99% by liquid chromatography–mass spectrometry analysis at 220 and 254 nm, and the final yield of etoposide quinone was 72%.

**DNA Cleavage Mediated by Topoisomerase IIβ.** DNA cleavage reactions were performed using the procedure described by Fortune and Osherson. Reaction mixtures contained 100 nM human topoisomerase IIβ and 10 nM negatively supercoiled pBR322 DNA in 20 μL of 10 mM Tris-HCl (pH 7.9), 5 mM MgCl₂, 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol. Final reaction mixtures contained ~0.4 μM DTT, which represents the residual DTT from the enzyme preparation. DNA cleavage reactions were carried out in the absence of the compound or in the presence of 0–30 μM etoposide or etoposide quinone as indicated. In some cases, 50 μM DTT or 1 mM ATP was added to reaction mixtures. Unless stated otherwise, assays were started by the addition of drug, and DNA cleavage mixtures were incubated for 6 min at 37 °C.

DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS followed by 1 μL of 375 mM Na₂EDTA (pH 8.0). Proteinase K was added (2 μL of a 0.8 mg/mL solution), and reaction mixtures were incubated for 30 min at 45 °C to digest topoisomerase IIβ. Samples were mixed with 2 μL of agarose gel loading buffer [60% sucrose in 10 mM Tris-HCl (pH 7.9)], heated for 2 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. Double-stranded DNA cleavage was monitored by the conversion of negatively supercoiled plasmid DNA to linear molecules. DNA bands were visualized by UV light and quantified using an Alpha Innotech digital imaging system.
To examine the potential effects of drug–DNA adduction on topoisomerase IIβ-mediated scission, 0.6 μg of pBR322 DNA was incubated with 30 μM etoposide quinone for 6 min at 37 °C in the absence of enzyme. Samples were then applied to a DNA Spin Column (Qiagen) and processed according to the manufacturer’s protocol. DNA was eluted and added to DNA cleavage reaction mixtures.

DNA Ligation Mediated by Topoisomerase IIβ. DNA cleavage–ligation equilibria were established as described above for 6 min at 37 °C in the absence or presence of 30 μM etoposide quinone.57 Ligation was initiated by cooling samples from 37 to 0 °C. Reactions were stopped at time points ranging from 0 to 30 s by the addition of 2 μL of 5% SDS followed by 1 μL of 375 mM Na2EDTA (pH 8.0). Samples were treated with Proteinase K, mixed with agarose gel loading buffer, processed, and analyzed as described above. The amount of linear DNA cleavage product at time zero was set to 100%, and DNA ligation was monitored by the loss of linear DNA.

### RESULTS AND DISCUSSION

Etoposide is one of the most well studied topoisomerase II-targeted agents in clinical use.1–6 The drug stabilizes covalent topoisomerase II-cleaved DNA complexes (i.e., cleavage complexes) by interacting at the enzyme–DNA interface in a noncovalent manner.2–6,59,60 Once the double helix is cut, the drug slips (i.e., intercalates) between the 3′- hydroxyl and the enzyme-linked 5′-phosphate at the cleaved scissile bond and acts as a physical block to topoisomerase II-mediated DNA ligation.60,61 Etoposide and other drugs that utilize this mechanism are termed “interfacial topoisomerase II poisons”.6,62

The effects of etoposide catechol and etoposide quinone on human topoisomerase IIα have been examined.27,30,41,57,63,64 The catechol displayed properties that were similar to those of the parent drug and appeared to be an interfacial poison. In contrast, the properties of the quinone metabolite differed from those of etoposide, and the quinone appeared to function by a different mechanism.57 Previous studies with quinones and other protein-reactive agents have found that some of these compounds increase levels of topoisomerase II-mediated DNA cleavage by covalently adding to the enzyme at residues that are distal to the active site.3,65–70 Thus, these agents are termed “covalent topoisomerase II poisons”.70 It is believed that covalent poisons enhance DNA cleavage, at least in part, by blocking the N-terminal gate of the protein.68,70–72 Several lines of evidence suggest that etoposide quinone poisons topoisomerase IIβ by this latter, covalent mechanism.57

As discussed above, topoisomerase IIβ appears to be the isoform largely responsible for initiating the chromosomal breaks that trigger MLL-associated t-AMLs.12,17,34,52 Because of the proposed role of etoposide quinone in this leukemogenic process, we characterized the effects of the metabolite on the DNA cleavage reaction mediated by human topoisomerase IIβ.

### Effects of Etoposide Quinone on DNA Cleavage Mediated by Human Topoisomerase IIβ

The activity of etoposide quinone against topoisomerase IIβ was considerably higher than that of etoposide (Figure 2, left). The quinone increased the relative level of double-stranded DNA cleavage ∼14-fold, which plateaued at ∼17.5 μM, while etoposide increased the level of cleavage only 3-fold at similar concentrations. The efficacy of etoposide quinone against topoisomerase IIβ was similar to that reported with the α isoform.57 However, the metabolite was ∼2 times more potent against topoisomerase IIβ. Furthermore, etoposide quinone reacted more rapidly with the β isoform, inducing maximal DNA cleavage in 2.5–5 min (as opposed to ∼10 min with topoisomerase IIα).57 (Figure 2, right).

Because covalent topoisomerase II poisons require protein-reactive groups, their activity can be suppressed by the presence of reducing agents.57,65,66,70,71,73 Therefore, the effects of 50 μM DTT on the activity of etoposide quinone (which should reduce the metabolite to the “unreactive” catechol) were examined. The ability of the quinone to induce topoisomerase IIβ-mediated DNA cleavage decreased precipitously in the presence of the reducing agent (Figure 2). In contrast, DTT had no effect on the activity of the parent drug, which acts by the interfacial mechanism (Figure 2, left). These findings imply that etoposide quinone is a covalent poison of topoisomerase IIβ.

Several control reactions were conducted to ensure that the DNA cleavage enhancement observed with etoposide quinone was mediated by topoisomerase IIβ (Figure 3). No DNA scission was seen in the presence of the quinone when the type II enzyme was omitted from reactions. Moreover, cleaved DNA products were covalently linked to topoisomerase IIβ. In the absence of proteinase K, the linear DNA band disappeared and was replaced by a band that remained at the origin of the gel (not shown). Finally, DNA cleavage induced by the drug metabolite was reversed when the active site Mg2+ ions were chelated with EDTA or the reaction mixture was treated with 0.5 M salt prior to trapping cleavage complexes with SDS.74 EDTA cannot chelate Mg2+ in the cleavage complex and can only sequester the metal ion after the DNA has been ligated. Similarly, the presence of an increased salt concentration will lead to dissociation of the enzyme–DNA complex only after the nucleic acid has been ligated. The fact that EDTA and salt can “reverse” cleavage demonstrates that the DNA scission observed reflects an enzyme-mediated cleavage–ligation equilibrium rather than an enzyme-independent reaction. These results establish that the DNA scission observed in the presence of etoposide quinone is mediated by topoisomerase IIβ.
Etoposide, as well as many covalent topoisomerase II poisons, increases the level of enzyme−DNA cleavage complexes primarily by inhibiting the DNA ligation activity of the enzyme.57,66,70,71,73 Like its parent compound, etoposide quinone severely inhibited DNA ligation mediated by human topoisomerase IIβ (Figure 4). Etoposide Quinone Acts Primarily as a Covalent Poison of Human Topoisomerase IIβ. The fact that a reducing agent severely diminishes the activity of etoposide quinone strongly suggests that the drug metabolite is a covalent poison of topoisomerase IIβ. However, the activity of etoposide is highly sensitive to changes in the substituents on the E-ring (Figure 1).59,75,76 Consequently, an alternative hypothesis is that the presence of the 3′- and/or 4′-carbonyl groups on etoposide quinone converts the metabolite into an interfacial poison that is more potent and efficacious than the parent drug (or the catechol).

Therefore, several experiments were performed to resolve this important issue. First, we examined the effects of ATP on the ability of etoposide quinone to induce DNA cleavage mediated by human topoisomerase IIβ. Previous studies have demonstrated that etoposide requires ATP for maximal DNA cleavage activity with the α isof orm.57,77 A similar result was seen with topoisomerase IIβ (Figure 5, left). Up to 30 μM etoposide, levels of drug-induced DNA cleavage were 2−5-fold higher in reaction mixtures that contained ATP compared to those that did not. In contrast, no such effect was seen with etoposide quinone and the β isof orm (Figure 5, right). In fact, levels of DNA cleavage induced in the presence of ATP were similar to or lower than those seen in the absence of the cofactor.

Second, we examined the ability of etoposide quinone to generate double-stranded versus single-stranded DNA breaks with topoisomerase IIβ (Figure 6). For the parent drug to stabilize topoisomerase II-generated double-stranded breaks, an etoposide molecule must intercalate between the newly formed DNA termini at each cleaved scissile bond.60,61,78 Because the two drug molecules appear to bind independently, a high

Figure 3. Etoposide quinone induces DNA cleavage via an enzyme-mediated mechanism. Control reactions were conducted in the absence of enzyme or drug (DNA Control), in the presence of 30 μM etoposide quinone without enzyme (+EQ−hTIIβ), or in the presence of topoisomerase IIβ without drug (−EQ+hTIIβ). All other reaction mixtures contained topoisomerase IIβ and 30 μM etoposide quinone. DNA cleavage reactions were terminated by the addition of SDS (+EQ+hTIIβ). To determine whether cleaved DNA was protein-linked, proteinase K treatment was omitted (−ProK). The reversibility of DNA cleavage was examined by adding EDTA (EDTA) or 0.5 M salt (NaCl) prior to SDS. The level of enzyme-mediated DNA cleavage in the absence of etoposide quinone was set to 1 in the bottom panel, and all other reactions were expressed relative to that value. Error bars represent standard deviations for three independent experiments. A representative agarose gel stained with ethidium bromide is shown at the top. The positions of supercoiled (form I, FII), nicked circular (form II, FII), and linear (form III, FII) molecules are indicated at the left.

Etoposide quinone does not require ATP to induce optimal DNA cleavage mediated by topoisomerase IIβ. DNA cleavage reactions of etoposide (left panel, Etop, red) or etoposide quinone (right panel, EQ, blue) were carried out in the absence (closed bars) or presence (open bars) of 0.25 mM ATP. Control reactions conducted in the absence of drug are shown (ND). Error bars represent the standard deviation of three independent experiments.

Figure 4. Etoposide quinone inhibits DNA ligation mediated by topoisomerase IIβ. DNA cleavage reactions were initiated in the absence (open circles, ND) or presence (closed circles, EQ) of 30 μM etoposide quinone. The DNA cleavage−ligation equilibrium was established at 37 °C, and ligation was initiated by cooling samples to 0 °C. The level of DNA cleavage observed at equilibrium for each reaction was set to 100% at time zero. Error bars represent the standard deviation of three independent experiments.

Figure 5. Etoposide quinone does not require ATP to induce optimal DNA cleavage mediated by topoisomerase IIβ. DNA cleavage reactions of etoposide (left panel, Etop, red) or etoposide quinone (right panel, EQ, blue) were carried out in the absence (closed bars) or presence (open bars) of 0.25 mM ATP. Control reactions conducted in the absence of drug are shown (ND). Error bars represent the standard deviation of three independent experiments.
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Figure 6. Etoposide quinone induces a high ratio of double-stranded DNA breaks (DSB) to single-stranded DNA breaks (SSB). DNA strand breaks generated by human topoisomerase IIβ were monitored in reaction mixtures containing no drug (ND, black), 30 μM etoposide (Etop, red), or 15 μM etoposide quinone (EQ, blue). Double- and single-stranded DNA cleavage was monitored by the conversion of negatively supercoiled plasmid DNA to linear and nicked molecules, respectively. Error bars represent the standard deviation of three independent experiments. Results of an unpaired two-tailed t test are shown (**p = 0.001).

Figure 7. Etoposide quinone inactivates human topoisomerase IIβ when incubated with the enzyme prior to the addition of DNA. The enzyme was incubated in the presence of 15 μM etoposide quinone (closed circles, blue) prior to a DNA cleavage assay. The inset shows cleavage levels established following incubation for 6 min in the absence of drug (ND, black bar) or in the presence of 30 μM etoposide (Etop, red bar), 15 μM etoposide quinone and DTT (EQ + DTT, open blue bar), or 15 μM etoposide quinone in the absence of DTT (EQ, blue bar). Error bars represent the standard deviation of three independent experiments.

Figure 8. Etoposide quinone is a covalent poison of topoisomerase IIβ. In the left panel, etoposide quinone enhancement of DNA cleavage is not reversed by the addition of reducing agents after DNA cleavage complexes have been established. DNA cleavage reactions were carried out in the absence (blue bars) or presence (open blue bars) of DTT. Reaction mixtures contained no drug (ND) or 30 μM etoposide quinone in mixtures that included DTT at the time of DNA cleavage (Pre EQ) or that was added (for an additional 6 min) after cleavage complexes were formed (Post EQ). In the right panel, etoposide quinone does not form DNA lesions that poison topoisomerase IIβ. DNA was incubated without (−EQ, open orange bars) or with (+EQ, orange bars) 30 μM etoposide quinone. DNA was purified from free drug and used in DNA cleavage reactions mediated by topoisomerase IIβ. DNA cleavage reactions were performed in the absence of drug (ND) or in the presence of 30 μM etoposide quinone (EQ). In all cases, error bars represent the standard deviation of three independent experiments.

The proportion of cleavage complexes established at clinically relevant (i.e., subsaturating) concentrations of etoposide contain only one cleaved DNA strand. Thus, under these conditions, etoposide routinely induces high levels of single-stranded DNA breaks. With human topoisomerase IIα, double-stranded:single-stranded break ratios as low as 0.5:1 frequently are observed in the presence of etoposide. Although the effect is less dramatic with the β isomor, etoposide still generates approximately equimolar levels of double- and single-stranded DNA breaks (Figure 6 shows results for 30 μM etoposide). In contrast, at a drug concentration (15 μM) that was lower than that used for etoposide, the quinone still generated approximately two double-stranded breaks for every single-stranded cut. Taken with the ATP results, these findings suggest that etoposide quinone induces topoisomerase IIβ-mediated DNA cleavage by a mechanism different than that of the parent drug.

Third, covalent topoisomerase II poisons display the hallmark characteristic of inactivating the enzyme when the two are incubated prior to the addition of DNA. This inactivation is not observed with interfacial poisons. As seen in Figure 7, incubation of 15 μM etoposide quinone with human topoisomerase IIβ rapidly inactivated the enzyme. The DNA cleavage activity was decreased by more than 90% following a 6 min incubation of the drug with the enzyme (t1/2 ≤ 1.5 min). Under parallel conditions (6 min incubation), little enzyme inactivation was observed in the presence of etoposide or a mixture of etoposide quinone and DTT (Figure 7, inset).

Fourth, once a covalent poison has adducted topoisomerase II and stimulated DNA cleavage, the redox state of the poison (quinone vs catechol) no longer appears to matter. Thus, if a reducing agent is added to reaction mixtures after the DNA cleavage–ligation equilibrium has been established in the presence of the poison, it will not reverse the cleavage enhancement. To determine whether this was the case for etoposide quinone, an order of addition experiment was carried out. As seen in Figure 8 (left), once cleavage complexes were established in the presence of the quinone, the addition of DTT did not affect levels of DNA scission. This is in contrast to results seen when DTT was added to reaction mixtures prior to the generation of cleavage complexes (Figure 2 and Figure 8, right).
left). Once again, these results are consistent with a topoisomerase II adduction mechanism for DNA cleavage enhancement by etoposide quinone.

In addition to modifying proteins, etoposide quinone also can form covalent nucleic acid adducts, especially with N7 of guanine residues.27 Because alkylated DNA lesions can enhance DNA cleavage mediated by human topoisomerase IIβ,28 it is possible that etoposide quinone stimulates the reaction by a mechanism that involves DNA, rather than protein, adduction. To address this possibility, DNA was incubated with 30 μM etoposide quinone for 6 min at 37 °C and then purified prior to cleavage assays. The incubation had no effect on the ability of topoisomerase IIβ to cleave DNA regardless of whether etoposide quinone was added to final reaction mixtures (Figure 8, right). Together with all of the data presented above, we conclude that etoposide quinone enhances DNA cleavage mediated by human topoisomerase IIβ primarily by a mechanism that involves addition to the enzyme.

■ CONCLUSIONS

Previous studies have identified a role for etoposide metabolites and topoisomerase IIβ in the initiation of t-AMLs associated with anticancer regimens that contain etoposide. However, the effects of etoposide quinone on the activity of the β isoform have not been described. Results indicate that the quinone is a potent topoisomerase IIβ poison that induces higher levels of enzyme-mediated DNA cleavage than does the parent drug. Etoposide quinone also displays higher reactivity toward topoisomerase IIβ than it does with topoisomerase Iiz. Finally, the metabolite induces DNA cleavage primarily by a mechanism that differs from that of etoposide and appears to involve covalent modification of the enzyme. These findings are consistent with the hypothesis that the oxidative environment of hematopoietic progenitor cells generates a highly reactive etoposide metabolite that contributes to the generation of DNA breakpoints with leukemogenic potential.

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Funding
This research was supported by Grants GM033944 (N.O.) and T35 ES016534 (N.A.S.) from the National Institutes of Health and funds from the Lipscomb University College of Pharmacy and Health Sciences (S.L.M. and J.E.D.).

Notes
The authors declare no competing financial interests.

■ ACKNOWLEDGMENTS

We are grateful to Katie J. Aldred, MaryJean Pendleton, Rachel E. Ashley, Kendra R. Vann, and Joel H. Everett for critical reading of the manuscript. N.A.S. was a participant in the Vanderbilt University School of Medicine Emphasis Program.

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