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Reciprocal DNA topoisomerase II cleavage events at 5′-TATTA-3′ sequences in *MLL* and *AF-9* create homologous single-stranded overhangs that anneal to form der(11) and der(9) genomic breakpoint junctions in treatment-related AML without further processing

Ryan J Whitmarsh[1], Charles Saginario[1], Ya Zhuo[1,2,10], Eva Hilgenfeld[3,11], Eric F Rappaport[4], Maureen D Megonigal[1,12], Martin Carroll[5], Mingli Liu[1,13], Neil Osheroff[6], Nai-Kong V Cheung[7], Diana J Slater[1], Thomas Ried[3], Turid Knutsen[3], Ian A Blair[8] and Carolyn A Felix*[,1,9]

[1]*Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA;* [2]*Thomas Jefferson University, Philadelphia, PA 19107, USA;* [3]*Genetics Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892, USA;* [4]*Joseph Stokes, Jr Research Institute, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA;* [5]*Division of Hematology/Oncology, Hospital of the University of Pennsylvania, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA;* [6]*Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232, USA;* [7]*Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA;* [8]*Center for Cancer Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA;* [9]*Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA*

Few t(9;11) translocations in DNA topoisomerase II inhibitor-related leukemias have been studied in detail and the DNA damage mechanism remains controversial. We characterized the der(11) and der(9) genomic breakpoint junctions in a case of AML following etoposide and doxorubicin. Etoposide-, etoposide metabolite- and doxorubicin-induced DNA topoisomerase II cleavage was examined in normal homologues of the *MLL* and *AF-9* breakpoint sequences using an *in vitro* assay. Induction of DNA topoisomerase II cleavage complexes in CEM and K562 cell lines was investigated using an *in vivo* complex of enzyme assay. The translocation occurred between identical 5′-TATTA-3′ sequences in *MLL* intron 8 and *AF-9* intron 5 without the gain or loss of bases. The 5′-TATTA-3′ sequences were reciprocally cleaved by DNA topoisomerase II in the presence of etoposide, etoposide catechol or etoposide quinone, creating homologous 4-base 5′ overhangs that would anneal to form both breakpoint junctions without any processing. der(11) and der(4) translocation breakpoints in a treatment-related ALL at the same site in *MLL* are consistent with a damage hotspot. Etoposide and both etoposide metabolites induced DNA topoisomerase II cleavage complexes in the hematopoietic cell lines. These results favor the model in which the chromosomal breakage leading to *MLL* translocations in DNA topoisomerase II inhibitor-related leukemias is a consequence of DNA topoisomerase II cleavage.
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Introduction

In the 1980s, the introduction of epipodophyllotoxins into clinical usage ushered in a form of treatment-related leukemia characterized by balanced translocations (Secker-Walker *et al*., 1985; Weh *et al*., 1986; Ratain *et al*., 1987), which most often are translocations of the *MLL* gene at chromosome band 11q23 (Rowley and Olney, 2002). The absence of PCR-detectable translocations before the start of chemotherapy (Megonigal *et al*., 2000; Blanco *et al*., 2001; Raffini *et al*., 2002a) and the general association of DNA topoisomerase II inhibitors, but not other cytotoxic chemotherapies, with leukemias with *MLL* translocations argue that DNA topoisomerase II inhibitors cause the translocations rather than selecting for translocations pre-existing in the marrow (Megonigal *et al*., 2000). However, the mechanism of damage to *MLL* and to its partner genes remains controversial and DNA topoisomerase II and/or an apoptotic nuclease have been implicated in the damage process (Betti *et al*., 2001, 2003; Felix, 2001; Sim and Liu, 2001).

*Correspondence: CA Felix, Division of Oncology, Abramson Research Center, Rm 902B, Children's Hospital of Philadelphia, 3615 Civic Center Blvd, Philadelphia, PA 19104, USA; E-mail: felix@email.chop.edu
[10]Current address: Department of Bone Biology, Merck, West Point, PA, USA
[11]Current address: Hematology/Oncology, Medizinische Klinik und Poliklinik A, University of Muenster, Muenster, Germany
[12]Current address: CSHL Press, Cold Spring Harbor, NY, USA
[13]Current address: Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA
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DNA topoisomerase II changes DNA topology from relaxed to supercoiled states by transiently cleaving and religating both strands of the double helix (Fortune and Osheroff, 2000). Chemotherapeutic DNA topoisomerase II inhibitors, more accurately referred to as DNA topoisomerase II poisons, increase the concentration of enzyme–DNA covalent complexes and have the overall effect of enhancing cleavage (Fortune and Osheroff, 2000). It has been suggested that the same DNA damage mechanism that forms the basis for their cytotoxic action may be relevant to translocations (Felix, 2001). The genotype of CYP3A4, which converts epipodophyllotoxin into its catechol metabolite, influences the risk of treatment-related leukemias with *MLL* translocations (Felix *et al*., 1998). This epidemiological association was recently validated (Ben-Yehuda *et al*., 2002). Epipodophyllotoxin catechol is readily oxidized to a quinone; these metabolites and reactive oxygen species produced during their redox cycling are genotoxins. Etoposide and its metabolites enhance DNA topoisomerase II cleavage at or near *MLL* translocation breakpoints (Lovett *et al*., 2001a, b).

The t(9;11) is the most common translocation disrupting band 11q23 in treatment-related leukemia (Bloomfield *et al*., 2002). The genomic sequences of both breakpoint junctions provide primary mechanistic insights into the translocation process, but both *MLL* genomic breakpoint junctions have been examined in few treatment-related cases (Domer *et al*., 1995; Megonigal *et al*., 1997, 2000; Lovett *et al*., 2001a; Raffini *et al*., 2002b; Langer *et al*., 2003). We characterized the genomic breakpoint junction sequences on both derivative chromosomes of a t(9;11) in the case of treatment-related AML and investigated whether the repair of DNA topoisomerase II-mediated breakage in *MLL* and *AF-9* could have formed both breakpoint junctions. *In vivo* complex of enzyme (ICE) bioassays were implemented to examine the induction of DNA topoisomerase II cleavage complexes by etoposide and its metabolites in hematopoietic cell lines. The induction of DNA topoisomerase II cleavage complexes by etoposide metabolites had not been previously studied in the cellular context.

Results

Case history

Patient t-24 was diagnosed at 15 years of age with paratesticular rhabdomyosarcoma metastatic to the lungs and bone. Treatment included surgical resection of the primary tumor, one cycle of phase II window therapy with topotecan, and intensive chemotherapy as per the NCI 93-C-0125 regimen with five cycles of vincristine, doxorubicin and cyclophosphamide followed by G-CSF. Three consolidation cycles followed, each including melphalan, ifosphamide, etoposide, G-CSF and autologous peripheral hematopoietic stem cell rescue. Cumulative leukemogenic chemotherapy included 450 mg/m^2 of doxorubicin, 12 000 mg/m^2 of cyclophosphamide, 180 mg/m^2 of melphalan, 40 800 mg/m^2 of ifosphamide and 2400 mg/m^2 of etoposide. At 2 years after the initial diagnosis and 18 months after the completion of this treatment, the patient presented with thrombocytopenia and mild inguinal adenopathy. The marrow was replaced with FAB M4 AML. The G-banded karyotype in 19 metaphase cells was 46,XY,t(9;11)(p21;q23). The patient succumbed rapidly to AML despite aggressive treatment.

Fluorescence in situ *hybridization analysis shows MLL translocation*

FISH analysis showed disruption of the *MLL* gene by the reciprocal t(9;11) translocation (Figure 1).

Genomic. cloning reveals precise interchromosomal DNA recombination

Southern blot analysis of *Bam*HI-digested DNA prepared from the bone marrow at AML diagnosis showed 20-kb and 2.1-kb *MLL* bcr rearrangements (Figure 2a). A 2.1-kb reverse panhandle PCR product indicated that the 2.1-kb rearrangement was from the der(9) chromosome (Figure 2b). The der(9) breakpoint in the partner DNA was position 4705, 4706, 4707, 4708, 4709 or 4710 in *AF-9* intron 5 (GenBank No. AC002053). The der(9) breakpoint in *MLL* was position 6588, 6589, 6590, 6591, 6592 or 6593 in intron 8. The same 5′-TATTA-3′ sequence and other identical short sequences were present at/near the breakpoints in both genes (Figure 2b).

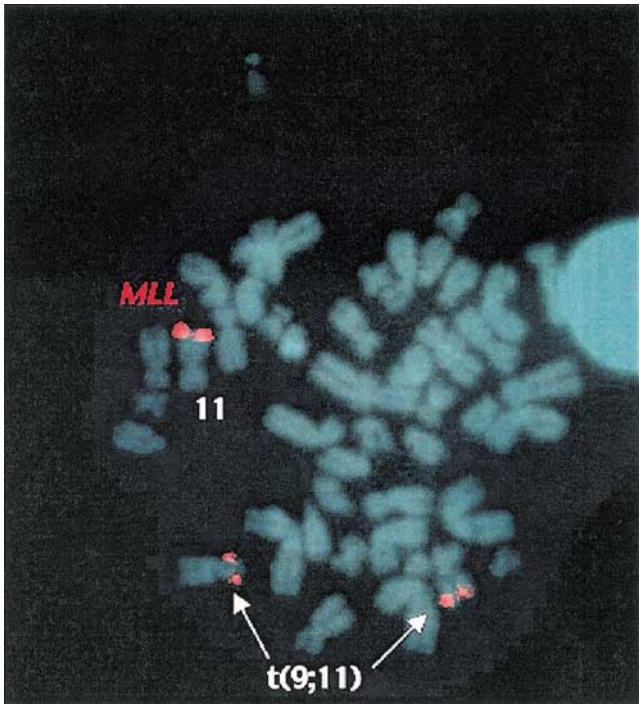

Figure 1 Metaphase FISH analysis of AML of patient t-24. FISH analysis of a metaphase cell with a probe for *MLL* (red) showed the disruption of *MLL* by the t(9;11) observed in the G-banded karyotype

PCR with *MLL*- and *AF-9*-specific primers amplified a 452-bp product containing the der(11) genomic breakpoint junction predicted by the der(9) sequence. The *MLL* breakpoint on the der(11) chromosome was position 6587, 6588, 6589, 6590, 6591 or 6592 in intron 8; the *AF*-9 breakpoint on the der(11) chromosome was position 4706, 4707, 4708, 4709, 4710 or 4711 in intron 5 (Figure 2c). The der(11) breakpoints in *MLL* and *AF-9* involved the exact same 5′-TATTA-3′ sequences as the der(9) breakpoints in both genes. It has been suggested that the relevant DNA double-strand break-repair mechanism in *MLL* translocations is nonhomologous end-joining (NHEJ) (Domer *et al*., 1995; Felix *et al*., 1997, 1999; Super *et al*., 1997; Megonigal *et al*., 1998, 2000; Gillert *et al*., 1999; Lovett *et al*., 2001a; Raffini *et al*., 2002b), but the 5′-TATTA-3′ sequences in *MLL* and *AF-9* (Figure 2b, c) suggested more bases of homology than have been observed at other *MLL* genomic breakpoint junctions (Domer *et al*., 1995; Felix *et al*., 1997, 1999; Super *et al*., 1997; Megonigal *et al*., 1998, 2000; Gillert *et al*., 1999; Lovett *et al*., 2001a; Raffini *et al*., 2002b). Comparison of the der(11) and the der(9) sequences showed that the translocation had occurred without the gain or loss of bases.

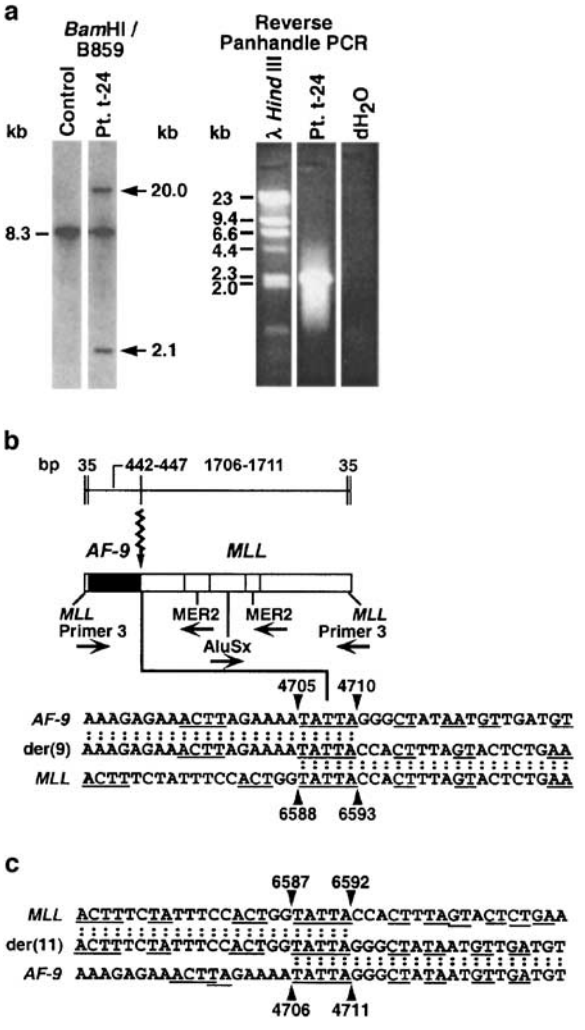

Figure 2 (**a**) *MLL* bcr rearrangements in AML of patient t-24 (left) and reverse panhandle PCR products (right). An 8.3-kb fragment on Southern blot is from normal *MLL* allele; arrows show rearrangements (left). (**b**) Summary of der(9) genomic breakpoint junction in recombination-PCR generated subclones from reverse panhandle PCR. The reverse panhandle PCR product was consistent with 2.1 kb *MLL* bcr rearrangement. One subclone was sequenced in entirety; the breakpoint junction was verified in seven other subclones. In reverse panhandle PCR, nested primer 3 from *MLL* exon 11/intron 10 anneals to both ends of the template. The 35 bp of 5′ sequence extend from *MLL* primer 3 through ligated oligonucleotide. The 442–447 bp of 5′ sequence are *AF-9*. The 3′ 1706–1711 bp include *MLL* bcr sequence through *MLL* primer 3. Arrowheads show *AF-9* and *MLL* breakpoint positions; identical 5′-TATTA-3′ sequences in *AF-9* intron 5 and *MLL* intron 8 precluded precise breakpoint assignments (bottom, underlined). Other short homologies are underlined (bottom). Repetitive sequences are shown (middle). The der(9) breakpoint junction involving the 5′-TATTA-3′ sequence was confirmed by sequencing the 390-bp products of 2 independent PCRs. (**c**) Summary of der(11) genomic breakpoint junction in products of conventional PCR. Gene-specific primers were designed from the der(9) sequence shown in (**b**) to amplify the predicted der(11) genomic breakpoint junction. The expected 452-bp product was obtained and the sequence of the der(11) genomic breakpoint junction was determined in the products from 4 PCRs. The 5′ sequence is *MLL* intron 8. The 3′ sequence is *AF-9* intron 5. The same 5′-TATTA-3′ sequence observed at the der(9) genomic breakpoint junction occurred at the breakpoint junction of the der(11) chromosome, indicating a precise translocation at the sequence level (i.e. a translocation without the gain or loss of bases). Arrowheads show *MLL* and *AF-9* breakpoint positions; 5′-TATTA-3′ sequence in both genes precluded precise breakpoint assignments (underlined). Other short sequence homologies are underlined. These genomic breakpoint junctions suggest more bases of homology than *MLL* genomic breakpoint junctions in other *de novo* or treatment-related leukemias where nonhomologous end-joining has been implicated (Felix *et al*., 1997, 1999; Super *et al*., 1997; Megonigal *et al*., 1998, 2000a; Gillert *et al*., 1999; Lovett *et al*., 2001a; Raffini *et al*., 2002b)

Reciprocal DNA topoisomerase II cleavage events at 5′-TATTA-3′ sequences in MLL and AF-9 create staggered DNA double-strand breaks with homologous single-stranded overhangs that anneal to form der(11) and der(9) genomic breakpoint junctions without any processing

The translocation breakpoints in *MLL* and *AF-9* coincided with functional drug-induced DNA topoisomerase II cleavage sites. Since prior therapy in patient t-24 included etoposide and doxorubicin, *in vitro* cleavage of substrate DNAs containing the normal homologues of the *MLL* and *AF-9* genomic breakpoints was analysed after incubation with human DNA topoisomerase IIα in the absence of drug and in the presence of etoposide, its catechol or quinone metabolites, or doxorubicin. In the absence of drug, little cleavage was detectable in either substrate (Figure 3a, b). Multiple DNA topoisomerase II cleavage sites were induced in both substrates by etoposide and its metabolites, but fewer sites were induced by doxorubicin (Figure 3a,b, no heat (+)topo). In the substrate comprising positions 6513 to 6778 in *MLL* intron 8 (Figure 3a), the sequence encompassing the der(11) and der(9) breakpoint was studied in detail. If the coordinate indicates the base at the 5′ side of the cleavage site

(−1 position), etoposide and both etoposide metabolites induced cleavage at positions 6587, 6588 and 6589. Detectable cleavage without drug indicates preferred sites of cleavage at each of these positions (Figure 3a, no heat (+)topo). Doxorubicin induced cleavage at position 6588. The strongest site in the absence of drug (position 6760) was used as a reference for phosphorimage quantitation (Figure 3a, no heat (+)topo). Position 6760 was the strongest site in the presence of etoposide and both etoposide metabolites; the strongest site in the presence of doxorubicin was position 6551. Table 1 shows the intensities of cleavage induced by etoposide, etoposide catechol, etoposide quinone and doxorubicin at *MLL* bcr positions 6587, 6588 and 6589 relative to the reference site, and rankings of these sites compared to other sites induced by the respective agent in this substrate. Etoposide-induced cleavage at position 6588 and etoposide catechol- and quinone-induced cleavage at positions 6587, 6588 and 6589 remained detectable after heating for 10 min at 75°C, indicating stability of the relevant enzyme–DNA covalent complexes (Figure 3a, heat (+)topo).

The sequence encompassing the der(11) and der(9) *AF-9* breakpoint was studied in detail in the substrate spanning *AF-9* intron 5 positions 4612 to 4905 (Figure 3b). Cleavage was induced at position 4706 by etoposide, both etoposide metabolites and doxorubicin, and etoposide catechol induced the greatest cleavage at this site (Figure 3b, no heat (+)topo). Cleavage was induced at position 4705 by etoposide and both etoposide metabolites and etoposide catechol again induced the greatest cleavage (Figure 3b, no heat (+)topo). Consistent with preferred cleavage sites, there was detectable cleavage without drug at both of these positions. Position 4831, the strongest site in the absence of drug, was used as the reference for phosphorimage quantitation (Figure 3b, no heat (+)topo). The strongest sites in the presence of etoposide, etoposide catechol, etoposide quinone and doxorubicin were positions 4770, 4831, 4831 and 4680, respectively. Table 2 shows the intensities of cleavage induced by these agents at *AF-9* positions 4705 and 4706 relative to the reference site and the ranking of these sites compared to other sites induced by each respective agent in this substrate. The DNA topoisomerase II covalent complexes formed at positions 4705 and 4706 in the presence of etoposide and both etoposide metabolites were heat stable and doxorubicin induced heat-stable cleavage at position 4706 (Figure 3b, heat (+)topo).

Since the translocation occurred without the gain or loss of bases (i.e. was precise at the sequence level), we explored how and which of the functional DNA

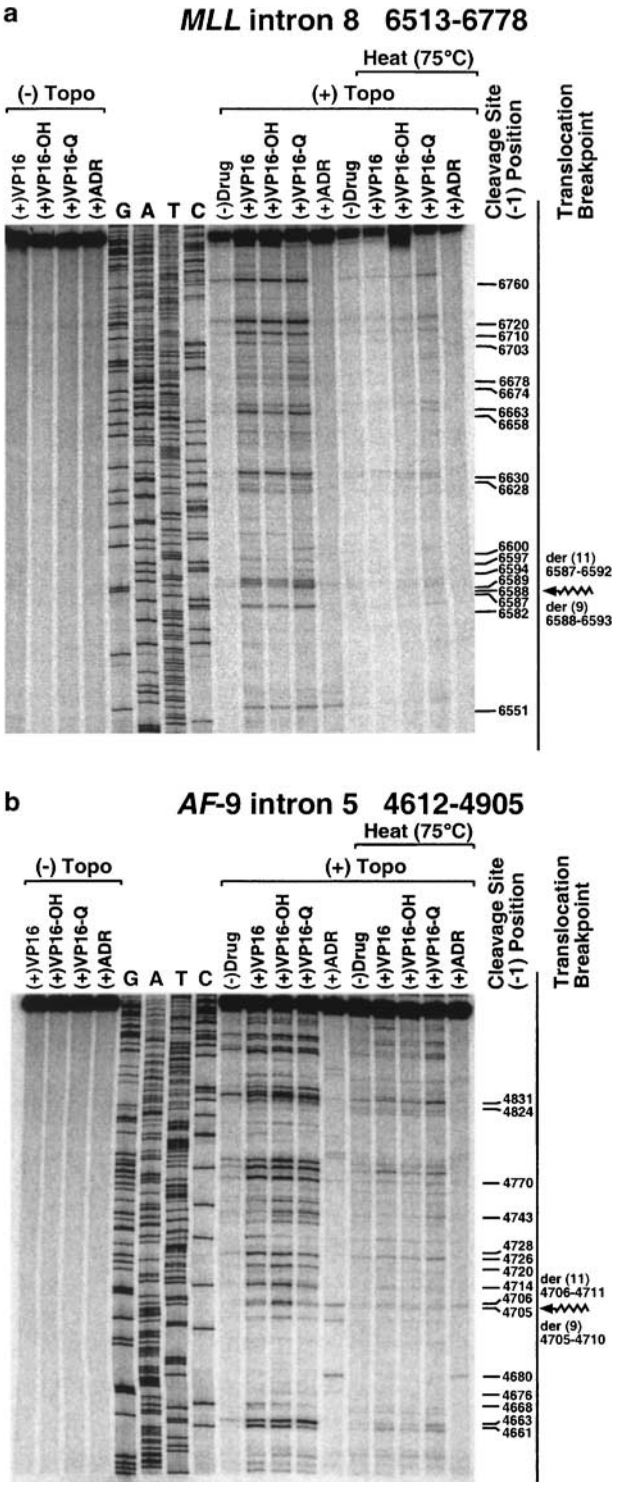

Figure 3 Analysis of normal homologues of *MLL* (**a**) and *AF-9* (**b**) genomic breakpoint sequences in DNA topoisomerase II *in vitro* cleavage assays. DNA topoisomerase II cleavage of *MLL* intron 8 coordinates 6513–6778 (**a**) and *AF-9* intron 5 coordinates 4612–4905 (**b**). Autoradiographs of cleavage products after 10 min incubation of 25 ng (30 000 c.p.m.) 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), etoposide quinone (VP16-Q), or doxorubicin (ADR) are shown. Heat indicates those reactions incubated for 10 min at 75°C before trapping of covalent complexes by adding SDS. 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 arrow at far right indicates translocation breakpoints

Table 1 Intensity of cleavage induced by DNA topoisomerase II inhibitors and heat stability of cleavage at specific *MLL* bcr positions relative to reference site (−1) position 6760 without heat

	Relative intensity/rank (−1) position 6587	*Relative intensity/rank (−1) position 6588*	*Relative intensity/rank (−1) position 6589*
No drug	0.152/7 of 8	0.183/5 of 8	0.116/8 of 8
Etoposide	0.291/16 of 18	1.440/5 of 18	1.231/7 of 18
Etoposide catechol	0.407/11 of 17	0.805/5 of 17	0.606/8 of 17
Etoposide quinone	1.399/5 of 18	1.794/3 of 18	1.045/7 of 18
Doxorubicin	Not applicable	0.068/2 of 2	Not applicable
No drug (+heat)	Not applicable	0.165/7 of 7	Not applicable
Etoposide (+heat)	Not applicable	0.096/7 of 7	Not applicable
Etoposide catechol (+heat)	0.188/11 of 12	0.222/9 of 12	0.175/12 of 12
Etoposide quinone (+heat)	0.209/9 of 12	0.163/11 of 12	0.118/ 12 of 12
Doxorubicin (+heat)	Not applicable	Not applicable	Not applicable

Table 2 Intensity of cleavage induced by DNA topoisomerase II inhibitors and heat stability of cleavage at specific *AF-9* intron 5 positions relative to reference site (−1) position 4831 without heat

	Relative intensity/rank (−1) position 4705	*Relative intensity/rank (−1) position 4706*
No drug	0.012/9 of 10	0.002/10 of 10
Etoposide	0.976/8 of 14	0.927/10 of 14
Etoposide catechol	1.042/10 of 14	1.668/4 of 14
Etoposide quinone	0.257/14 of 14	0.426/12 of 14
Doxorubicin	Not applicable	0.851/2 of 4
No drug (+heat)	0.447/5 of 10	0.434/8 of 10
Etoposide (+heat)	0.702/4 of 11	0.597/6 of 11
Etoposide catechol (+heat)	0.353/9 of 9	0.392/8 of 9
Etoposide quinone (+heat)	0.547/8 of 10	0.748/6 of 10
Doxorubicin (+heat)	Not applicable	0.658/1 of 3

topoisomerase II cleavage sites in *MLL* and *AF-9* could be resolved to form the observed breakpoint junctions. It has been shown before that DNA topoisomerase II introduces 4-base staggered nicks in duplex DNA and creates 4-base 5′ overhangs by introducing coordinated nicks on opposite strands of the double helix (Burden and Osheroff, 1998; Fortune and Osheroff, 2000). Each enzyme subunit forms a covalent phosphodiester bond between its active site tyrosine residue and the 5′-phosphate terminus created in the scission (Burden and Osheroff, 1998; Fortune and Osheroff, 2000). In the model shown in Figure 4, the 4-base 5′ overhangs created by DNA topoisomerase II cleavage at *MLL* position 6587 and *AF-9* position 4705 are perfectly homologous and preserved completely. Repair of the overhangs occurs without any processing. Figure 4 details the simplest model for the creation of both breakpoint junctions; different models derived from the other proximal cleavage sites require added processing (not shown).

MLL translocation breakpoints in treatment-related ALL of patient t-120 occur at DNA topoisomerase II cleavage site in the same genomic region

The der(11) and der(4) genomic breakpoint junctions of a t(4;11) in the treatment-related ALL of patient t-120 have been described (Raffini *et al*., 2002b). The *MLL* der(11) genomic breakpoint (position 6588 or 6589) and the *MLL* der(4) genomic breakpoint (position 6594, 6595 or 6596) were in the exact same region of the bcr as the breakpoints in the case of patient t-24. The der(11) breakpoint was at the functional DNA topoisomerase II cleavage sites at *MLL* bcr positions 6588 and 6589 (Figure 3a). Exonucleolytic nibbling of a few bases from the 3′ side of these cleavage sites would generate the *MLL* breakpoint site (position 6594, 6595 or 6596) on the der(4).

Etoposide and etoposide metabolites induce the formation of DNA topoisomerase II cleavage complexes in human hematopoietic cell lines

ICE bioassays demonstrated significant induction of DNA topoisomerase II cleavage complexes in CEM cells by 20 μM etoposide or its catechol or quinone metabolites (Figure 5a). The cleavage complexes induced by etoposide catechol were comparable in amount to the parent drug. The induction of DNA cleavage complexes was observed in K562 cells treated with 20 μM etoposide or etoposide quinone (Figure 5b). Consistent with the presence of DNA bound to the enzyme, the DNA topoisomerase IIα protein ran higher than its known molecular weight of 170 kDa. However, no induction of DNA topoisomerase II cleavage complexes was detected in either CEM or K562 cells after treatment with doxorubicin at the same concentration. Percentages of viable CEM and K562 cells after etoposide, etoposide catechol, etoposide quinone or doxorubicin exposure for 2 h at 20 μM concentration were similar to untreated cells (Table 3).

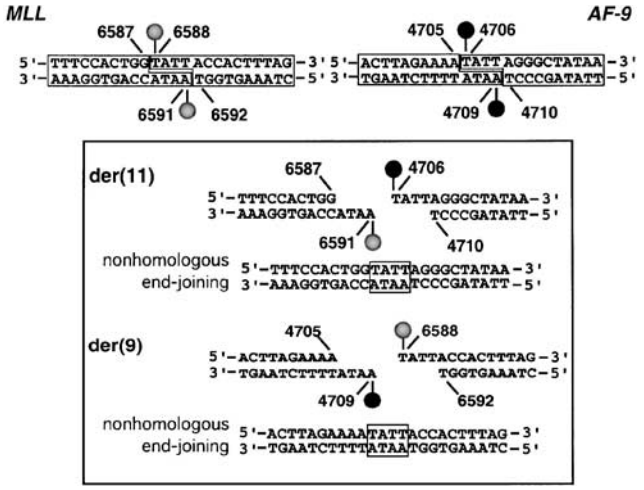

Figure 4 Model for formation of der(11) and der(9) genomic breakpoint junctions. Functional DNA topoisomerase II *in vitro* cleavage sites from which the model was derived are shown at the top. The DNA topoisomerase II homodimer creates 4-base staggered nicks in duplex DNA. *MLL* position 6587 is the 5′ side (−1 position) of a cleavage site on the sense strand. The corresponding cleavage site (+1 position) on the antisense strand staggered by 4 bases from *MLL* position 6587 is position 6591 (Left). *AF-9* position 4705 is the 5′ side (−1 position) of a cleavage site on the sense strand. The corresponding cleavage site (+1 position) on the antisense strand is position 4709 (Right). In the model shown (box), each 4-base 5′ sense and antisense strand overhang of both respective cleavage sites is preserved completely. The 4-base 5′ antisense-strand overhang (positions 6588–6591) of *MLL* and the 4-base 5′ sense-strand overhang (positions 4706–4709) of *AF-9* are perfectly homologous and anneal without any processing to create the der(11). The der(11) forms by fusion of position 6587 on the *MLL* sense strand to position 4706 on the *AF-9* sense strand. Conversely, the entire 4-base 5′ antisense-strand overhang (positions 4706–4709) of *AF-9* and the entire 4-base 5′ sense-strand overhang (positions 6588–6591) of *MLL* are perfectly homologous and anneal without any processing to create the der(9). The der(9) forms by fusion of position 4705 on the *AF-9* sense strand to position 6588 on the *MLL* sense strand

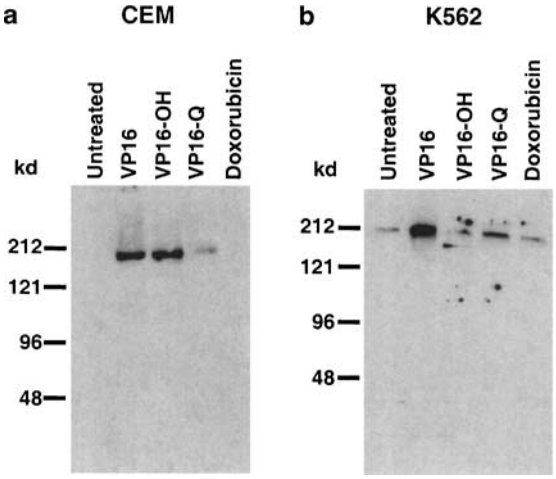

Figure 5 Modified ICE assay. CEM (**a**) and K562 (**b**) cells were treated for 2 h with the indicated drugs at 20 μM final concentration. Untreated CEM and K562 cells were controls. Total DNA including any protein-bound DNA was isolated using a CsCl cushion and 5 μg per lane were loaded on a 4–12% Bis-Tris gradient gel. Western blot was performed using an anti-human DNA topoisomerase IIα antibody to detect DNA topoisomerase II-bound DNA

Table 3 Cell viability (%) without drug or upon treatment with 20 μM DNA topoisomerase II inhibitors for 2 h

	CEM	*K562*
No drug	83–84	88–89
Etoposide	78	83
Etoposide catechol	81	86
Etoposide quinone	79	84
Doxorubicin	81	78

Discussion

The mechanism of damage to *MLL* and its many partner genes from chemotherapeutic DNA topoisomerase II inhibitors that results in translocations remains controversial. This work provides mounting evidence that DNA topoisomerase II can mediate this damage. Patient t-24 had received prior sarcoma therapy with two different DNA topoisomerase II inhibitors. Reverse panhandle PCR (Raffini *et al*., 2002b) enabled cloning of the der(9) genomic breakpoint junction of the t(9;11) and PCR with gene-specific primers amplified the predicted der(11) genomic breakpoint junction. Approaches for cloning both genomic breakpoint junctions are prerequisite to localizing the genomic regions where DNA damage has occurred and has been resolved to form translocations. Figure 6 summarizes *MLL* genomic breakpoint locations in the bcr in treatment-related leukemias where either or both genomic breakpoint junctions have been cloned. Including the AML of patient t-24, both genomic breakpoint junctions have been cloned in nine treatment-related cases (Domer *et al*., 1995; Megonigal *et al*., 1997, 2000; Lovett *et al*., 2001a; Raffini *et al*., 2002b; Langer *et al*., 2003). Nonetheless, patterns are emerging that are relevant to the damage and its resolution: (1) the interchromosomal DNA recombination occurs with overall gains or losses of either no or, most often, relatively few bases in the formation of both breakpoint junctions, suggesting minimal processing (i.e. the interchromosomal DNA recombination is precise or near-precise) (Domer *et al*., 1995; Megonigal *et al*., 1997, 2000; Lovett *et al*., 2001a; Raffini *et al*., 2002b; Langer *et al*., 2003); (2) the *MLL* genomic breakpoint sites are distributed heterogeneously in introns in the bcr with emerging breakpoint hotspots (Domer *et al*., 1995; Megonigal *et al*., 1997, 2000; Sobulo *et al*., 1997; Atlas *et al*., 1998; Daheron *et al*., 2001; Lovett *et al*., 2001a; Raffini *et al*., 2002b; Langer *et al*., 2003); (3) short sequence homologies between *MLL* and partner genes are characteristic of the breakpoint junctions (Domer *et al*., 1995; Megonigal *et al*., 1997, 2000; Sobulo *et al*., 1997; Atlas *et al*., 1998; Daheron *et al*., 2001; Lovett *et al*., 2001a; Raffini *et al*., 2002b; Langer *et al*., 2003).

Microhomologies at and near the breakpoint junctions in *de novo* and treatment-related *MLL*-rearranged leukemias are consistent with the DNA double-strand break-repair mechanism of NHEJ (Domer *et al*., 1995; Felix *et al*., 1997, 1999; Super *et al*., 1997; Megonigal

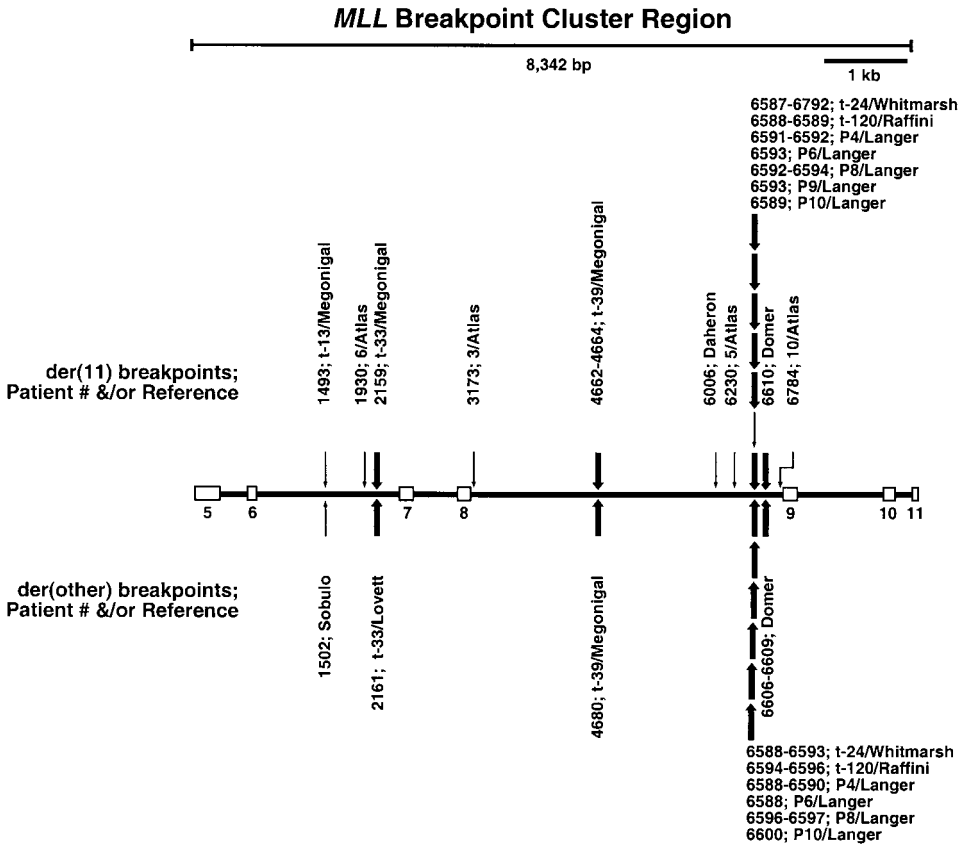

Figure 6 *MLL* genomic breakpoint sites in the bcr in treatment-related leukemias where only the der(11) (fine arrows, top), only the der(other) (fine arrows, bottom) or both genomic breakpoint junctions (bold arrows, top and bottom) have been cloned (Domer *et al*., 1995; Megonigal *et al*., 1997, 2000a; Sobulo *et al*., 1997; Atlas *et al*., 1998; Daheron *et al*., 2001; Lovett *et al*., 2001a; Raffini *et al*., 2002b)

et al., 1998, 2000; Gillert *et al*., 1999; Lovett *et al*., 2001a; Raffini *et al*., 2002b). However, the treatment-related leukemia of patient t-24 is novel inasmuch as precise interchromosomal DNA recombination occurred without the gain or loss of bases and without additional processing. Most DNA double-strand breaks from mutagenic agents cannot be directly ligated and some limited processing, including small deletions and/or DNA polymerization is required before NHEJ occurs (Liang *et al*., 1998; Jackson, 2002). This is consistent with several small deletions (Megonigal *et al*., 2000; Raffini *et al*., 2002b) and a templated single-base insertion (Lovett *et al*., 2001a) that we reported at various breakpoint junctions in treatment-related leukemias shown in Figure 6 (c.f. cases t-39, t-120 and t-33). The rearrangement between identical 5′-TATTA-3′ sequences in *MLL* intron 8 and *AF-9* intron 5 without the gain or loss of any bases in the treatment-related leukemia of patient t-24 is compelling evidence that the sites of damage and the translocation breakpoints in *MLL* and *AF-9* were the same. Although NHEJ repair events generally ensue after small deletions or insertions at the break site (Liang *et al*., 1998), the formation of breakpoint junctions in the treatment-related leukemias with minimal or no processing indicates relationships between the damage regions and observed translocation breakpoints.

In vitro assays demonstrating DNA topoisomerase II cleavage sites at the 5′-TATTA-3′ sequences in *MLL* and *AF-9* provide functional evidence that the repair of DNA topoisomerase II-mediated breakage in *MLL* and *AF-9* could have formed both breakpoint junctions. The formation of the observed breakpoint junctions by the annealing of perfectly homologous 4-base 5′ overhangs of cleavage sites also may suggest the alternative repair mechanism of DNA topoisomerase II subunit exchange, whereby DNA strands undergo recombination via the exchange of enzyme subunits (Bae *et al*., 1988; Charron and Hancock, 1991; Zhou *et al*., 1997). Previously, the DNA topoisomerase II subunit exchange mechanism followed by repair of mismatches in the cohesive ends was proposed for the precise interchromosomal translocation between hotspots for DNA topoisomerase II cleavage sites in the *aprt* locus and an unrelated sequence in amsacrine-treated Chinese hamster ovary cells (Zhou *et al*., 1997). However, this is the first precise drug-induced recombination that has been reported in a patient and, unlike in the experimental model (Zhou *et al*., 1997), no further processing was needed. Nevertheless, induction by the relevant agents of functional DNA topoisomerase II cleavage sites *in vitro*, repair of which could lead to both genomic breakpoint junctions, supports disruption of the DNA topoisomerase II

cleavage-religation equilibrium as the damage mechanism in this translocation.

The more precise *MLL* recombinations in the treatment-related leukemias and heterogeneity in breakpoint distribution (Figure 6) together incriminate a damaging event that occurs at many different sites. DNA topoisomerase II-mediated damage at multiple different sites in these and prior DNA topoisomerase II cleavage assays (Felix *et al*., 1995; Lovett *et al*., 2001a, b) is consistent with the heterogeneity in translocation breakpoint distribution, although cleavage sites in the cellular chromatin context should be more restricted (Capranico *et al*., 1990a). The similar *MLL* breakpoint sites in cases t-120 (Raffini *et al*., 2002b) and t-24 may indicate a preferred damage site in the heterogeneous background (Fortune and Osheroff, 2000), and may be consistent with the damage hotspot previously suggested by the biased *MLL* translocation breakpoint distribution 3′ in the bcr from Southern blot analysis of treatment-related leukemias in adults (Broeker *et al*., 1996). We also reported on the treatment-related leukemia of patient t-13 with a breakpoint at *MLL* intron 6 position 1493 (Megonigal *et al*., 1997; Lovett *et al*., 2001b) near another published breakpoint (Sobulo *et al*., 1997) (Figure 6), possibly suggesting a more 5′ damage hotspot.

While this paper was in review, Langer *et al*. (2003) described both genomic breakpoint junctions of t(9;11) translocations in four cases of treatment-related AML, and the der(11) breakpoint junction in another case. The genomic cloning in each of these five cases indicates involvement of the exact same genomic region of the *MLL* bcr as in the AML of patient t-24 (Figure 6). In cases P8 and P10, there were 1–4-bp and 10-bp deletions with respect to the normal sequence of the *MLL* bcr, and in cases P4 and P6 there were 2–5-bp and 6-bp duplications (Langer *et al*., 2003). Therefore, the additional cases further indicate that the breakpoint site in *MLL* in the AML of patient t-24 is a DNA damage hotspot; however, precise recombination without the gain or loss of any bases still appears uncommon. Although there were two different 5′-TATTA-3′ motifs proximal to the *AF-9* genomic breakpoints in case P10, none of the *AF-9* breakpoints in the cases reported on by Langer (Langer *et al*., 2003) were in the same genomic region of *AF-9* as in the case of patient t-24.

The DNA topoisomerase II cleavage assays described herein also suggest a potential role for DNA topoisomerase II cleavage in the five treatment-related leukemias that Langer recently described (Langer *et al*., 2003), the translocation breakpoints in which are shown in Figure 6. In four of the five cases there was exposure to the DNA topoisomerase II inhibitor doxorubicin (Patients P4, P6, P9, P10). There was additional daunorubicin exposure in patients P6 and P9, and exposure to the DNA topoisomerase I/II inhibitor dactinomycin in patient P10. In case P8, the prior genotoxic chemotherapeutic agents were not DNA topoisomerase II inhibitors (Langer *et al*., 2003). The cleavage assay shown in Figure 3a indicates that doxorubicin induced functional, although weaker DNA topoisomerase II cleavage, at *MLL* bcr position 6588 and that there were several enzyme-only cleavage sites proximal to these translocation breakpoints. It is possible that enzyme-only DNA topoisomerase cleavage may have been important in case P8 and possibly other cases. DNA topoisomerase II catalyses the relaxation of supercoiled DNA for many important cellular process, including replication, transcription, chromosome condensation and mitosis (Wang, 1996). Cytotoxic chemotherapy forces bone marrow repopulation and recovery, which creates a situation where native DNA topoisomerase II cleavage should be active since DNA topoisomerase IIα expression is highest in proliferating tissues (Isaacs *et al*., 1998). Therefore, several cleavage sites shown in Figure 3a may have been of relevance in the cases reported on by Langer.

In *de novo MLL*-rearranged leukemias, there is also heterogeneity in breakpoint distribution (Negrini *et al*., 1993; Felix *et al*., 1997, 1999; Super *et al*., 1997; Megonigal *et al*., 1998; Reichel *et al*., 2001; Raffini *et al*., 2002b; Slater *et al*., 2002). In an analysis of *de novo* ALLs with t(4;11), two breakpoint clusters were suggested in the *MLL* bcr, cluster I of breakpoints in ALLs in children and adults corresponding to intron 7, and cluster II of breakpoints in ALLs in infants corresponding to the region of 3′ intron 8 (Reichel *et al*., 2001), the latter of which coincides with the breakpoint locations in cases t-24, t-120 and the treatment-related leukemias recently described by Langer (Langer *et al*., 2003). (N.B. Alternative designations for *MLL* bcr exons/introns in the cited reference (Reichel *et al*., 2001) where exons 5 through 11 were called exons 8 through 14, respectively.) In leukemia in infants, classical epidemiological studies suggest that maternal exposure to dietary DNA topoisomerase II inhibitors may increase the risk (Ross *et al*., 1996; Ross, 2003). However, *de novo MLL*-rearranged leukemias often contain duplications or deletions of several hundred bases or inverted sequences (Super *et al*., 1997; Felix *et al*., 1999; Reichel *et al*., 2001; Raffini *et al*., 2002b), which differs from treatment-related cases. DNA topoisomerase II introduces single-stranded nicks in duplex DNA as kinetic intermediates of double-stranded breaks (Muller *et al*., 1988; Andersen *et al*., 1989; Osheroff, 1989; Zechiedrich *et al*., 1989; Lee and Hsieh, 1992). It was also recently shown that double occupancy by etoposide at either scissile bond between the DNA and DNA topoisomerase II subunit is needed for a double-stranded break; this observation may further explain how certain DNA topoisomerase II inhibitors can induce single-stranded nicks (Bromberg *et al*., 2003). In the infant cases, the epidemiology and breakpoint junction sequences may suggest a mechanism in which two type II DNA topoisomerases introduce separate single-stranded nicks in duplex DNA that are staggered by up to several hundred bases, and template-directed polymerization of the single-stranded overhangs between the staggered nicks generates duplications. The large deleted regions observed in other infant cases are consistent with multiple

sites of breakage or, alternatively, more extensive processing (Super *et al*., 1997; Gillert *et al*., 1999; Raffini *et al*., 2002b; Slater *et al*., 2002).

The specific homologous 5′-TATTA-3′ sequence identified at the *MLL* breakpoint 3′ in intron 8 in the case of patient t-24 is not a previously recognized DNA topoisomerase II target. An alternative mechanism has been proposed, whereby cleavage by an apoptotic nuclease within the *MLL* bcr is responsible for the damage (Betti *et al*., 2001, 2003; Sim and Liu, 2001). After exposure of hematopoietic cell lines to chemotherapeutic DNA topoisomerase II inhibitors, Southern blot experiments detect a breakage site 3′ in the *MLL* bcr (Aplan *et al*., 1996; Stanulla *et al*., 1997; Strissel *et al*., 1998; Strick *et al*., 2000; Sim and Liu, 2001). This site is induced by several other general cytotoxic agents that cause apoptosis (Stanulla *et al*., 1997; Sim and Liu, 2001). However, in several treatment-related leukemias shown in Figure 6, the *MLL* der(11) and or the der(other) genomic breakpoints are more 5′ in the bcr. The breakage site 3′ in the *MLL* bcr would not account for translocations in which both the *MLL* der(11) and der(other) genomic breakpoints are more 5′ in the bcr. It is possible that the *MLL* bcr is susceptible to site-specific cleavage by DNA topoisomerase II and an apoptotic nuclease as others have suggested (Sim and Liu, 2001), since DNA topoisomerase II cleavage both initiates apoptosis (Kaufmann, 1998) and is associated with recombination (Bae *et al*., 1988; Wang *et al*., 1990; Charron and Hancock, 1991; Pommier and Bertrand, 1993; Zhou *et al*., 1997; Sim and Liu, 2001).

The recently validated association of *CYP3A4-W* genotype with an increased risk of treatment-related leukemia with *MLL* translocations (Felix *et al*., 1998; Ben-Yehuda *et al*., 2002), the conversion of etoposide to genotoxic metabolites by CYP3A, prior demonstration that etoposide catechol and etoposide quinone induce DNA topoisomerase II cleavage (Lovett *et al*., 2001a, b) and the specific drug exposures formed the basis to investigate the potential role of etoposide metabolites in the genesis of the t(9;11) in the AML of patient t-24. The simplest model for the formation of both breakpoint junctions was derived from functional DNA topoisomerase II cleavage sites at *MLL* intron 8 position 6587 and *AF-9* intron 5 position 4705. Doxorubicin did not induce detectable cleavage at either *MLL* bcr position 6587 or *AF-9* position 4705, possibly suggesting that exposure to etoposide and/or its metabolites, but not doxorubicin, was relevant to the leukemia of this patient.

We modified the ICE bioassay to determine whether etoposide metabolites induce DNA–DNA topoisomerase II covalent complexes in the chromatin context of hematopoietic cell lines. The assay entails trapping the DNA covalently bound to DNA topoisomerase II by phosphotyrosine linkage using protein denaturants, and immunodetection (Subramanian *et al*., 1995, 2001; Pourquier *et al*., 2000; Byl *et al*., 2001). Isolation of the protein-bound DNA using a CsCl cushion streamlines the methodology compared to prior versions, and the change from slot blot analysis to Western blot analysis enables size-separation of the DNA–protein complexes. Etoposide induced significant DNA topoisomerase II cleavage complexes in CEM and K562 cells as expected from existing data (Byl *et al*., 2001). Induction of DNA topoisomerase II cleavage complexes was also observed when CEM and K562 cells were treated with etoposide metabolites, which had not been previously studied in this assay. Although the ICE assay does not address the enzyme–DNA interaction at the sequence level, detection of DNA topoisomerase II cleavage complexes in hematopoietic cell lines corroborates the induction of DNA topoisomerase II cleavage complexes by etoposide catechol and etoposide quinone that was observed *in vitro*. Doxorubicin is an intercalative DNA topoisomerase II inhibitor that induces DNA topoisomerase II cleavage with different sequence site-selectivity (Capranico *et al*., 1990b). Doxorubicin is also associated with leukemia as a treatment complication, albeit less often (Sandoval *et al*., 1993). Doxorubicin induced cleavage at fewer sites in the *in vitro* assay, and no increased formation of DNA topoisomerase II cleavage complexes was appreciable within the limits of sensitivity of the ICE assay when CEM and K562 cells were exposed to doxorubicin.

The treatment-related AML with t(9;11) in this report is the first case where functional DNA topoisomerase II cleavage sites induced by etoposide and its metabolites formed reciprocal overhangs for interchromosomal DNA recombination between *MLL* and its partner gene without any processing or repair of mismatched bases. That etoposide metabolites disrupt the DNA–DNA topoisomerase II cleavage–religation equilibrium in hematopoietic cell lines also is new information. Enhanced DNA topoisomerase II cleavage is the accepted mechanism of cytotoxicity of epipodophyllotoxin anticancer drugs (Fortune and Osheroff, 2000). The gravity and increasing incidence of DNA topoisomerase II inhibitor-related leukemias mandates a better understanding of the relationship between the mechanism of cytotoxicity and the mechanism whereby these drugs cause translocations. These results favor the model in which DNA topoisomerase II mediates chromosomal breakage that results in translocations, particular DNA topoi somerase II inhibitors contribute to the breakage in specific cases, and translocations form when this breakage is repaired.

Materials and methods

The IRB at The Children's Hospital of Philadelphia approved this research.

Fluorescence in situ *hybridization analysis (FISH)*

A DNA probe for *MLL* (Ventana Medical Systems, Tuscon, AZ, USA) was hybridized to slides prepared for cytogenetic analysis at the time of AML diagnosis. *FISH* was performed according to standard procedures. Images were acquired

using Leica Q-FISH software (Leica Imaging Systems, Cambridge, UK).

Southern blot analysis

The *MLL* bcr was examined in *Bam*HI-digested DNA using the B859 fragment of *ALL-1* cDNA (Gu *et al*., 1992).

PCR analysis of translocation breakpoint junctions

Reverse panhandle PCR analysis of the der(9) genomic breakpoint junction was performed as described (Raffini *et al*., 2002b). The reaction products were subcloned by recombination PCR (Raffini *et al*., 2002b) and sequenced. Forward and reverse primers 5′-GGGTCATTTGAATAG-CAGCAG-3′ from positions 4559 to 4579 in *AF-9* intron 5 (GenBank No. AC002053) and 5′-AAAACTTGTG-GAAGGGCTCA-3′ from positions 6811 to 6830 in exon 9 in the *MLL* bcr (GenBank No. U04737) were used in conventional PCRs to confirm the der(9) genomic breakpoint junction. The *AF-9* sequence in clone AC002053 is the reverse complement of the *AF-9* genomic sequence; therefore, the reverse complement of AC002053 is used for reference to positions. Forward and reverse primers 5′-TGGAAAGGA-CAAACCAGACC-3′ from positions 6403 to 6422 in intron 8 in the *MLL* bcr and 5′-AGATCACATACCCCACCCCT-3′ from positions 4972 to 4953 in *AF-9* intron 5, respectively, were used in conventional PCRs to isolate the der(11) genomic breakpoint junction.

DNA topoisomerase II in vitro cleavage assays

DNA fragments containing the normal homologues of the *MLL* and *AF-9* genomic breakpoints in the AML of patient t-24 were subcloned into the *Bam*HI and *Eco*RI sites of pBluescript II SK (Stratagene; La Jolla, CA, USA). Singly 5′ end-labeled, double-stranded DNA substrates were prepared from these plasmids exactly as described (Lovett *et al*., 2001a). A total of 25 ng of substrate DNA was incubated with human DNA topoisomerase IIα, ATP and $MgCl_2$ either in the absence of drug or in the presence of etoposide, etoposide catechol, etoposide quinone or doxorubicin at 20 μM final concentration as per the same reaction conditions used for other cleavage assays (Lovett *et al*., 2001a). Covalent complexes were then irreversibly trapped by adding SDS, without or following incubation for 10 min at 75°C, the latter to evaluate heat stability (Bromberg and Osheroff, 2001). Cleavage products were deproteinized and electrophoresed in a denaturing polyacrylamide gel in parallel with a dideoxy sequencing ladder to map the sites of cleavage (Lovett *et al*., 2001a).

Cell lines

The CCRF-CEM cell line originally was derived from the peripheral blood lymphoblasts of a patient with T-cell ALL (Uzman *et al*., 1966). CEM cells were grown in RPMI-1640 medium (Bio-Whittaker; Walkersville, MD, USA) with 10% fetal bovine serum that was heat-inactivated at 70°C (HyClone; Logan, UT, USA) supplemented with 10 mM HEPES and 1 mM sodium pyruvate. The K562 CML-derived cell line (Lozzio and Lozzio, 1979) was grown in RPMI-1640 medium (Bio-Whittaker, Walkersville, MD, USA) with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA). Stock cultures were maintained at 37°C in a humidified 5% CO_2 incubator. Both cell lines were grown in suspension and maintained at $2–3 \times 10^5$ viable cells/ml.

Modified ICE bioassay

The ICE bioassay (Subramanian *et al*., 1995, 2001; Pourquier *et al*., 2000; Byl *et al*., 2001) was modified to examine the induction of DNA–DNA topoisomerase II covalent complexes in CEM and K562 cell lines. Cells (4×10^6) were incubated without drug or with 20 μM final concentration of etoposide, etoposide catechol, etoposide quinone or doxorubicin at 37°C in 2 ml of medium for 2 h in a T25 flask. Percentages of viable cells were then assessed by trypan blue exclusion. Treated and untreated cells were pelleted by centrifugation at 1200 r.p.m. × 10 min and lysed in 3.5 ml of nuclear lysis buffer containing 50 mM Tris pH 7.5, 0.5 M NaCl, 1% NP-40, 1% DOC, 0.1% SDS, 2 mM EDTA and complete protease inhibitors. The lysates were flash-frozen in liquid nitrogen and thawed at 37°C before passage through a 25 G needle to shear the DNA. After centrifugation at 14 000 r.p.m. for 15 min, the supernatants were layered onto 1.5 ml CsCl (1.5 mg/ml) cushions and spun in an NVT90 rotor (Beckman; Palo Alto, CA, USA) × 6 h at 80 000 r.p.m. in an ultracentrifuge to obtain a pellet containing the total genomic DNA and any protein-bound DNA according to the *In vivo Link Kit* protocol (Topogen, Inc.; Columbus, OH, USA) (Zinkevich and Beech, 2000). The pellet was dissolved in 250 μl of 10 mM Tris-HCl (pH 8.0) 1 mM EDTA buffer. The DNA was quantified by measuring the OD_{260} and 0.05 μg of DNA was visualized on an agarose gel. Five μg of DNA was analysed on a Western blot with a Western Breeze kit according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA, USA). DNA topoisomerase II-bound DNA was detected with a mouse anti-human DNA topoisomerase IIα antibody (DAKO, Carpinteria, CA, USA) used at 1 μg/ml in blocking solution.

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Accession numbers

The sequences described in this study were deposited in the Gen Bank database (Accession Nos. AY387662, AY387663).

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