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Etoposide Metabolites Enhance DNA Topoisomerase II Cleavage near Leukemia-Associated *MLL* Translocation Breakpoints†

Brian D. Lovett,[‡,§] Dirk Strumberg,[||,⊥] Ian A. Blair,[#,▽] Shaokun Pang,[#,▽,○] Donald A. Burden,[×,□] Maureen D. Megonigal,[‡,§] Eric F. Rappaport,[◇,§] Timothy R. Rebbeck,[△,▽] Neil Osheroff,[×] Yves G. Pommier,[||] and Carolyn A. Felix*[,‡,§]

Division of Oncology, Department of Pediatrics, Joseph Stokes, Jr., Research Institute, The Children's Hospital of Philadelphia, Center for Cancer Pharmacology, Department of Biostatistics and Epidemiology, Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania Cancer Center, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, Laboratory of Molecular Pharmacology, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, and Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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ABSTRACT: Chromosomal breakage resulting from stabilization of DNA topoisomerase II covalent complexes by epipodophyllotoxins may play a role in the genesis of leukemia-associated *MLL* gene translocations. We investigated whether etoposide catechol and quinone metabolites can damage the *MLL* breakpoint cluster region in a DNA topoisomerase II-dependent manner like the parent drug and the nature of the damage. Cleavage of two DNA substrates containing the normal homologues of five *MLL* intron 6 translocation breakpoints was examined in vitro upon incubation with human DNA topoisomerase IIα, ATP, and either etoposide, etoposide catechol, or etoposide quinone. Many of the same cleavage sites were induced by etoposide and by its metabolites, but several unique sites were induced by the metabolites. There was a preference for G(−1) among the unique sites, which differs from the parent drug. Cleavage at most sites was greater and more heat-stable in the presence of the metabolites compared to etoposide. The *MLL* translocation breakpoints contained within the substrates were near strong and/or stable cleavage sites. The metabolites induced more cleavage than etoposide at the same sites within a 40 bp double-stranded oligonucleotide containing two of the translocation breakpoints, confirming the results at a subset of the sites. Cleavage assays using the same oligonucleotide substrate in which guanines at several positions were replaced with N7-deaza guanines indicated that the N7 position of guanine is important in metabolite-induced cleavage, possibly suggesting N7-guanine alkylation by etoposide quinone. Not only etoposide, but also its metabolites, enhance DNA topoisomerase II cleavage near *MLL* translocation breakpoints in in vitro assays. It is possible that etoposide metabolites may be relevant to translocations.

Epipodophyllotoxins and other agents that target DNA topoisomerase II are associated with leukemia with various translocations as a treatment complication (reviewed in ref *1*). Interference of these agents with the religation reaction catalyzed by DNA topoisomerase II has led to a model of *MLL*[1] gene translocations in which DNA topoisomerase II-mediated chromosomal breakage occasionally is resolved by translocation (reviewed in ref *1*). We have shown that etoposide and teniposide enhance DNA topoisomerase II cleavage proximal to *MLL* translocation breakpoints in vitro, which may support this model (*2*). Thus, the chromosomal breakage induced by DNA topoisomerase II-targeted anticancer drugs may render them leukemogenic.

We have also shown an association between cytochrome P-450 (CYP) 3A4 genotype and epipodophyllotoxin-related leukemias with *MLL* gene translocations in which the wild-

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* To whom correspondence should be addressed. Phone: (215) 590-2831. Fax: (215) 590-3770. E-mail: felix@email.chop.edu.

‡ Division of Oncology, Department of Pediatrics; The Children's Hospital of Philadelphia.

§ The Children's Hospital of Philadelphia.

|| Laboratory of Molecular Pharmacology.

⊥ Present addresses: Dirk Strumberg, Department of Internal Medicine and Medical Oncology, West German Cancer Center, University of Essen, Essen, Germany.

Center for Cancer Pharmacology.

▽ University of Pennsylvania Cancer Center.

○ Present addresses: Shaokun Pang, Bristol-Myers Squibb, New Brunswick, NJ 08903.

× Department of Biochemistry.

□ Present addresses: Donald A. Burden, Department of Chemistry, Middle Tennessee State University, Murphreesboro, TN 37132.

◇ Joseph Stokes, Jr., Research Institute.

△ Department of Biostatistics and Epidemiology.

[1] Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; bcr, breakpoint cluster region; CYP3A4, cytochrome P-450 3A4; dpm, disintegrations per minute (1 dpm = $\sim 4.5 \times 10^{-7}$ μCi); HPLC, high-performance liquid chromatography; *MLL*, *Mixed Lineage Leukemia* (*Myeloid/Lymphoid Leukemia*) gene, also named *ALL-1*, *HRX*, *Htrx-1*; PCI, 25:24:1 v/v phenol:chloroform:isoamyl alcohol; SV40, simian virus 40; Topo, DNA topoisomerase IIα (p170); VP16, etoposide; VP16-OH, etoposide catechol; VP16-Q, etoposide quinone.

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type genotype increases and variant promoter genotypes decrease the risk (*3*). CYP3A converts epipodophyllotoxin to a catechol metabolite, which is further oxidized to a quinone (*4*−*6*). These epipodophyllotoxin metabolites may have genotoxic properties (*7*−*9*). Redox cycling between the catechol and quinone generates reactive oxygen species that may damage DNA (*5*, *10*). In addition, other quinone moieties produce depurinating N7-guanine adducts (*11*). This DNA damage may be relevant to translocations. Previously, it was shown that etoposide catechol and etoposide quinone are potent enhancers of DNA topoisomerase II cleavage within simian virus 40 (SV40) and pBR322 (*7*−*9*). The objective of this work was to determine whether these metabolites can damage the *MLL* breakpoint cluster region in a DNA topoisomerase II-dependent manner and to characterize the nature of the damage.

MATERIALS AND METHODS

Purification of Human DNA Topoisomerase IIα. Human DNA topoisomerase IIα was isolated from *Saccharomyces cerevisiae* containing the human TOP2α gene in the inducible overexpression plasmid YEpWOB6 (*12*, *13*). Alternatively, for analysis of DNA topoisomerase II cleavage in 40 bp double-stranded oligonucleotides, human DNA topoisomerase IIα, overexpressed and purified from yeast according to the same purification protocol, was provided by J. L. Nitiss.

Preparation of Etoposide Metabolites. Etoposide (Sigma, St. Louis, MO) was dissolved in dioxane and treated with sodium periodate in the dark at 10 °C. The quinone was extracted into organic solvent and recrystallized from dichloromethane/diethyl ether. The corresponding catechol was prepared by reducing etoposide quinone with sodium borohydride. The final product was again recrystallized from dichloromethane/diethyl ether. Structural confirmation was made by mass spectrometry and nuclear magnetic resonance. Purity was determined as >99% by HPLC analysis on a cyano column, which allowed baseline separation of the two compounds.

Preparation of Singly 5′ End-Labeled Substrates. Fragments of the *MLL* breakpoint cluster region (bcr) spanning positions 1358−1858 in intron 6 or positions 1600−2587 in intron 6, exon 7 and intron 7 (*14*) were subcloned into the *Eco*RI−*Bam*HI sites of pBluescript II SK (Stratagene; La Jolla, CA). Singly 5′ end-labeled DNA fragments were prepared as previously described (*2*) with some modifications. Twenty micrograms of plasmid DNA first was treated with 400 units T4 DNA ligase (New England Biolabs; Beverly, MA) for 1 h to ensure that the substrates were not nicked. Following extraction with 25:24:1 phenol:chloroform: isoamyl alcohol (PCI) and ethanol precipitation, plasmids were digested with 32 units of *Bss*HII (New England Biolabs) and dephosphorylated with 20 units of calf intestinal phosphatase (New England Biolabs). Calf intestinal phosphatase was inactivated in 5 mM EDTA (pH 8.0) and the DNA was PCI-extracted and ethanol precipitated. The DNA then was 5′ end-labeled with [γ-^{32}P]ATP (Amersham; Arlington Heights, IL) and T4 polynucleotide kinase (New England Biolabs) as described (*2*). Alternatively, to prepare cold substrate DNA, 20 μg of *Bss*HII-digested, dephosphorylated plasmid were phosphorylated with 10 μM ATP (Boehringer Mannheim; Indianapolis, IN) in otherwise similar reactions. After heat inactivation of the kinase at 70 °C for 20 min, the DNA was digested with 80 units of *Sac*I (New England Biolabs) to generate either singly 5′ end-labeled or cold *Bss*HII−*Sac*I fragments containing the normal homologues of *MLL* translocation breakpoints. The DNA fragments were resolved in 4% polyacrylamide gels, excised upon visualization either by autoradiography or brief ethidium bromide staining, eluted overnight in a solution of 500 mM NH_4-OAc, 10 mM MgOAc, 1 mM EDTA (pH 8.0), and ethanol-precipitated. DNA recovery was quantitated by optical density measurement and scintillation counting.

DNA Topoisomerase II in Vitro Cleavage Assays. A total of 25 ng of substrate DNA containing 30 000 cpm labeled DNA and the remainder cold DNA were incubated at 37 °C for 10 min in 50 μL reactions containing 147 nM human DNA topoisomerase IIα, 1 mM ATP, 137.5 mM KCl, 12.5 mM Tris, pH 7.6, 5 mM $MgCl_2$, 105 μM EDTA, 25 μM dithiothreitol, 4.5% (v/v) glycerol, 8% (v/v) DMSO, and either 20 μM etoposide, 20 μM etoposide catechol, or 20 μM etoposide quinone. Additional reactions were performed in the absence of drug or DNA topoisomerase IIα and with the drug and drug metabolites at 50 and 100 μM final concentrations. Covalent complexes were irreversibly trapped by adding 5 μL of 10% SDS without or with prior incubation for 10 min at 65 °C, the latter to evaluate the heat stability of the complexes. Following addition of 3.75 μL of 250 mM EDTA, cleavage products were deproteinized by incubation with proteinase K at 60 μg/mL final concentration (Amersham) for 30 min at 45 °C. Twenty micrograms of yeast tRNA was added, and the cleavage products were ethanol precipitated twice, resuspended in 6 μL of Sequenase Stop Solution (Amersham), and resolved in an 8% polyacrylamide−7.0 M urea gel in parallel with dideoxy sequencing reactions primed at the same 5′ end. Cleavage products were visualized by autoradiography and quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics; Sunnyvale, CA).

Preparation and 5′ End Labeling of DNA Oligonucleotides. Single-stranded sense and antisense 40 bp oligonucleotides spanning positions 1474−1513 in the *MLL* bcr were synthesized and HPLC-purified by Integrated DNA Technologies, Inc. (Coralville, IA) with and without N7-deaza guanine substitutions for guanine. In the N7-deaza guanine-containing oligonucleotides, with g indicating substitution; the sequence of the sense strand was 5′-GCG GGC GGA TCA TGA ggg CAg GAg ATC GAG ACC ATC CTG G-3′ and the sequence of the antisense strand was 5′-CCA GGA TGG TCT CGA TCT CCT gCC CTC ATG ATC CGC CCG C-3′.

5′-end labeling was accomplished by incubating 5 pmol of single-stranded oligonucleotide at 37 °C for 60 min with 10 units of T4 polynucleotide kinase and 100 μCi [γ-^{32}P]-ATP in 1× kinase buffer (70 mM Tris-HCl, pH 7.6, 0.1 M KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.5 mg/mL bovine serum albumin). Reactions were stopped by heating at 70 °C for 15 min. After purification using Sephadex-G25 columns (Boehringer Mannheim; Indianapolis, IN), the radiolabeled, single-stranded DNA oligonucleotides were annealed to 5 pmol unlabeled, complementary strands in 1× annealing buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, and 1 mM EDTA) to create singly 5′ end-labeled, double-stranded oligonucleotides.

Analysis of Double-Stranded Oligonucleotides in DNA Topoisomerase II Cleavage Assays. Singly 5′ end-labeled, double-stranded oligonucleotides (5×10^4 dpm/reaction) were equilibrated with or without etoposide and etoposide catechol or etoposide quinone in 1% DMSO, 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM dithiothreitol, 0.1 mM Na_2EDTA, 15 μg/mL bovine serum albumin, and 1 mM ATP for 5 min before adding 8 units (80 ng) of human DNA topoisomerase IIα to final 10 μL reactions. Reactions were incubated at 37 °C for 30 min and then stopped by adding 1% SDS (v/v) and digesting with 0.4 mg/mL proteinase K at 55 °C for 30 min. To evaluate the relative stability of cleavage complexes formed in the presence of etoposide and etoposide catechol, salt reversal experiments were performed in which the cleaved oligonucleotides were incubated in the presence of 0.5 M NaCl final concentration for 0, 2, 5, 10, and 30 min before adding SDS and proteinase K. After ethanol precipitation, the samples were resuspended in 5 μL of loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue), heated at 95 °C for 5 min and resolved by electrophoresis at 2500 V (60 W) for 3 h in a 20% polyacrylamide−7.0 M urea gel. The purine ladder used to map the DNA topoisomerase II cleavage sites was obtained after formic acid reaction as described (*15*). The gels were visualized with a PhosphorImager and ImageQuant software (Molecular Dynamics).

RESULTS

Cleavage of singly 5′ end-labeled *MLL* DNA substrates was analyzed in vitro after incubation with human DNA topoisomerase IIα in absence of drug and in the presence of 20, 50, and 100 μM etoposide and its catechol and quinone metabolites. The 501 bp *MLL* DNA substrate corresponding to nucleotide positions 1358−1858 in intron 6 in the bcr contained the normal homologues of previously described *MLL* translocation breakpoints in two cases of treatment-related leukemia. These included the breakpoint at nucleotide 1493 of an *MLL* intron 6−intron 1 fusion resulting from a partial tandem duplication where nucleotide 1493 was the last base of intron 6 sequence 5′ of the fused sequence from intron 1 (*16*), and the der(16) *MLL* breakpoint at position 1502 resulting from a t(11;16) translocation between *MLL* and the *CBP* gene from chromosome band 16p13.3 where nucleotide 1502 was the start of *MLL* intron 6 sequence in the der(16) (*17*). The 988 bp *MLL* DNA substrate corresponding to positions 1600−2587 of the bcr contained the normal homologues of der(11) *MLL* translocation breakpoints at position 1725 in a case of infant acute myeloid leukemia (AML) where karyotype analysis was not performed (Lo Nigro and C.A.F., unpublished material), at position 1737 in a case of infant acute lymphoblastic leukemia (ALL) with a t(4;11) translocation (*18*) and at position 1929 or 1930 in a case of treatment-related AML with a t(9;11) translocation (*19*).

In absence of drug, little cleavage was detectable in either substrate (Figures 1 and 2). In both substrates, fragments of various sizes and intensities indicated that multiple DNA topoisomerase II cleavage sites were induced by etoposide and by the metabolites.

Etoposide Catechol and Quinone Metabolites Enhance DNA Topoisomerase II Cleavage in the MLL bcr between Positions 1384 and 1585. For the *MLL* intron 6 substrate that spanned positions 1358−1858, the sequence between positions 1384 and 1585 encompassing the normal homologues of known translocation breakpoints at positions 1493 and 1502 was resolved and studied in detail in the gel in Figure 1, which shows results of cleavage assays in absence of drug and in the presence of 20 μM etoposide, 20 μM etoposide catechol, and 20 μM etoposide quinone. If the coordinate indicates the base at the 5′ side of the cleavage site, also called the −1 position, some cleavage was detected in absence of drug at positions 1390, 1452, 1492, 1510, and 1530 [Figure 1, no heat, (+) Topo]. We used nucleotide 1390, the strongest cleavage site in absence of drug, as a reference site for phosphorimage quantitation. Nucleotide 1390 also was the strongest cleavage site in assays performed with etoposide and with both metabolites; cleavage at this site was enhanced 2.4-fold in comparison with the reference site in the presence of etoposide, but 3.6- and 15.0-fold in the presence of etoposide catechol and etoposide quinone, respectively [Figure 1, no heat, (+) Topo]. The same patterns of multiple cleavage sites, but some variations in relative intensities, were observed in assays performed with etoposide and its metabolites at 50 and 100 μM concentrations, indicating reproducibility and confirming the same site-selectivity (data not shown).

In general, metabolite-induced cleavage at each specific site was quantitatively greater than etoposide-induced cleavage and etoposide quinone resulted in greater cleavage than the catechol [Figure 1, no heat, (+) Topo]. This was true of positions 1390, 1422, 1425, 1438, 1452, 1479, 1492, 1500, 1510, 1529, 1530, and 1534. At positions 1432 and 1484, cleavage was greatest in the presence of etoposide quinone, but etoposide resulted in more cleavage than the catechol metabolite.

While the parent drug and the metabolites induced many of the same cleavage sites, for example, positions 1390, 1422, 1425, 1432, 1438, 1452, 1479, 1484, 1492, 1500, 1510, 1529, 1530, and 1534, some unique cleavage sites were induced by the metabolites, especially the quinone [Figure 1, no heat, (+) Topo]. Cleavage was detected in the presence of the quinone but not the parent drug or the catechol metabolite at positions 1395, 1434, 1436, 1446, 1464, 1489, 1494, 1505, 1508, and 1523. Cleavage was detected both in the presence of the catechol and the quinone but not the parent drug at position 1585 [Figure 1, no heat, (+) Topo].

If the base at the 5′ side of the cleavage site is position −1 and the base at the 3′ side +1, C was the most common residue at the −1 position in absence of drug and in the presence of etoposide and both metabolites (Table 1). This is consistent with previously reported data on etoposide (*2*, *20*). In contrast, among the 11 sites induced by etoposide quinone but not etoposide, G was the most common residue at the −1 position; five sites (45.4%) had G(−1), four (36.4%) had T(−1), and only two (18.2%) had C(−1) (Table 1).

To evaluate the relative heat-stability of the complexes, cleavage was assessed upon incubation for 10 min at 65 °C after the reactions were complete. Detectable cleavage at many sites decreased significantly or was eliminated after heating [Figure 1, heat, (+) Topo]. However, even in absence of drug, cleavage was detectable after heating at positions 1390, 1452, 1492, and 1510, indicating that these sites were

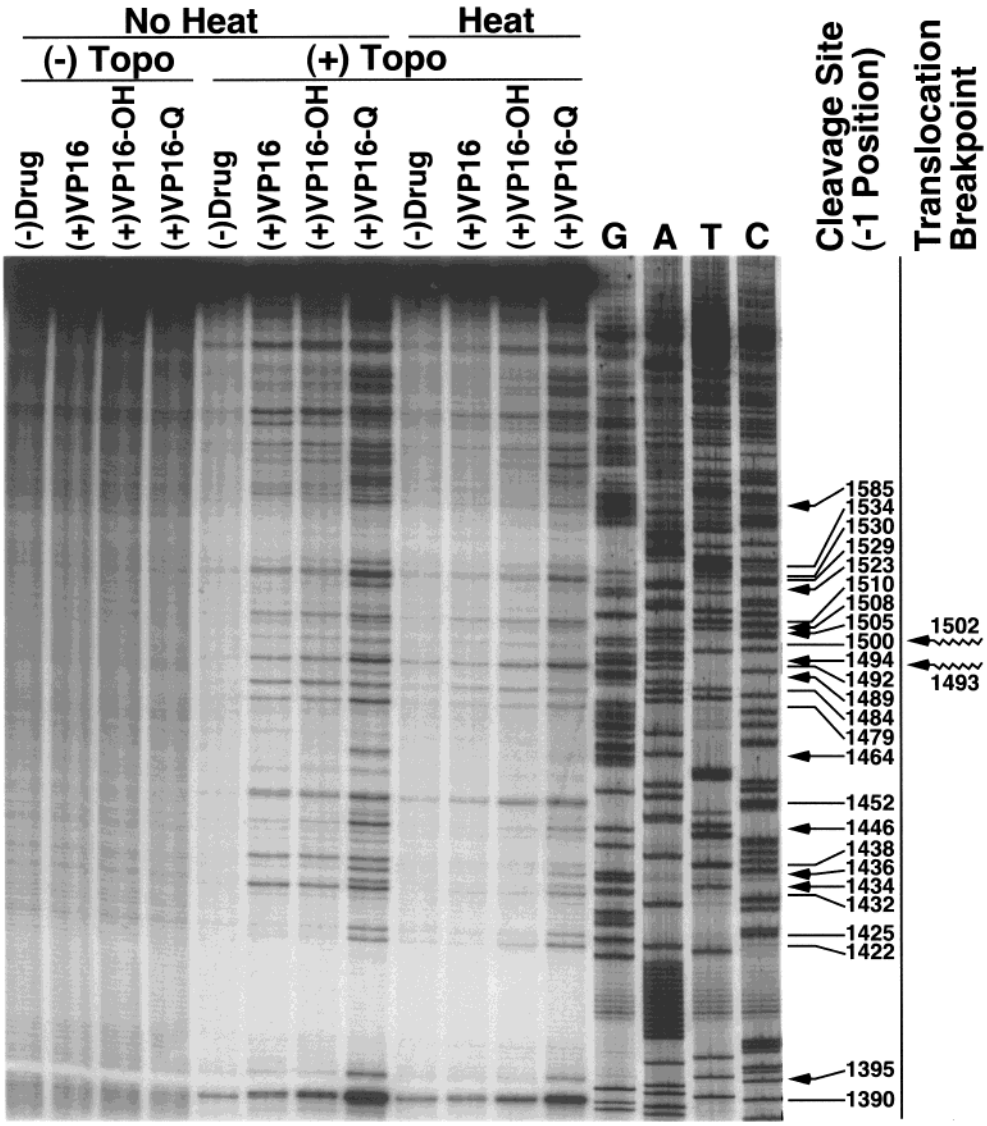

FIGURE 1: Etoposide and etoposide metabolite-induced DNA topoisomerase II cleavage of *MLL* bcr coordinates 1384−1585. Autoradiograph of cleavage products after 10 min incubation of 25 ng (30 000 cpm) singly 5′ end-labeled DNA with 147 nM human DNA topoisomerase IIα, 1 mM ATP and, where indicated, 20 μM etoposide (VP16), etoposide catechol (VP16-OH), or etoposide quinone (VP16-Q). Heat indicates that reactions were incubated for 10 min at 65 °C before trapping of covalent complexes by adding SDS. Similar results were obtained with reaction mixtures containing 50 and 100 μM VP16, VP16-OH or VP16-Q (data not shown). Lines indicate sites where cleavage in the presence of VP16-OH and/or VP16-Q was greater than in the presence of VP16 by phosphorimage quantitation; arrows, unique sites of cleavage with VP16-Q or VP16-OH and VP16-Q. The indicated nucleotide is the 5′ side or −1 position of the cleavage site; the cleaved phosphodiester bond is 3′ to the indicated nucleotide. Corkscrew arrows at far right indicate positions of *MLL* translocation breakpoints contained within the substrate.

particularly stable. Heat stability at these sites was increased in reactions performed with etoposide or either of the metabolites compared to the reaction without drug except at position 1492 where heat stability was increased only in reactions with etoposide catechol and quinone. In absence of drug and in the presence of both of the metabolites, the most heat-stable cleavage site was nucleotide 1390 and heat stability at position 1492 ranked second. Relative to the designated reference site [position 1390, no heat, (+) Topo, (−) drug], cleavage at position 1492 after heating was enhanced 0.69-fold without drug but 1.28- and 2.9-fold in the presence of etoposide catechol and etoposide quinone, respectively. Heat stability at any given site was greatest in the presence of etoposide quinone [Figure 1, heat, (+) Topo]. In reactions performed with etoposide quinone, each unique cleavage site described above also was heat stable.

Etoposide Catechol and Quinone Metabolites Enhance DNA Topoisomerase II Cleavage in the MLL bcr between Positions 1702 and 1965. In cleavage assays of the substrate that spanned positions 1600−2587 of the *MLL* bcr, the sequence between positions 1702 and 1965 in intron 6 was resolved and analyzed in detail because this region contained the normal homologues of known translocation breakpoints. Results of cleavage assays of this substrate in absence of drug and in the presence of 50 μM etoposide, 50 μM etoposide catechol, or 50 μM etoposide quinone are shown in Figure 2. In absence of drug, some cleavage was detectable at positions 1719, 1724, 1729, 1787, 1858, 1863, 1887, 1913, 1924, 1927, and 1933 [Figure 2, no heat, (+) Topo]. Nucleotide 1724 was the strongest cleavage site in absence of drug and was used as the reference site for phosphorimage quantitation of cleavage in this substrate. Position 1724 also

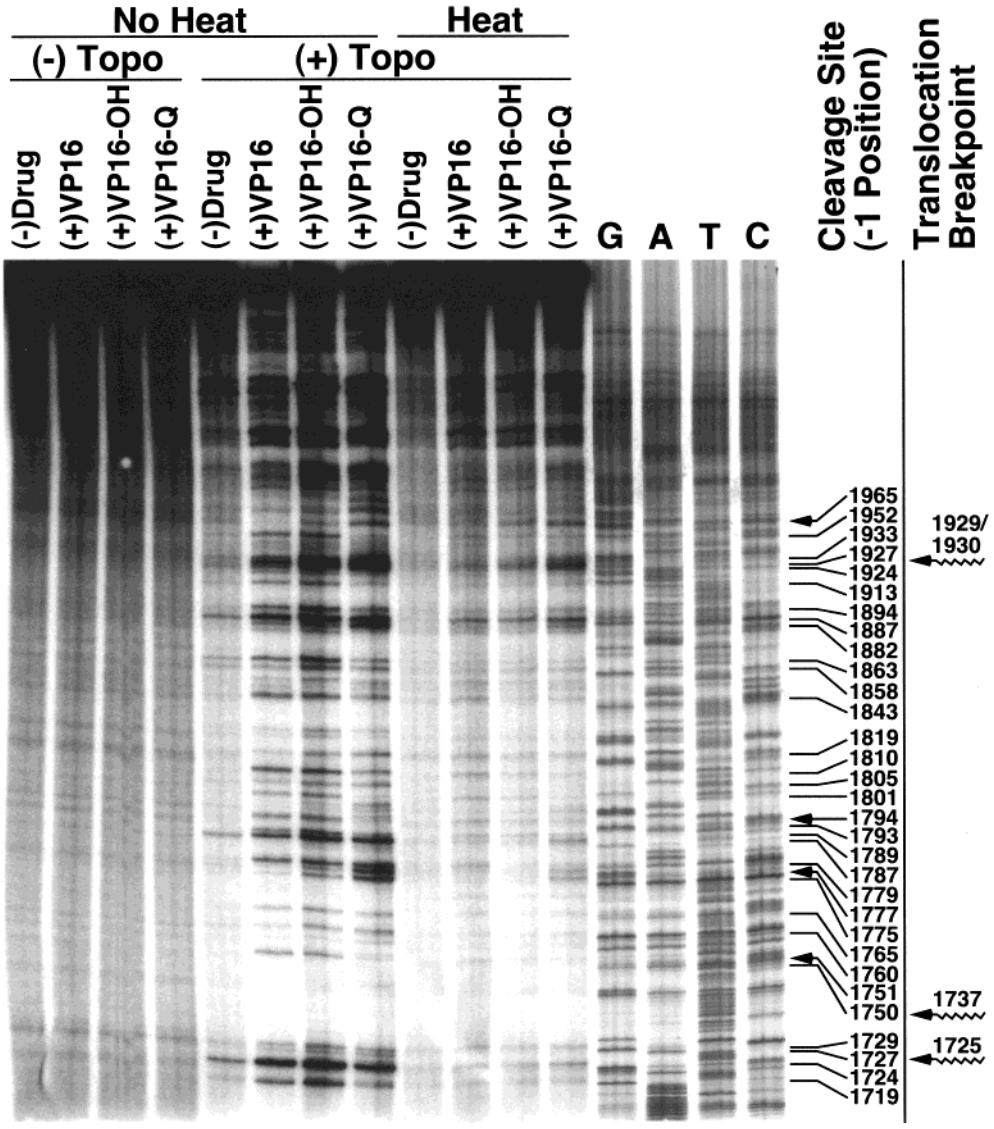

FIGURE 2: Etoposide and etoposide metabolite-induced DNA topoisomerase II cleavage of *MLL* bcr coordinates 1702−1965. Autoradiograph of cleavage products after 10 min incubation of 25 ng (30 000 cpm) singly 5′ end-labeled DNA with 147 nM human DNA topoisomerase IIα, 1 mM ATP, and where indicated, 50 μM etoposide (VP16), etoposide catechol (VP16-OH), or etoposide quinone (VP16-Q). Heat indicates that reactions were incubated for 10 min at 65 °C before trapping of covalent complexes by adding SDS. Similar results were obtained with reaction mixtures containing 20 and 100 μM VP16, VP16-OH or VP16-Q (data not shown). Lines indicate sites where cleavage in the presence of VP16-OH and/or VP16-Q was greater than in the presence of VP16; arrows, unique sites of cleavage with VP16-OH and/or VP16-Q. The indicated nucleotide is the 5′ side or −1 position of the cleavage site; the cleaved phosphodiester bond is 3′ to the indicated nucleotide. Corkscrew arrows at far right indicate positions of *MLL* translocation breakpoints contained within the substrate.

was the strongest cleavage site in assays performed with etoposide and with the catechol metabolite [Figure 2, no heat, (+) Topo]. In the presence of etoposide quinone, the strongest and second strongest cleavage sites were nucleotides 1927 and 1933, while cleavage at position 1724 ranked fifth [Figure 2, no heat, (+) Topo]. In the presence of etoposide and the catechol and quinone metabolites, cleavage at position 1724 was enhanced 4.1-fold, 7.1- and 3.4-fold, respectively, relative to the designated reference site [position 1724, no heat, (+) Topo, (−) drug]. In the presence of etoposide quinone, cleavage at positions 1927 and 1933 was enhanced 6.1- and 3.9-fold, respectively, relative to the reference site [Figure 2, no heat, (+) Topo]. In assays performed with etoposide or its metabolites at 20 and 100 μM concentrations, the patterns of multiple cleavage sites were identical, with some variations in relative intensities (data not shown), again indicating that the DNA cleavage was reproducible and site-selective.

At positions 1727, 1760, 1775, 1779, 1787, 1789, 1882, 1887, 1924, 1927, and 1933, metabolite-induced cleavage was greater than etoposide-induced cleavage, and cleavage with the quinone was greater than with the catechol [Figure 2, no heat, (+) Topo]. At several other sites including positions 1719, 1729, 1765, 1805, 1819, 1843, 1858, 1894, and 1913, metabolite-induced cleavage was greater than etoposide-induced cleavage, but cleavage with the catechol was greater than with the quinone [Figure 2, no heat, (+) Topo]. Cleavage was less in the presence of the quinone relative to etoposide catechol and etoposide at nucleotides 1724, 1750, 1793, 1801, 1810, 1863, and 1952, suggesting differences in site selectivity [Figure 2, no heat, (+) Topo]. Unique cleavage sites were induced by etoposide quinone at positions 1751 and 1794. Cleavage was detectable in the presence of both the catechol and the quinone but not the

Table 1: Sequence Preferences at 5′ Side of Cleavage Sites (−1 Position)[a]

	no. of sites/total (%)	(−) drug	VP16	VP16-OH	VP16-Q	VP16-OH and/or VP16-Q only
MLL bcr positions	**G**	0/5 (0%)	0/14 (0%)	0/15 (0%)	5/25 (20%)	**5/11 (45.4%)**
1384−1585	**A**	0/5 (0%)	0/14 (0%)	0/15 (0%)	0/25 (0%)	0/11 (0%)
	T	0/5 (0%)	1/14 (7.1%)	2/15 (13.3%)	5/25 (20%)	4/11 (36.4%)
	C	**5/5 (100%)**	**13/14 (92.9%)**	**13/15 (86.7%)**	**15/25 (60%)**	2/11 (18.2%)
MLL bcr positions	**G**	2/11 (18.2%)	2/27 (7.4%)	4/29 (13.8%)	4/31 (12.9%)	**2/4 (50%)**
1702−1965	**A**	2/11 (18.2%)	2/27 (7.4%)	2/29 (6.9%)	2/31 (6.5%)	0/4 (0%)
	T	**4/11 (36.4%)**	10/27 (37.0%)	10/29 (34.5%)	11/31 (35.5%)	1/4 (25%)
	C	3/11 (27.3%)	**13/27 (48.1%)**	**13/29 (44.8%)**	**14/31 (45.2%)**	1/4 (25%)
composite	**G**	2/16 (12.5%)	2/41 (4.9%)	4/44 (9.1%)	9/56 (16.1%)	**7/15 (46.7%)**
	A	2/16 (12.5%)	2/41 (4.9%)	2/44 (4.5%)	2/56 (3.6%)	0/15 (0%)
	T	4/16 (25%)	11/41 (26.8%)	12/44 (27.3%)	16/56 (28.6%)	5/15 (33.3%)
	C	**8/16 (50%)**	**26/41 (63.4%)**	**26/44 (59.1%)**	**29/56 (51.8%)**	3/15 (20%)

[a] Composite indicates sum of results for cleavage sites analyzed between *MLL* bcr positions 1384−1585 and between *MLL* bcr positions 1702−1965. Fractions and percentages in bold indicate preferred nucleotides at −1 position.

parent drug at positions 1777 and 1965 [Figure 2, no heat, (+) Topo].

The most common base at the −1 position in this substrate was T in absence of drug, but C in the presence of etoposide, etoposide catechol and etoposide quinone at frequencies of 36.4, 48.1, 44.8, and 45.2%, respectively, of the sites examined (Table 1). However, of the four sites described above that were unique to the metabolites, half had G(−1) and G was the most common base at this position (Table 1).

At most sites in this substrate, detectable cleavage also decreased significantly after heating [Figure 2, heat, (+) Topo]. Heat stability of the complexes formed in the presence of etoposide was greatest at position 1887 [Figure 2, heat, (+) Topo]. In the presence of either metabolite, heat stability was greatest at position 1927, and the complexes generally were more heat stable in the presence of the quinone than the catechol [Figure 2, heat, (+) Topo].

Leukemia-Associated Translocation Breakpoints Are near Strong or Stable Cleavage Sites. The translocation breakpoint at position 1493 in a case of treatment-related AML (*16*) coincided with the site of enzyme attachment or the +1 or 3′ side of a heat-stable cleavage site that was detectable in absence of drug and enhanced by both metabolites as described above. The der(16) *MLL* translocation breakpoint at position 1502 (*17*) was also near this site. The −1 positions of additional cleavage sites were mapped nearby at *MLL* bcr nucleotides 1494 and 1500, the former of which was a unique site induced by the quinone (Figure 1).

The translocation breakpoint at position 1725 in the case of infant AML (Lo Nigro and C.A.F., unpublished material) was the site of enzyme attachment or the +1 position of the strongest cleavage site in the second substrate in assays performed in absence of drug and in the presence of etoposide and the catechol metabolite. Cleavage also occurred at −1 positions 1727 and 1729; the cleavage at position 1729 was detectable in absence of drug and was heat resistant (Figure 2). The translocation breakpoint at position 1737 in the case of infant ALL (*18*) was between these cleavage sites and the cleavage site with nucleotide 1750 at the −1 position (Figure 2). The translocation breakpoint at position 1929 or 1930 in a case of treatment-related AML (*19*) was within cluster of cleavage sites with −1 positions 1924, 1927, and 1933 that were detectable without drug and enhanced by etoposide and by both metabolites. Cleavage at position 1927 was especially heat resistant in the presence of the metabolites as described above (Figure 2).

DNA Topoisomerase II Cleavage Assays Using Oligonucleotide DNA Substrates Suggest Potential Mechanism of Increased Cleavage by Etoposide Metabolites. Since position 1493 was the site of enzyme attachment in a DNA topoisomerase II cleavage site that coincided with an *MLL* translocation breakpoint (*16*), we performed DNA topoisomerase II cleavage assays using double-stranded oligonucleotide DNA substrates to examine cleavage at position 1493 in isolation, analyze cleavage on both strands, and test the effects of chemical modification on this cleavage.

The 40 bp oligonucleotide containing the breakpoint at nucleotide 1493 spanned positions 1474−1513 in intron 6 of the *MLL* bcr and also contained the translocation breakpoint of the der(16) of the t(11;16) that was described above (*17*). Figure 3 shows the cleavage pattern for each DNA strand in absence of drug and in the presence of etoposide, etoposide catechol, and etoposide quinone. Consistent with results just described, in the sense strand, the translocation breakpoint at position 1493 was the site of enzyme attachment, i.e., the +1 position, of a strong DNA topoisomerase II cleavage site in absence of drug. Nucleotide 1485 was the site of enzyme attachment of a weaker cleavage site in absence of drug (Figure 3A). In the presence of etoposide, cleavage at position 1493 was greater than at position 1485. DNA topoisomerase II induces four-base staggered nicks in duplex DNA (*21*). At +1 positions 1493 and 1485 on the sense strand and at the corresponding +1 positions, 1496 and 1488, respectively, on the complementary DNA strand, which were staggered by four bases from positions 1493 and 1485, DNA topoisomerase II cleavage was enhanced by both metabolites in comparison to the parent drug (Figure 3).

Salt reversal experiments were performed in the presence of NaCl at 0.5 M final concentration to compare the stability of the ternary drug−DNA−enzyme complexes. Figure 4 shows the results of salt-reversal assays in the presence of etoposide and the catechol metabolite for the double-stranded oligonucleotide with the sense strand labeled. Salt stability at the cleavage sites where nucleotides 1485 and 1493 coincided with +1 positions was greater with etoposide catechol than with the parent compound. Enhanced salt stability at these sites also was observed with etoposide quinone (data not shown). Thus, the catechol and quinone metabolites enhanced DNA topoisomerase II cleavage at both sites compared to the parent compound and the metabolite-induced cleavage complexes showed increased salt-stability.

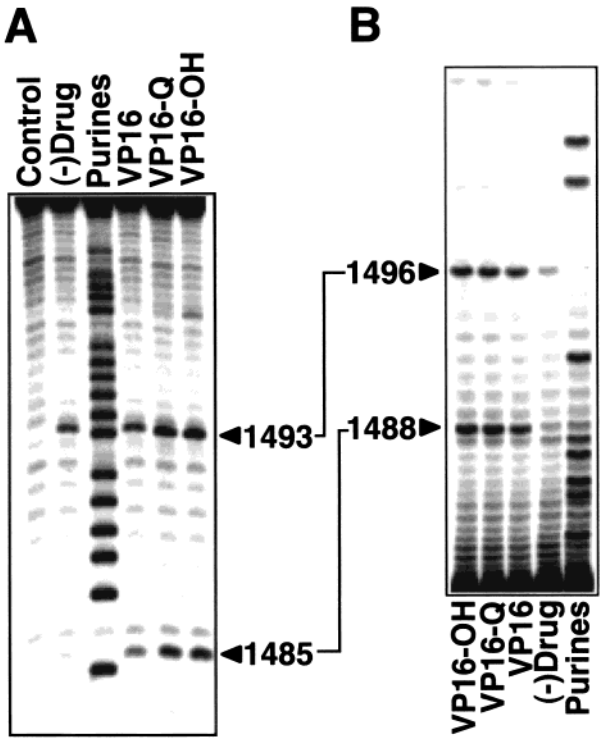

FIGURE 3: Analysis of etoposide (VP16)-, etoposide catechol (VP16-OH)-, and etoposide quinone (VP16-Q)-induced DNA topoisomerase II cleavage in double-stranded DNA oligonucleotide spanning *MLL* bcr positions 1474−1513. The oligonucleotide was singly 5′ end-labeled with [γ-^{32}P]ATP on the upper (panel A) or the lower strand (panel B). DNA topoisomerase II cleavage reactions were performed at 37 °C for 30 min in the presence of 100 μM VP16, 100 μM VP16-OH, or 100 μM VP16-Q. Control indicates reaction without drug or enzyme. The purine ladder was used to map the DNA topoisomerase II cleavage sites (*15*). Arrowheads indicate the 3′ sides, i.e., sites of enzyme attachment, of cleavage sites with 4-base stagger in upper and lower strands.

Since other quinone moieties are known to form N7 guanine adducts (*11*), we began to investigate N7-alkylation of guanine as a possible mechanism of the enhanced DNA topoisomerase II cleavage in the presence of the drug metabolites at the cleavage site with nucleotide 1493 corresponding to the +1 position. Here we utilized double-stranded DNA oligonucleotides spanning the same positions of the *MLL* bcr as described above, but containing N7-deaza guanine substitutions at positions 1489, 1490, 1491, 1494, and 1497 in the sense strand or at position 1492 in the complementary strand. In the presence of the catechol and quinone metabolites, substitution of N7-deaza guanine residues for guanine at the designated positions in the sense strand was associated with reduced DNA topoisomerase II cleavage at the site where nucleotide 1493 corresponded with the +1 position, but cleavage induced by the parent drug at this site was not affected (Figures 3A and 5A). In contrast, at the site with nucleotide 1485 corresponding to the +1 position, which was more distant from the N7-deaza guanine substitutions, cleavage in the modified oligonucleotide was not reduced in the presence of the drug metabolites. Assays on the unmodified and modified oligonucleotides were performed in triplicate and cleavage at the site with nucleotide 1493 corresponding to the +1 position was quantitated using phosphorimaging. Figure 5B compares the amount of cleavage at this site in the unmodified and modified oligonucleotides in the presence of etoposide and the catechol and quinone metabolites. The same results were obtained when both DNA strands contained N7-deaza guanine residues as described above (data not shown). These results suggest that the N7 position of guanine was critical for enhancement of DNA topoisomerase II cleavage by the catechol and quinone metabolites at the site with nucleotide 1493 corresponding to the +1 position.

DISCUSSION

The epidemiological association between chemotherapeutic agents that target DNA topoisomerase II and leukemias with translocations of the *MLL* gene at chromosome band 11q23 and other translocations is well-established, but whether the drugs directly cause the translocations or select for cells with translocations still remains unknown. The specificity of the association of DNA topoisomerase II inhibitors with leukemias with balanced translocations (*22*) supports the former possibility. The clinical efficacy of anticancer drugs that target DNA topoisomerase II depends on chromosomal breakage that results from stabilization of enzyme−DNA covalent complexes (*23*−*26*). If the role of chemotherapy in leukemogenesis is to directly cause the translocations, it is plausible that the chromosomal breakage that results from the drug-stabilized enzyme−DNA covalent complexes occasionally is resolved in translocations and that the translocations are a form of DNA repair.

The association of *CYP3A4* genotype with epipodophyllotoxin-related leukemias (*3*) provided the rationale to investigate whether the catechol and quinone metabolites of etoposide are relevant to the DNA damage that results in translocations involving *MLL*. Moreover, pharmacokinetic studies have suggested that patients receiving anticancer treatment with etoposide are exposed to measurable plasma levels of etoposide catechol (*27*, *28*), providing further rationale to address this question. For example, peak plasma etoposide catechol concentrations of 1.3 ± 0.5 μg/mL ($\sim$2.1 $\pm$ 0.8 μM) have been observed on the last day of a 1 h intravenous infusion of 600 mg/m^2 etoposide daily $\times$ 4 days (*27*). Similary, in pediatric patients peak plasma etoposide catechol levels of $\sim$0.6 μM have also been achieved (*28*).

Prior to this study, the biochemistry of etoposide metabolite−*MLL* gene interactions had not been defined. Therefore, we used in vitro DNA topoisomerase II cleavage assays to begin to understand the possible mechanistic role of etoposide catechol and etoposide quinone in the translocation process. The purpose was to compare the effects of etoposide and these potentially DNA-damaging metabolites on DNA topoisomerase II cleavage in two regions of the *MLL* bcr containing translocation breakpoints. Functional in vitro studies of DNA topoisomerase II cleavage of these regions had not been performed before at the level of the sequence. The salient findings were that etoposide catechol and etoposide quinone can damage the *MLL* bcr in a DNA topoisomerase II-dependent manner, as is true of etoposide, and that the metabolite-induced damage possibly may involve alkylation, although the evidence for the latter is indirect as discussed below. Not only were etoposide metabolites more active than the parent drug in inducing cleavage, but metabolite-induced cleavage was more stable. The sequence preferences of DNA topoisomerase II in the presence the metabolites were addressed for the first time and found to

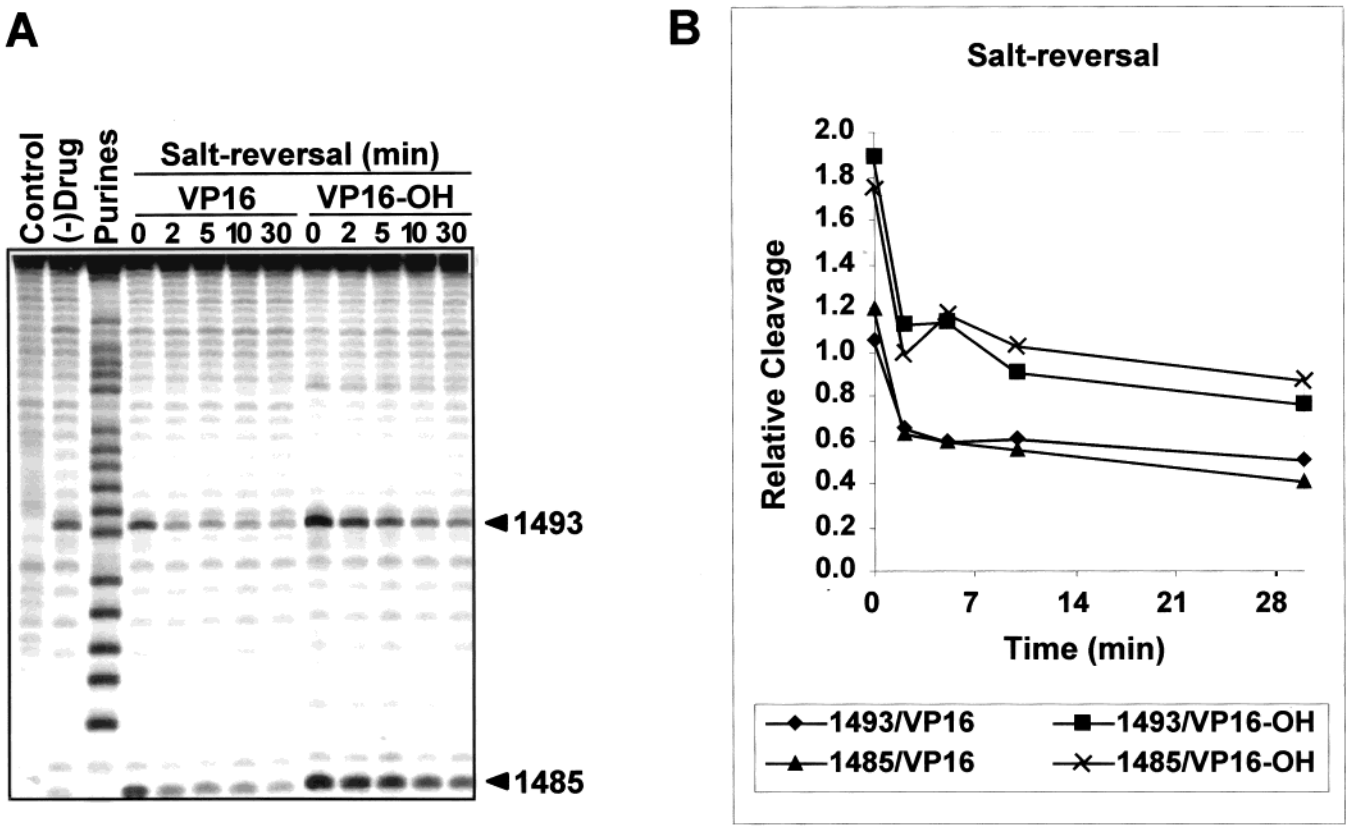

FIGURE 4: (A) Analysis of salt reversibility of DNA topoisomerase II cleavage complexes formed in the presence of etoposide (VP16) and etoposide catechol (VP16-OH). The DNA oligonucleotide was the same as in Figure 3. The upper strand was 5′ end-labeled with [γ-^{32}P]-ATP. VP16 and VP16-OH concentrations were 100 μM. DNA topoisomerase II cleavage reactions were performed at 37 °C for 30 min. After addition of NaCl at 0.5 M final concentration, the reactions were incubated at 37 °C for the indicated times before the addition of SDS and proteinase K. Control indicates reaction without drug or enzyme. Arrowheads indicate bases at 3′ sides of cleavage sites. (B) Kinetics of salt reversal of DNA topoisomerase II cleavage complexes in Figure 4A. Cleavage is relative to strongest site without drug (second lane), in which nucleotide 1493 is the +1 position; this reference site was assigned a value of 1.0.

be unique. These experiments in naked DNA establish the sites where drug−DNA topoisomerase II−*MLL* or metabolite−DNA topoisomerase II−*MLL* interactions, which could lead to translocations, are possible.

Comparable to these results for *MLL*, previous in vitro cleavage assays of DNA substrates not involved in therapy-related translocations suggested local base sequence selectivity in the presence of various DNA topoisomerase II-targeted anticancer drugs and differences in the frequency of cleavage sites depending on the drug and the region (*29*−*32*). Other studies mapping DNA topoisomerase II cleavage sites in naked versus nucleosome-reconstituted SV40 DNA suggested that many DNA topoisomerase II cleavage sites observed in vitro would not be found in vivo because chromatin structure would restrict the sites of cleavage (*33*). Thus, while many in vitro DNA topoisomerase II cleavage sites were detected in both substrates in the present study, fewer cleavage sites would be expected in the context of the cell. It is possible that *MLL* translocation breakpoints in vivo are a subset of DNA topoisomerase II cleavage sites that are seen in vitro and that, in vivo, the sites are further processed to form translocations.

In absence of drug, we observed less cleavage than in the presence of etoposide or either of the metabolites. The enzyme-only sites may be relevant to translocations because they indicate preferred cleavage sites that may be enhanced by specific agents with various sequence preferences (*20*, *34*−*36*). There was heat-resistant cleavage even in absence of drug, as well as salt-resistant cleavage, at the site with *MLL* bcr nucleotide 1493 at the +1 position. The genomic breakpoint of an *MLL* intron 6−intron 1 fusion resulting from partial tandem duplication in a case of AML following rhabdomyosarcoma therapy with the DNA topoisomerase II poison, dactinomycin, was nucleotide 1493 (*16*). The der-(16) *MLL* translocation breakpoint of a t(11;16) at position 1502 in a case of treatment-related myelodysplastic syndrome/AML following therapy for primary AML with doxorubicin and etoposide (*17*, *37*) was also proximal to nucleotide 1493. The more stable cleavage and decreased tendency toward religation, indicated by both heat and salt resistance, may have been important in these translocations.

The site of enzyme attachment in the strongest cleavage site in absence of drug in the second substrate corresponded with the der(11) *MLL* translocation breakpoint at position 1725 in a case of infant AML (Lo Nigro and C.A.F., unpublished material). The der(11) *MLL* translocation breakpoint in a case of AML with a t(9;11) following chemotherapy that included doxorubicin was either nucleotide 1929 or 1930 (*19*) but the position could not be ascertained precisely. In absence of drug, there was heat-resistant cleavage at sites with −1 positions 1927 and 1933, both near this translocation breakpoint.

It previously was suggested that the simultaneous trapping of two DNA topoisomerase II cleavage complexes on different homologous DNA fragments might facilitate enzyme−DNA subunit exchange and lead to precise interchro-

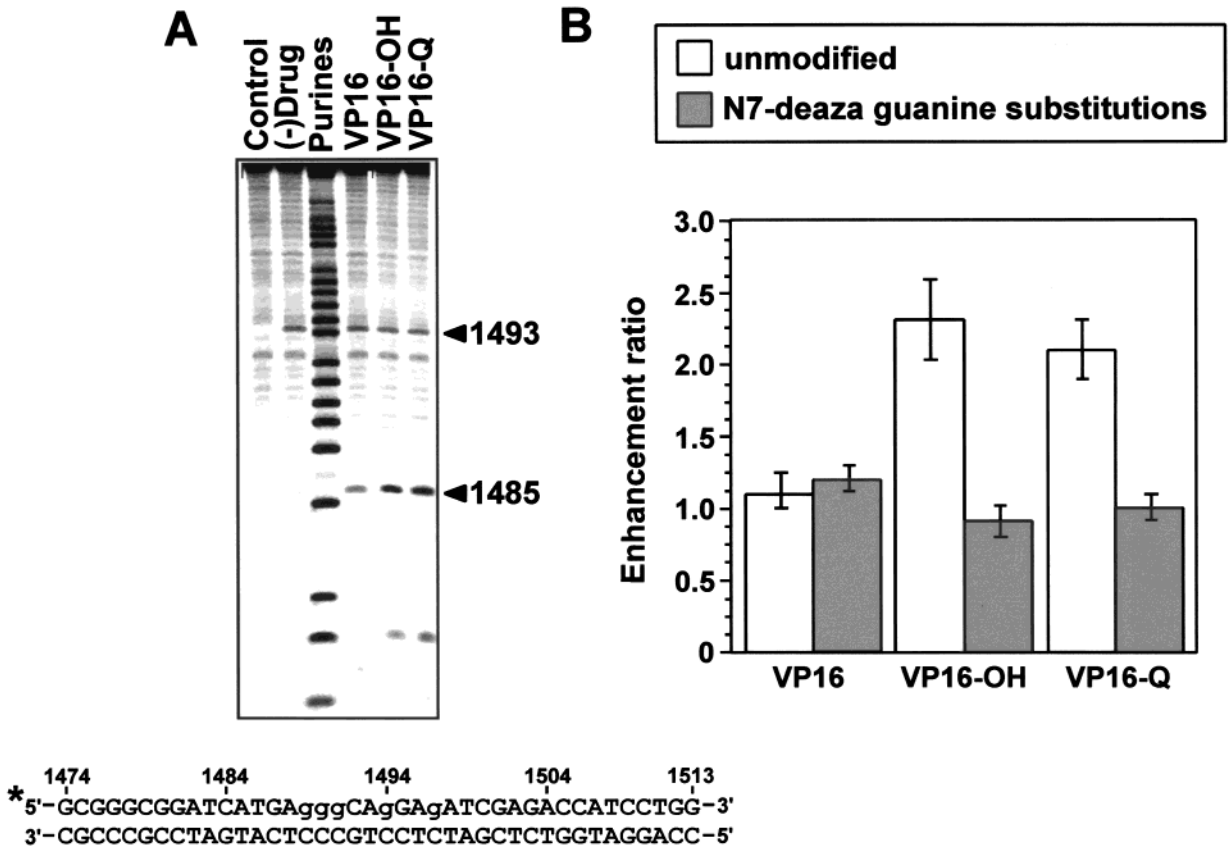

FIGURE 5: (A) Analysis of effects of N7-deaza guanine substitutions on DNA topoisomerase II cleavage. The DNA sequence of the oligonucleotide spanning *MLL* bcr positions 1474−1513 was the same as in Figures 3 and 4, except that guanine residues at positions 1489, 1490, 1491, 1494, and 1497 in the sense strand were replaced with N7-deaza guanine (g). The upper strand was 5′ end-labeled with [γ-^{32}P]ATP. DNA topoisomerase II cleavage assays were performed at 37 °C for 30 min. VP16, VP16-OH, and VP16-Q concentrations were 100 μM. Control indicates reaction without drug or enzyme. Arrowheads indicate 3′ sides of cleavage sites. The same results were obtained when guanine residues at positions at positions 1489, 1490, 1491, 1494, and 1497 in the sense strand and at position 1492 in the complementary strand were replaced with N7-deaza guanine and the upper strand was 5′ end-labeled (data not shown). (B) Relative enhancement of DNA topoisomerase II cleavage at site with +1 position 1493 by 100 μM VP16, VP16-OH or VP16-Q in unmodified oligonucleotide (cf. Figure 3A) versus oligonucleotide containing N7-deaza guanine substitutions in the sense strand (cf. Figure 5A). Cleavage was quantitated in three independent experiments using phosphorimaging.

mosomal DNA recombination (*38*, *39*). While the translocation breakpoints at positions 1493 and 1725 coincided with strong, heat- and/or salt-resistant cleavage sites, the cleavage was between positions 1492 and 1493 or between positions 1724 and 1725, and positions 1493 and 1725 were the sites of enzyme attachment in each respective cleavage site. Positions 1493 and 1725 were the last bases of *MLL* 5′ of the fused partner DNAs in the respective translocation breakpoint junctions in the leukemias, but these bases were 3′ of the cleavage sites in the in vitro assays. These data are not consistent with DNA topoisomerase II-mediated religation via simple reciprocal exchange of enzyme subunits and DNA strand transfer (*38*, *39*) because further processing would be required to generate to the observed translocation breakpoint junction sequences before resealing of the ends. Instead, however, these data may be consistent with a translocation mechanism that involves DNA topoisomerase II-mediated chromosomal breakage, processing of the DNA free ends and then recombination. Other translocation breakpoints and strong and/or stable cleavage sites in this study were adjacent but did not coincide exactly. Prior studies showed small deletions, insertions, and duplications at DNA topoisomerase II cleavage sites in teniposide-treated Chinese hamster ovary cells in vivo compared to drug-stimulated cleavage sites in vitro (*40*, *41*). The cloning of *MLL* genomic breakpoint junctions indicates that small insertions and deletions also may occur during the translocation process (*2*, *42*, *43*).

In other cases, the cloning of *MLL* genomic breakpoint junctions has revealed regions up to several hundred bases long from *MLL* or from the partner gene on both derivative chromosomes (*44*−*47*), indicating duplication. The duplicated sequences are consistent with staggered single-stranded breakage and DNA repair in which each single-stranded overhang serves as a template for the complementary strand of the duplicated region (*44*, *45*, *48*). Several additional in vitro studies using eukaryotic DNA topoisomerase II showed that the enzyme often introduces single-stranded nicks in DNA in addition to double-stranded breaks (*49*−*53*). The single-stranded nicks may be relevant to *MLL* breakpoint junctions where there are duplicated regions. In yet other cases, deletions of several hundred bases from *MLL* and from its partner genes have also been observed, suggesting further processing after breakage has occurred or, alternatively, multiple sites of breakage (*46*, *47*).

In the present study, many of the same cleavage sites were induced by etoposide and by its metabolites, but cleavage at most sites was quantitatively greater and more heat-stable in the presence of the metabolites compared to the parent drug. Salt reversal experiments utilizing the oligonucleotide

substrate showed that the cleavage complexes formed with the metabolites were also salt resistant.

Consistent with previous results (*2*, *36*), in the presence of etoposide, DNA topoisomerase II cleaved preferentially at C(−1). The sequence preferences of DNA topoisomerase II in the presence of the catechol and quinone metabolites had not been addressed before. Here we showed a preference for C(−1) among the sites cleaved with both the parent drug and with the metabolites. However, there was a preference for G(−1) among the sites unique to the metabolites. A composite analysis of DNA topoisomerase II cleavage sites examined in both substrates indicates that G(−1) was more common in sites that were unique to the metabolites (7 of 15) compared to sites induced by the parent drug (2 of 41) ($p = 0.005\,26$, two-tailed Fisher's Exact Test). These data provide new information on the site selectivity of the enzyme in the presence of these compounds. This may prove highly relevant in determining the specific agent responsible for the damage as more breakpoints are sequenced.

Etoposide catechol is oxidized to the quinone moiety (*4*, *5*), which potentially could produce depurinating N7-guanine adducts (*11*). Since position 1493 was not only the location of one of the translocation breakpoints, but also the 3′ side of a cleavage site enhanced more by the drug metabolites than the parent drug, we constructed double-stranded oligonucleotides with N7-deaza guanine substitutions to begin to investigate the mechanism of increased cleavage. In the presence of the drug metabolites, but not etoposide, the introduction of N7-deaza guanine substitutions was associated with reduced cleavage compared to cleavage in the unmodified oligonucleotide. These data may be consistent with the formation of N7-guanine adducts and DNA alkylation by etoposide quinone. Although we do not have direct evidence for covalent binding of etoposide quinone to DNA, the reduced cleavage in the *MLL* DNA substrate with N7-deaza guanine substitutions may suggest that the nature of the damage by etoposide quinone involves alkylation since the modified base could not be alkylated at the N7 position due to the replacemant of the nitrogen by a carbon atom. Spontaneous depurination of the N7-guanine adducts would leave abasic sites, which are associated with increased DNA topoisomerase II cleavage (*13*, *54*−*56*). Although N7-guanine adducts would readily be formed as a result of alkylation by the quinone metabolite, etoposide catechol could not alkylate DNA directly. The similar data obtained in the presence of the catechol and quinone metabolites (Figure 5B) may suggest that redox cycling of the catechol to the quinone may have been occurring under the conditions of the assay. Alternatively, substitution with the N7-deaza guanine analogue simply may have altered ordinary DNA topoisomerase poisoning in the presence of the metabolites.

In the present study, we used the p170 (α) isozyme of human DNA topoisomerase II, but there also is a p180 (*β*) isozyme encoded by a different gene (*57*−*59*). DNA topoisomerase IIα expression is maximal in proliferating cells and during the G2 phase of the cell cycle (*58*, *59*). In contrast, the p180 isozyme is expressed in quiescent cells and p180 expression is constant during the cell cycle (*58*, *59*). Both DNA topoisomerase II isozymes may be sensitive in vivo targets of the epipodophyllotoxin anticancer drugs (*60*). There may be minor differences in their sequence preferences (*60*).

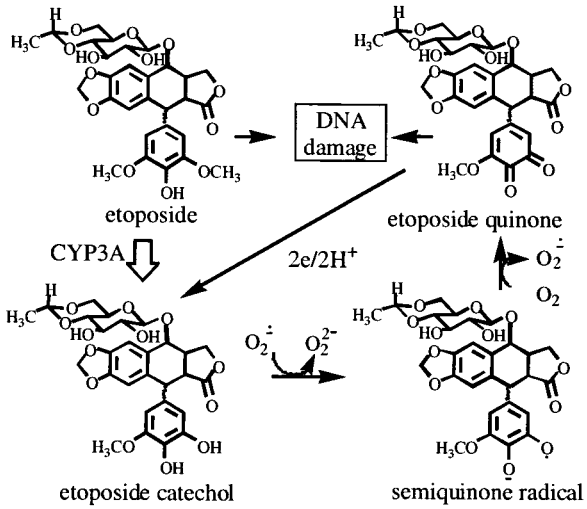

FIGURE 6: Chemical structure of etoposide and its catechol and quinone metabolites. CYP3A4 converts etoposide to its catechol metabolite (*6*), which is further oxidized via a semiquinone free radical to the quinone moiety (*4*). There is redox cycling between the catechol and quinone (*4*). DNA damage from etoposide and its metabolites may be relevant to translocations. Indirect evidence suggests that the nature of the damage by etoposide quinone possibly may involve N7 guanine alkylation (cf. text).

We have shown that etoposide catechol and etoposide quinone, like the parent drug, can induce DNA topoisomerase II-mediated damage in the *MLL* bcr in vitro and suggested indirectly that the nature of the damage may involve alkylation (Figure 6). A possible relationship between several translocation breakpoints and DNA topoisomerase II in vitro cleavage sites also was observed. Additional analysis of the role of the catechol and quinone metabolites is warranted because the genotype of CYP3A4, which metabolizes epipodophyllotoxin to the catechol metabolite, is associated with treatment-related leukemias with translocations involving *MLL* (*3*). Prior demonstration of low plasma levels ofetoposide catechol in patients receiving chemotherapy with etoposide (*27*, *28*) may suggest that etoposide catechol and its quinone metabolite, which is derived from further oxidation of the catechol, may be relevant to translocations. It is conceivable that low metabolite levels may induce sub lethal damage and allow for DNA repair, which could lead to translocations. There are no data yet from patients on the effect of the *CYP3A4* polymorphism on etoposide or etoposide metabolite pharmacokinetics. CYP3A4 is the most abundant liver cytochrome (*61*); there also are no data on the effect of the polymorphism on the concentration of etoposide or its metabolites in hematopoietic cells. The definitive link of etoposide, etoposide metabolites, and other DNA topoisomerase II inhibitors to the translocation process has yet to be determined. While a sensitive in vivo experimental system to directly examine DNA topoisomerase II cleavage within *MLL* at the sequence level would be highly relevant, no such system is currently in place. For example, DNA topoisomerase II immunodetection experiments using the in vivo complex of enzyme assay (*62*) do not examine cleavage at specific sequences, and Southern blot analyses do not directly examine the DNA−enzyme interaction (*63*−*66*). Southern blot analyses also lack the sensitivity to detect rare events in a single-copy gene. Moreover, the breakage site 3′ in the *MLL* bcr detected by Southern blot analyses (*63*−*66*) does not account for translocations where the *MLL*

der(11) and der(other) breakpoints both are more 5′ in the bcr (*43*). The detailed analyses of the biochemistry of etoposide−DNA topoisomerase II−*MLL* interactions and metabolite−DNA topoisomerase II−*MLL* interactions in the present study provide the framework to develop more sensitive assays to examine how the frequently occurring cleavage sites observed in vitro are related to sites of DNA topoisomerase II-mediated breakage within *MLL* in the context of the cell.

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