



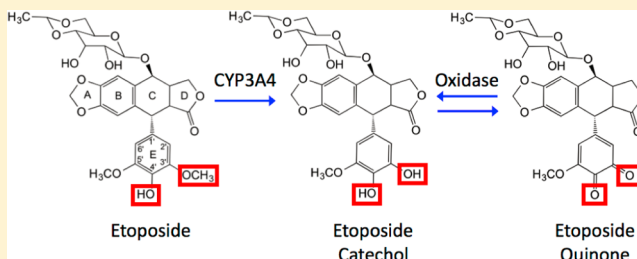
## Etoposide Quinone Is a Covalent Poison of Human Topoisomerase II $\beta$

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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 II $\beta$  in the leukemogenic process. Although interactions between etoposide quinone and topoisomerase II $\alpha$  have been characterized, the effects of the drug metabolite on the activity of human topoisomerase II $\beta$  have not been reported. Thus, we examined the ability of etoposide quinone to poison human topoisomerase II $\beta$ . The quinone induced  $\sim 4$  times more enzyme-mediated DNA cleavage than did the parent drug. Furthermore, the potency of etoposide quinone was  $\sim 2$  times greater against topoisomerase II $\beta$  than it was against topoisomerase II $\alpha$ , and the drug reacted  $\sim 2$ – $4$  times faster with the  $\beta$  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 II $\beta$  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 II $\beta$ .



Etoposide is an integral component of chemotherapeutic regimens that are used to treat hematological malignancies, somatic tumors, germ cell tumors, and other human cancers.<sup>1–6</sup> The drug targets type II topoisomerases, enzymes that generate transient double-stranded breaks in the double helix.<sup>3,5,7–11</sup> 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.<sup>1–6</sup> These breaks induce DNA recombination–repair processes and have the potential to activate apoptosis.<sup>3,5,8,12</sup> However, if cells survive drug treatment, they may carry stable chromosomal translocations or other rearrangements.<sup>3,8,12–17</sup>

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.<sup>12–14,17–24</sup> Initially, as many as 12% of patients treated with etoposide developed t-AMLs.<sup>19–22,25</sup> Once high-risk schedules were identified and eliminated, that number subsequently dropped to  $\sim 2$ – $3$ %.<sup>19–22,26</sup>

Several studies suggest that therapy-related leukemic translocation breakpoints in *MLL* are derived directly from chromosomal breaks generated by type II topoisomerases.<sup>3,12–15,17,27</sup> 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.<sup>12,28–32</sup> 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.<sup>12,33,34</sup>

Etoposide can be metabolized by a number of cellular pathways.<sup>35–38</sup> 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).<sup>36–41</sup> The high concentration of myeloperoxidase in hematopoietic cells ( $\sim 3$ % of the cell by weight) is consistent with a role for etoposide quinone in the generation of leukemic chromosomal translocations.<sup>27,33,42,43</sup> 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*

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To examine the potential effects of drug–DNA adduction on topoisomerase II $\beta$ -mediated scission, 0.6  $\mu$ g of pBR322 DNA was incubated with 30  $\mu$ M etoposide quinone for 6 min at 37  $^{\circ}$ 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 II $\beta$ .** DNA cleavage–ligation equilibria were established as described above for 6 min at 37  $^{\circ}$ C in the absence or presence of 30  $\mu$ M etoposide quinone.<sup>57</sup> Ligation was initiated by cooling samples from 37 to 0  $^{\circ}$ C. Reactions were stopped at time points ranging from 0 to 30 s by the addition of 2  $\mu$ L of 5% SDS followed by 1  $\mu$ L of 375 mM Na<sub>2</sub>EDTA (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.

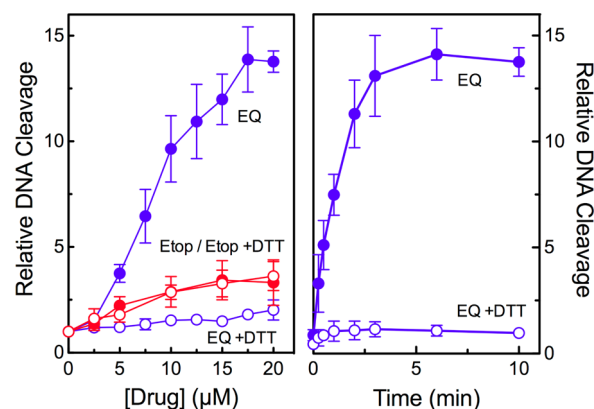
## RESULTS AND DISCUSSION

Etoposide is one of the most well studied topoisomerase II-targeted agents in clinical use.<sup>1–6</sup> The drug stabilizes covalent topoisomerase II-cleaved DNA complexes (i.e., cleavage complexes) by interacting at the enzyme–DNA interface in a noncovalent manner.<sup>2–6,59,60</sup> 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.<sup>60,61</sup> Etoposide and other drugs that utilize this mechanism are termed "interfacial topoisomerase II poisons".<sup>6,62</sup>

The effects of etoposide catechol and etoposide quinone on human topoisomerase II $\alpha$  have been examined.<sup>27,40,41,57,63,64</sup> 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.<sup>57</sup> 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 adducting to the enzyme at residues that are distal to the active site.<sup>3,65–70</sup> Thus, these agents are termed "covalent topoisomerase II poisons".<sup>70</sup> It is believed that covalent poisons enhance DNA cleavage, at least in part, by closing the N-terminal gate of the protein.<sup>68,70–72</sup> Several lines of evidence suggest that etoposide quinone poisons topoisomerase II $\alpha$  by this latter, covalent mechanism.<sup>57</sup>

As discussed above, topoisomerase II $\beta$  appears to be the isoform largely responsible for initiating the chromosomal breaks that trigger MLL-associated t-AMLs.<sup>12,17,34,52</sup> 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 II $\beta$ .

**Effects of Etoposide Quinone on DNA Cleavage Mediated by Human Topoisomerase II $\beta$ .** The activity of etoposide quinone against topoisomerase II $\beta$  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  $\mu$ M, while etoposide increased the level of cleavage only 3-fold at similar concentrations. The efficacy of etoposide quinone against topoisomerase II $\beta$  was similar to that reported with the  $\alpha$  isoform.<sup>57</sup> However, the metabolite was ~2 times more potent



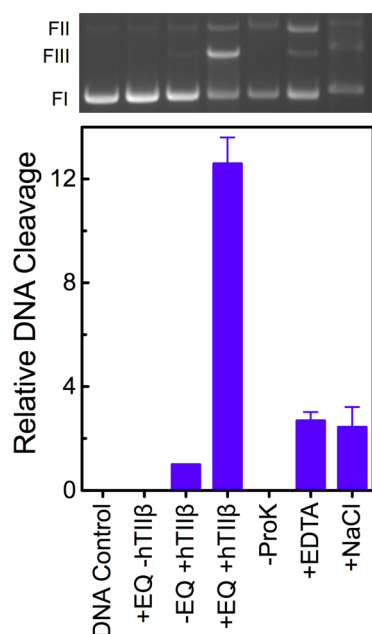
**Figure 2.** Etoposide quinone enhances DNA cleavage mediated by human topoisomerase II $\beta$ . DNA cleavage was carried out in the presence of etoposide (Etop, red) or etoposide quinone (EQ, blue) in the absence (closed circles) or presence (open circles) of 50  $\mu$ M dithiothreitol (DTT). The left panel shows drug titrations, and the right panel shows a time course for DNA cleavage in the presence of 15  $\mu$ M etoposide quinone. Error bars represent the standard deviation of three or more independent experiments.

against topoisomerase II $\beta$ . Furthermore, etoposide quinone reacted more rapidly with the  $\beta$  isoform, inducing maximal DNA cleavage in 2.5–5 min (as opposed to ~10 min with topoisomerase II $\alpha$ <sup>57</sup>) (Figure 2, right).

Because covalent topoisomerase II poisons require protein-reactive groups, their activity can be suppressed by the presence of reducing agents.<sup>57,65,66,70,71,73</sup> Therefore, the effects of 50  $\mu$ M 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 II $\beta$ -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 II $\beta$ .

Several control reactions were conducted to ensure that the DNA cleavage enhancement observed with etoposide quinone was mediated by topoisomerase II $\beta$  (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 II $\beta$ . 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 Mg<sup>2+</sup> ions were chelated with EDTA or the reaction mixture was treated with 0.5 M salt prior to trapping cleavage complexes with SDS.<sup>74</sup> EDTA cannot chelate Mg<sup>2+</sup> 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 II $\beta$ .



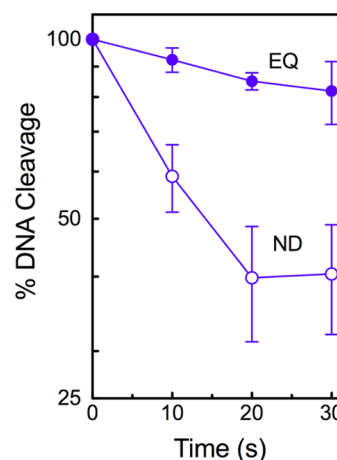


**Figure 3.** Etoposide quinone induces DNA cleavage via an enzyme-mediated mechanism. Control reactions were conducted in the absence of enzyme or drug (DNA Control), in the presence of 30  $\mu$ M etoposide quinone without enzyme (+EQ -hTII $\beta$ ), or in the presence of topoisomerase II $\beta$  without drug (-EQ +hTII $\beta$ ). All other reaction mixtures contained topoisomerase II $\beta$  and 30  $\mu$ M etoposide quinone. DNA cleavage reactions were terminated by the addition of SDS (+EQ +hTII $\beta$ ). 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, FI), nicked circular (form II, FII), and linear (form III, FIII) molecules are indicated at the left.

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.<sup>57,66,70,71,73</sup> Like its parent compound, etoposide quinone severely inhibited DNA ligation mediated by human topoisomerase II $\beta$  (Figure 4).

**Etoposide Quinone Acts Primarily as a Covalent Poison of Human Topoisomerase II $\beta$ .** 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 II $\beta$ . However, the activity of etoposide is highly sensitive to changes in the substituents on the E-ring (Figure 1).<sup>59,75,76</sup> 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).

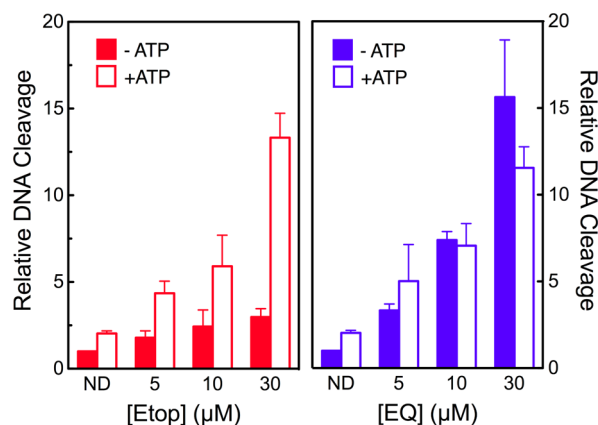
Therefore, several experiments were performed to resolve this important issue. First, we examined the effects of ATP on the ability of etoposide quinone to induce DNA cleavage mediated by human topoisomerase II $\beta$ . Previous studies have demonstrated that etoposide requires ATP for maximal DNA cleavage activity with the  $\alpha$  isoform.<sup>57,77</sup> A similar result was



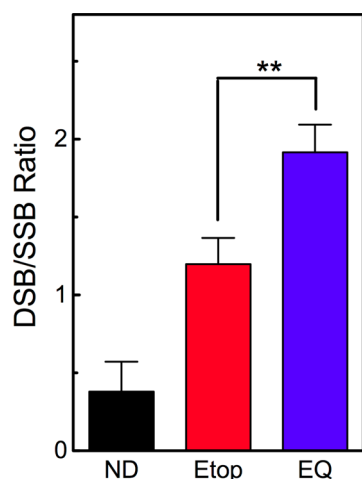
**Figure 4.** Etoposide quinone inhibits DNA ligation mediated by topoisomerase II $\beta$ . DNA cleavage reactions were initiated in the absence (open circles, ND) or presence (closed circles, EQ) of 30  $\mu$ M etoposide quinone. The DNA cleavage–ligation equilibrium was established at 37 °C, and ligation 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.

seen with topoisomerase II $\beta$  (Figure 5, left). Up to 30  $\mu$ M 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  $\beta$  isoform (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 II $\beta$  (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.<sup>60,61,78</sup> Because the two drug molecules appear to bind independently, a high



**Figure 5.** Etoposide quinone does not require ATP to induce optimal DNA cleavage mediated by topoisomerase II $\beta$ . 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.

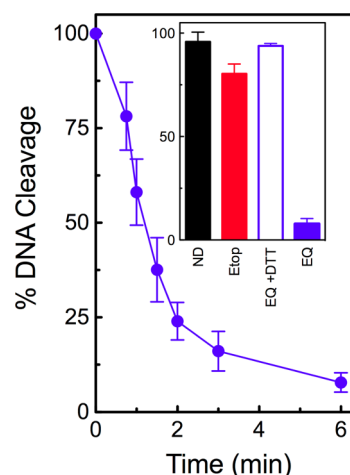


**Figure 6.** Etoposide quinone induces a high ratio of double-stranded DNA breaks (DSB) to single-stranded DNA breaks (SSB). DNA strand breaks generated by human topoisomerase II $\beta$  were monitored in reaction mixtures containing no drug (ND, black), 30  $\mu$ M etoposide (Etop, red), or 15  $\mu$ M 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 (\*\* $p = 0.001$ ).

proportion of cleavage complexes established at clinically relevant (i.e., subsaturating) concentrations of etoposide contain only one cleaved DNA strand.<sup>78–80</sup> Thus, under these conditions, etoposide routinely induces high levels of single-stranded DNA breaks. With human topoisomerase II $\alpha$ , double-stranded: single-stranded break ratios as low as 0.5:1 frequently are observed in the presence of etoposide.<sup>57,78</sup> Although the effect is less dramatic with the  $\beta$  isoform, etoposide still generates approximately equimolar levels of double- and single-stranded DNA breaks (Figure 6 shows results for 30  $\mu$ M etoposide). In contrast, at a drug concentration (15  $\mu$ M) 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 II $\beta$ -mediated DNA cleavage by a mechanism different than that of the parent drug.

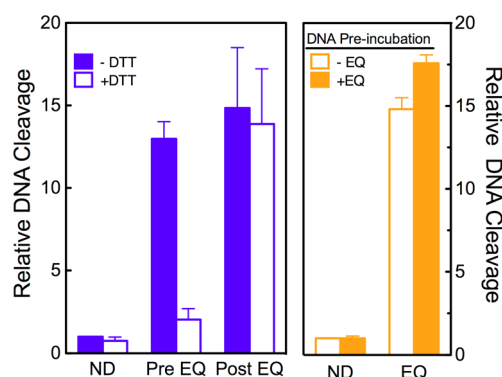
Third, covalent topoisomerase II poisons display the hallmark characteristic of inactivating the enzyme when the two are incubated prior to the addition of DNA.<sup>65,66,70,71</sup> This inactivation is not observed with interfacial poisons. As seen in Figure 7, incubation of 15  $\mu$ M etoposide quinone with human topoisomerase II $\beta$  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 ( $t_{1/2} \leq 1.5$  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.<sup>57,66</sup> To determine whether this was the case for etoposide quinone, an order of addition experiment was carried



**Figure 7.** Etoposide quinone inactivates human topoisomerase II $\beta$  when incubated with the enzyme prior to the addition of DNA. The enzyme was incubated in the presence of 15  $\mu$ M 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  $\mu$ M etoposide (Etop, red bar), 15  $\mu$ M etoposide quinone and DTT (EQ + DTT, open blue bar), or 15  $\mu$ M etoposide quinone in the absence of DTT (EQ, blue bar). Error bars represent the standard deviation of three independent experiments.

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,



**Figure 8.** Etoposide quinone is a covalent poison of topoisomerase II $\beta$ . 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  $\mu$ M etoposide quinone in mixtures that included DTT at the time of DNA cleavage (Pre EQ) or DTT 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 II $\beta$ . DNA was incubated without (–EQ, open orange bars) or with (+EQ, orange bars) 30  $\mu$ M etoposide quinone. DNA was purified from free drug and used in DNA cleavage reactions mediated by topoisomerase II $\beta$ . DNA cleavage reactions were performed in the absence of drug (ND) or in the presence of 30  $\mu$ M etoposide quinone (EQ). In all cases, error bars represent the standard deviation of three independent experiments.

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.<sup>27</sup> Because alkylated DNA lesions can enhance DNA cleavage mediated by human topoisomerase II $\beta$ ,<sup>81</sup> 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  $\mu$ M 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 II $\beta$  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 II $\beta$  primarily by a mechanism that involves adduction to the enzyme.

## CONCLUSIONS

Previous studies have identified a role for etoposide metabolites and topoisomerase II $\beta$  in the initiation of t-AMLs associated with anticancer regimens that contain etoposide. However, the effects of etoposide quinone on the activity of the  $\beta$  isoform have not been described. Results indicate that the quinone is a potent topoisomerase II $\beta$  poison that induces higher levels of enzyme-mediated DNA cleavage than does the parent drug. Etoposide quinone also displays higher reactivity toward topoisomerase II $\beta$  than it does with topoisomerase II $\alpha$ . 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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### Notes

The authors declare no competing financial interests.

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