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Bioflavonoids promote stable translocations between *MLL-AF9* breakpoint cluster regions independent of normal chromosomal context: Model system to screen environmental risks

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Abstract

Infant acute leukemias are aggressive and characterized by rapid onset after birth. The majority harbor translocations involving the *MLL* gene with *AF9* as one of its most common fusion partners. *MLL* and *AF9* loci contain breakpoint cluster regions (bcrs) with sequences hypothesized to be targets of topoisomeraseII inhibitors that promote translocation formation. Overlap of *MLL* bcr sequences associated with both infant acute leukemia and therapy-related leukemia following exposure to the topoisomeraseII inhibitor etoposide led to the hypothesis that exposure during pregnancy to biochemically similar compounds may promote infant acute leukemia. We established a reporter system to systematically quantitate and stratify the potential for examine the potential for such compounds to promote chromosomal translocations between the *MLL* and *AF9* bcrs analogous to those in infant leukemia. We show bioflavonoids genistein and quercetin most biochemically similar to etoposide have strong association with *MLL-AF9* bcr translocations, while kaempferol, fisetin, flavone, and myricetin have weak but consistent association, and other compounds have minimal association in both embryonic stem and hematopoietic stem cell populations. The frequency of translocations induced by bioflavonoids at later stages of myelopoiesis is significantly reduced by more than one log. The *MLL* and *AF9* bcrs are sensitive to these agents and recombinogenic independent of their native context suggesting bcr sequences themselves are drivers of illegitimate DNA repair reactions and translocations, not generation of functional oncogenic fusions. This system provides for rapid systematic screening of relative risk, dose dependence, and combinatorial impact of multitudes of dietary and environmental exposures on *MLL-AF9* translocations.

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Author Contributions

BB and CGV contributed equally to the design of experiments, collection and interpretation of data, and preparation of manuscript. BD contributed to the interpretation of data and preparation of the manuscript. DG contributed to data collection and interpretation. MU, RWE, and MS contributed to data collection. CR contributed to hypothesis and design of experiments, collection and interpretation of data interpretation of data, and preparation of manuscript.

Conflict of Interest

None

Keywords

infant acute leukemia; AML; environmental toxicology; *in utero* exposure; bioflavonoids; DNA repair; topoisomerase II; chromosomal translocations; genome instability

Introduction

Infant acute leukemias are characterized by rapid onset less than one year of age, progress rapidly, and are aggressively invasive. Acute lymphoblastic leukemia (ALL) is the more predominant form of infant leukemia while acute myeloblastic leukemia (AML) accounts for about 18% of cases. Approximately 85% of infant ALL and 50% of infant AML cases involve rearrangements of the *MLL* gene at chromosomal position 11q23 that may initially form *in utero* (Gale et al. 1997; Felix et al. 2006). The particularly broad spectrum of rearrangements is a hallmark of *MLL* and may reflect an inherent recombinogenic nature of the locus, or the centrality of the MLL protein in hematopoietic development and differentiation such that rearrangements often possess leukemogenic potential. *MLL-AF9* t(9;11)(p22;q23) fusion is one of the most common *MLL* translocations associated with infant ALL, therapy-related AML (t-AML) and a smaller portion of adult ALL and AML. Leukemias with the MLL-AF9 fusion are clinically aggressive, difficult to treat, often resistant to traditional therapy regimens with the intensive therapies generally being toxic, and have poor prognosis.

The significant majority of *MLL* translocation breakpoints in clinical samples occur in the well-characterized 8.2 kb breakpoint cluster region (bcr) fragment (Gu et al. 1994) containing multiple topoisomeraseII (topoII) cleavage sites, Alu repetitive sequences, DNaseI hypersensitive sites, and high affinity scaffold associated region (SAR) (Strissel et al. 2000; Le et al. 2009). The *AF9* locus has two bcr regions identified from clinical samples of t-AML, infant AML and adult ALL patients (Negrini et al. 1993); bcr1 within intron 4 and bcr2 spanning introns 7 and 8 (Strissel et al. 2000). Elements similar to the *MLL* bcr have been identified including a topoII cleavage site and a DNaseI hypersensitivity site located within intron 7 in bcr2 and two SARs at the borders of both AF9 bcrs (Strissel et al. 2000).

Exposure to topoII inhibitors is associated with t-*MLL*-rearranged leukemias. TopoII is an essential cellular enzyme that catalyzes changes in DNA topology via its cleavage-religation equilibrium. Inhibitors stabilize topoII-DNA covalent complexes, most often by decreasing the rate of religation in a dose-dependent manner, resulting in accumulation of double-strand breaks (DSBs), p53 activation, and induction of repair or apoptosis (Osherooff et al. 1994; Clifford et al. 2003). Etoposide is a well-established inhibitor of topoII, promotes DSBs across the genome as well as specifically within the *MLL* bcr, and promotes *MLL* chromosomal translocations (Blanco et al. 2003; Libura et al. 2005; Felix et al. 2006). *MLL* breakpoint sequences associated with infant acute leukemia are similar to those in t-AML following etoposide-containing regimens (Super et al. 1993; Broeker et al. 1996). Thus, it has been hypothesized that exposure to biochemically similar compounds including

bioflavonoids may contribute to infant leukemia from *in utero* exposure from maternal diet (Ross 2000; Vanhees et al. 2011; Bariar et al. 2013).

Similar to etoposide, bioflavonoids genistein and quercetin contain pendant rings that feature a 4'-OH group essential for activity. Genistein and quercetin and other bioflavonoids inhibit topoII α and topoII β activity (Bandelet and Osheroff 2007; Azarova et al. 2010), induce cleavage in the *MLL* gene locus, and result in some detection of *MLL* rearrangements by inverse PCR and karyotype analysis (Strick et al. 2000; Barjesteh van Waalwijk van Doorn-Khosrovani et al. 2007). The most common sources of bioflavonoids are fruits, vegetables, soy, tea, coffee, wine, and over-the-counter supplements. Due to their antioxidant capacity, bioflavonoids are used for their presumed health benefits such as protection against cardiovascular diseases, cancer and inflammation. However, they have pleiotropic effects on cells, and their direct role in any single pathway has been less clear (Bariar et al. 2013). Further, clear quantitation of the relative potency of compounds or their additive, synergistic, or even quenching effects on the potential to promote translocations remains unclear. *MLL* bcr cleavage and rearrangements have also been detected following exposure of cells to multiple non-genotoxic agents, possibly due to apoptotic DNA fragmentation (Betti et al. 2001; Ploski and Aplan 2001), underscoring the importance of screening a large number of compounds to determine their relative potential for initiating *MLL* translocation events.

We established a stem cell reporter system that can systematically examine, quantitate, and stratify the potential for a large panel of compounds to promote DNA DSBs and chromosomal translocations between the *MLL* and *AF9* bcrs analogous to those observed in infant leukemia. For this we generated stem cell reporter cell lines such that illegitimate DNA repair by nonhomologous end-joining (NHEJ) producing a chromosomal translocation between *MLL* and *AF9* bcrs results in a functional full-length green fluorescent protein (GFP) transcript. This system enabled us to determine (1) if *MLL* and *AF9* bcrs that undergo DSBs are recombinogenic independent of chromosomal context or fusion protein; (2) if a panel of bioflavonoids have the potential to promote these translocations analogous to those observed in infant leukemia; and (3) if a panel of bioflavonoids demonstrates quantifiably stratifiable abilities to promote such events.

These studies demonstrate that bioflavonoids genistein and quercetin are potent promoters that directly lead to *MLL*-*AF9* bcr translocations, while kaempferol, fisetin, flavone, and myricetin consistently but weakly lead to *MLL*-*AF9* bcr translocations. Cells at the earliest hematopoietic stem cell stage are the most at risk for translocations following exposure to bioflavonoids genistein and quercetin, whereas myeloid differentiated cells demonstrated a significantly lower frequency of translocations. The sensitivity of the *MLL* and *AF9* bcrs to bioflavonoids was independent of their normal chromatin context and independent of the formation of any leukemic fusion protein. These studies provide a stratified relative range of the susceptibility of these two bcrs to these agents and their susceptibility to illegitimate NHEJ repair that results in translocations. This reporter system provides for rapid, reproducible, and systematic screening of relative risk, dose dependence, and combinatorial impact of multitudes of compounds in diet and environment that may have direct potential to promote leukemogenic translocations.

Materials and Methods

Transgene constructs:

Two green fluorescent protein (*GFP*) gene reporters were generated (Figure 1A). NZE-GFP fragment of GFP open reading frame (orf) was inserted into pCAGGs (Niwa et al. 1991). To engineer GFP into two exons, adenovirus intron sequence 246 nucleotides was inserted into the GFP orf between the GG doublet (nucleotides 241/242). This GFP-intron plasmid generated two separate constructs. **GFPe1**: contained the chicken β -actin promoter, 5' engineered exon 1 with the first 233 nucleotides of the *GFP* orf, splice donor (sd), 160 nucleotides of adenovirus intron, an I-*SceI* endonuclease recognition site within a linker (S). **GFPe2**: contained an I-*SceI* endonuclease recognition site within a linker (S), 76 nucleotides of adenovirus intron to a splice acceptor (sa), 3' engineered exon 2 of the GFP gene, and β -globin polyA. Blunt-ended XhoI-XhoI fragment of GFPe1 was inserted into intron 11 in blunt-ended XbaI site of the hu*MLL* bcr 8.2 kb BamHI-BamHI (Gu et al. 1994) (Figure 1B). Blunt-ended XhoI-PstI fragment of GFPe2 was inserted into intron 7 of the hu*AF9* bcr2 region 8.94 kb (1276–10221 of AL513498GenBank) between a strong topoII cleavage site and exon 8 (4015 1.18 kb 5' of exon 8) (Strissel et al. 2000) (Figure 1B). Constructs were verified by sequencing (Sequetech Corporation, CA, USA).

Generation of cell lines:

HPRT- EtG2a mouse embryonic stem (ES) cells (ATCC) (Hooper et al. 1987) were maintained at 37°C 5% CO₂ on tissue culture dishes pre-treated with 0.2% gelatin (Sigma). Cells were maintained in non-selective medium consisting of Dulbecco's Modified Eagle Medium (DMEM; Gibco), 15% ES qualified STASIS fetal bovine serum (FBS; Gemini), 100U/mL penicillin/streptomycin (Gemini), 2mM L-glutamine (Gemini), 0.1 mM non-essential amino acids (Gibco), 1000U/ml ESGRO® leukemia inhibitory factor (LIF; Gemini), 100 μ M β -mercaptoethanol (Sigma). Constructs were transfected into EtG2a cells to generate a hu*MLL*-hu*AF9* bcr reporter system. Co-transfection with either *puromycin* or *neomycin* resistance gene-containing plasmids was used for drug selection. 2×10^7 cells were transfected by electroporation with the linearized GFPe1-hu*MLL* bcr construct and pMCneo containing the *neomycin* gene. 24 hours post-electroporation, cells were placed in 200 μ g/mL G418 (Sigma) 10 days. DNA extracted from G418^R clones was screened for single copy GFPe1-hu*MLL* construct by Southern blotting using a GFPe1 fragment as probe on DNA digested with appropriate endonucleases (BamHI/XbaI, XbaI; New England Biolabs Inc.)(Figure 1D). 2×10^7 GFPe1-hu*MLL* cells were transfected by electroporation with the linearized GFPe2-hu*AF9* bcr construct and pgk-puro. 24 hours post-electroporation, cells were placed in 120 μ g/mL puromycin (Sigma) 10 days. DNA extracted from Puro^R clones was screened for single copy of the GFPe2-*AF9* construct by Southern blotting using a GFPe2 fragment as probe on DNA digested with appropriate endonucleases (BglII, XhoI; New England Biolabs Inc.)(Figure 1D). Inserts were confirmed by PCR and sequencing (Sequetech Corporation, California). Single copy clones MAG1, MAG2 and MAG3 were used. MAG cell lines containing stably transfected constructs were maintained in non-selective ES medium. At the conclusion of the studies described, all MAG clones were placed into selective medium for 10 days and confirmed that MAG cell lines had maintained resistance to both puromycin and G418.

DSB induction and translocation formation by IScel expression:

DSBs were induced by IScel expression of the pCBASce expression vector (Richardson and Jasin 2000). 2×10^7 MAG cells per cohort were electroporated (250mV/950μF) with (a) no DNA, (b) 40μg NZE-GFP expression plasmid, or (c) 20μg pCBASce expression plasmid. Cells were plated at 4×10^6 cells per 10 cm tissue culture dish (Falcon). Surviving cells were determined 24 hours post electroporation. All cohorts were screened every 24hrs for GFP expression at 400× magnification on an inverted Zeiss Axiovert25 microscope with images recorded by Zeiss AxioCam MRc digital camera. The interchromosomal NHEJ repair and translocation frequency was calculated as the number of GFP+ colonies per number of cells surviving electroporation (24 hrs) with a minimum of 1×10^7 cells per experiment. To determine significance of translocation frequencies from all three cell lines, a one-way ANOVA was performed using Bonferroni post-hoc test. To determine significance of differences in translocation frequencies between each of the three cell lines, a two-tailed unpaired t-test was used (1vs2, 2vs3, 1vs3).

Compounds:

Etoposide, all flavonoids, and vitamins were obtained from LKT Laboratories. Dipyrone and benzoquinone were obtained from Sigma. Compounds were diluted in dimethyl sulfoxide (DMSO) and stored as 20mM stock solutions. Stock solutions were diluted in 1× phosphate buffered saline pH 7 (PBS; Fisher) prior to each experiment.

Cell viability following exposure to potential topoll inhibitors:

Cytotoxicity of all compounds was determined by Vybrant MTT Cell Proliferation Assay kit (Invitrogen) according to manufacturers' protocol described here. 5×10^3 MAG ES or HSC cells were plated into 96 well plates and incubated 48 hr 37°C 5%CO₂. Each compound at 0, 10, 25, 50, 75, 100, 150, 200, and 250μM in 50μL volume was added to 150μL appropriate medium and incubated 1 hr 37°C 5%CO₂. Media was aspirated and cells washed with 1× PBS then cultured in media. Cells were assayed at 0, 12, and 24 hr post-treatment. 10μL MTT stock solution was added and plates incubated 4 hr 37°C 5%CO₂, followed by 100μL sodium dodecyl sulfate-hydrochloric acid and incubated 4 hr 37°C 5%CO₂, followed by vigorous pipetting and measurement of optical density at 570nm using a Multiskan GO Microplate Reader (ThermoScientific). Each concentration and each time point was assayed in three wells (technical replicates) per experiment. Each experiment was repeated three times (biological replicates, n=3). These data were used to estimate LD50 (Supplemental Figure 1).

Cell proliferation recovery following exposure to potential topoll inhibitors:

Cell recovery and proliferation over time following exposure to all compounds was determined. Serial dilutions of MAG cells (1×10^6 to 1×10^2 cells) were plated on gelatin-coated 10cm tissue culture dishes and incubated 12 hr 37°C 5%CO₂. Cells were treated with each compound at 0, 10, 25, 50, 75, 100, 150, 200, and 250μM by addition to appropriate medium and incubated 1 hr 37°C 5%CO₂. Media was aspirated and cells washed with 1× PBS three times then cultured in media. Cells were assayed at 0, 48, 72, and 96 hr post-treatment by hemacytometer, excluding dead cells with trypan blue exclusion. Each

concentration and each time point was assayed in three culture dishes (technical replicates) per experiment. Each experiment was repeated a minimum of three independent times (biological replicates, n=3). (Supplemental Figure 2).

γ -H2AX foci detection following exposure to potential topoll inhibitors:

1×10^6 MAG cells were plated on 15 mm gelatin-coated coverslips (Neuvitro Corporation) and 1mL media, and incubated 12 hrs 37°C 5%CO₂. Cells were treated with each compound at 0, 10, 25, 50, 75, 100, 150, 200, and 250 μ M by addition to media and incubated 1 hr 37°C 5%CO₂. Media was aspirated and cells washed with 1 $\times$ PBS, then cells incubated in media 4 hr 37°C 5% CO₂. Media was aspirated and cells washed with 1 $\times$ PBS, then cells on coverslips fixed with 3% paraformaldehyde (Sigma) 15 min room temperature with rocking. Then coverslips were incubated with 0.2% Triton-X (Sigma) 15 min room temperature. Then coverslips were blocked with 5% non-fat milk resuspended in 1 $\times$ Tris-Buffered Saline-Tween 20 (TBS-T, Fisher) 30 min at room temperature with rocking. Anti-phospho-Histone H2A.X antibody conjugated to Alexa Fluor 488 (Millipore) was diluted 1:100 in 5% non-fat milk/TBS-T and applied to coverslips 1 hr room temperature in the dark. Coverslips were washed once with 1 $\times$ PBS and mounted to slides (VWR) with ProLong Gold Antifade Mountant with DAPI (Life Technologies). Slides were stored at -20°C until confocal images recorded with an Olympus FV1000 microscope.

DSB induction and translocation formation by potential topoll inhibitors in embryonic stem (ES) cells:

MAG cells were plated at a density of 2×10^7 cells per 10 cm tissue culture plate. Each compound at doses ranging 0, 10, 25, 50, 75, 100, 150, 200, and 250 μ M was added to MAG cells in nonselective ES media and incubated 1 hr 37°C 5% CO₂. Following exposure, cells were washed with 1 $\times$ PBS, cells were trypsinized, washed with 1 $\times$ PBS, and plated on 10cm culture dishes with ES media. All treatment cohorts were washed with 1 $\times$ PBS twice daily on the first two days after treatment, then incubated in media. For each experiment, all cells on all plates in all treatment groups were screened every 24hrs for GFP+ fluorescence at 400 $\times$ magnification on an inverted Zeiss Axiovert25 microscope with images recoded by Zeiss AxioCam MRc digital camera. Each complete experiment including controls was repeated a minimum of three independent times (n=3). To determine the significance of promoting translocations from all the tested compounds, one-way ANOVA tests comparing frequencies using Bonferroni post-hoc tests compared colony numbers/frequency of translocations stimulated by compounds from mock-treated (DMSO only) cells and from other compounds.

DSB induction and translocation formation by potential topoll inhibitors in hematopoietic stem cell (HSC) populations:

We used a previously described differentiation protocol (Francis and Richardson 2007). MAG1 cells were plated at a density of 2×10^6 cells per 10cm tissue culture dish in Iscoves Modified Dulbecco's Medium (IMDM, GIBCO), 15% ES qualified STASIS FBS (Gemini), 1mM sodium pyruvate (Sigma), 100U/ml penicillin/streptomycin (Gemini), 2mM L-glutamine (Gemini), 0.1 mM non-essential amino acids (GIBCO), 1000U/ml ESGRO® LIF (Gemini), 100 μ M monothioglycerol (MTG, Sigma) incubated 48 hr 37°C 5%CO₂. Media

was aspirated and cells washed once in $1\times$ PBS, cells were trypsinized, washed once with $1\times$ PBS, and all cells were replated at 1×10^4 cells per 3.5cm low adherence tissue culture dishes in IMDM, 1% methylcellulose (Stemcell Technologies), 15% BenchMark FBS (Gemini), 2mM L-glutamine (Gemini), 100U/mL penicillin/streptomycin (Gemini), 150 μ M MTG (Sigma), and 40ng/ml murine stem cell factor (mSCF, Stemcell Technologies). By day 7, cultures were given medium containing IMDM, 0.5% methylcellulose, 15% BenchMark FBS, 100U/mL penicillin/streptomycin, 150 μ M MTG, 160 ng/mL mSCF, 30 ng/mL interleukin-3 (mIL-3, Stemcell Technologies), 20 ng/mL human interleukin-6 (hIL-6, Stemcell Technologies), and 3U/mL human erythropoietin (hEpo, Stemcell Technologies) referred to IMDM/HSC media. By day 14, colony bodies that differentiated into hematopoietic stem cells (HSC) were isolated, collected, and centrifuged 10 min 1000 rpm. Resultant cell pellets were disrupted with 0.25% collagenase (Gibco) 1 hr 37°C 5%CO₂. Cells were further disrupted by passage through a 21G 1.5" needle 3 $\times$ and collagenase neutralized by addition of equal volume of IMDM/5% FBS. Cells were treated 1 hr 37°C 5%CO₂ with compounds. Following exposure, cells were centrifuged 10 min 1000 rpm and resuspended in $1\times$ PBS. Cells were centrifuged 10 min 1000 rpm and replated with IMDM/HSC medium at 1×10^6 cells per 3.5cm low adherence tissue culture dishes. For each experiment, all cells on all plates in all treatment groups were screened every 24hrs for GFP + fluorescence at 400 $\times$ magnification on an inverted Zeiss Axiovert25 microscope with images recoded by Zeiss AxioCam MRc digital camera. Each complete experiment including controls was repeated a minimum of three independent times (n=3). To determine the significance of promoting translocations from all the tested compounds, one-way ANOVA tests comparing frequencies using Bonferroni post-hoc tests compared colony numbers/frequency of translocations stimulated by compounds from mock-treated (DMSO only) cells and from other compounds.

DSB induction and translocation formation by potential topoll inhibitors in hematopoietic myeloid progenitor cell populations:

We used a previously described differentiation protocol (Francis and Richardson 2007). MAG1-derived HSC (see above) were isolated and centrifuged 10 min 1000 rpm. Resultant cell pellets were disrupted with 0.25% collagenase 1hr 37°C 5%CO₂ then by passage through a 21G 1.5" needle 3 $\times$ and collagenase neutralized by addition of equal volume of IMDM/5% FBS. To generate myeloid progenitors, cells were plated at a density of 1×10^6 cells per 3.5 cm, low adherence tissue culture dish in medium containing 1% methylcellulose, 15% Benchmark FBS, 100 U/mL penicillin/streptomycin, 2mM L-glutamine, 150 μ M MTG, 1% bovine serum albumin (BSA, Sigma), 10 μ g/mL insulin (Stemcell Technologies), 200 μ g/mL transferrin BIT9500 serum substitute (Stemcell Technologies), 150 ng/mL mSCF, 30 ng/mL mIL-3, 30 ng/mL hIL-6, 3 U/mL hEpo -- referred to as culture forming units-myeloid cell (CFU-M) media. At day 7 progenitors were isolated and centrifuged 10 min 1000 rpm. Resultant cell pellets were disrupted with 0.25% collagenase 1hr 37°C 5%CO₂ then disrupted by passage through a 21G 1.5" needle 3 $\times$ and collagenase neutralized by addition of equal volume of IMDM/5% FBS. Cells were treated 1 hr 37°C 5%CO₂ with compounds. Following exposure, cells were centrifuged 10 min 1000 rpm, washed once in $1\times$ PBS. Cells were centrifuged 10 min 1000 rpm. Cells were resuspended in CFU-M media and added in a drop-wise fashion on top of methylcellulose at

1×10^6 cells per 3.5cm low adherence tissue culture dish. Cells were allowed to generate CFU-M for 12–14 days when CFU-M were scored from all treatment groups. (n=3).

Molecular analysis of translocation events.

For chromosome analysis to confirm translocations, 1×10^6 cells of individual ES cell repaired clones were expanded and chromosome metaphase spreads prepared as described previously (Richardson and Jasin 2000). At least 10 spreads of each were analyzed by the Columbia University (New York, NY) Spectral karyotyping (SKY) core facility using the Applied Spectral Imaging software. MAG1 showed t(2;5), MAG2 showed t(4;17), and MAG3 showed t(2;7). For polymerase chain reaction (PCR), 1×10^6 cells of individual ES repaired clones were expanded and DNA extracted. Alternatively, individual HSC repaired clones were isolated and cells pelleted by centrifugation. DNA extractions and whole genome amplification (WGA) were performed using Qiagen Repli-G kit (Qiagen) according to manufacturer's protocol. For PCR reactions amplifying GFP1-GFP2 or GFP3-GFP4, each 25 μ L PCR reaction contained 1.0 μ g template DNA, 15 pmol each primer, and a PuReTaq Ready-To-Go PCR bead (GE Healthcare) reconstituted in H₂O. Program parameters: 94°C 5 min; 94°C 30 sec, 57°C 30 sec, 72°C 45 sec 35 cycles; 72°C 10 min; 4°C hold.

For PCR reactions amplifying GFP1-GFP4 across breakpoint junctions, PCR reactions were performed using Phusion High Fidelity PCR kit (New England Biolabs) according to manufacturer's protocol. Each 25 μ L PCR reaction contained 1.0 μ g template DNA, 1 $\times$ HF buffer, 15 pmol each primer, 10mM dNTPs, 1 uL Phusion Polymerase. Additional primer pairs within the two GFP exons and intron boundaries were used in combinations to independently confirm results (data not shown). Program parameters: 98°C 5 min; 98°C 30 sec, 57°C 30 sec, 72°C 45 sec 35 cycles; 72°C 15 min; 4°C hold. PCR primers: GFP 1-- 5'AAGGCCAAGAGGGCCAA3'; GFP 2-- 5'TTCCTGCTCTGCCTGATC3'; GFP 3-- 5'TGGACGGCGACGTAAAC3'; GFP 4-- 5'GTGCTCAGGTAGTGGTTG3'. PCR sequencing was performed by Eurofins Genomics LLC.

Results

Independently derived reporter cell lines MAG1, MAG2, and MAG3 with single copy insertions of the GFP₁-hu*MLL* bcr and GFP₂-hu*AF9* bcr constructs were utilized. *GFP* reporters were upstream (GFP₁) or downstream (GFP₂) of mapped breakpoints identified in clinical samples of infant- and t-AML-related containing strong topoII cleavage sites (Broeker et al. 1996; Strissel et al. 2000). Construct design allowed for determining if the compounds promote DSBs in the bcrs independent of their endogenous chromatin context and promote illegitimate NHEJ to result in translocations. A translocation by this mechanism will ligate the GFP₁ exon and the GFP₂ exon onto the same DNA duplex and generate a functional *GFP* gene (GFP+; Figure 1). To validate the system and confirm a lack of cryptic splice sites within the intervening bcr regions, I-*SceI* endonuclease-induced DSBs were generated. DSB induction significantly stimulated readily detectable GFP+ fluorescent cells/colonies in all three parental cell lines demonstrating illegitimate NHEJ between the *MLL* and *AF9* bcrs with targeted DSBs independent of their endogenous chromosomal loci (Table 1; Figure 2). The frequencies of interchromosomal illegitimate NHEJ translocations

were calculated to be highest in MAG1, intermediate in MAG2, and lowest in MAG3 ($2.6 \pm 0.23 \times 10^{-6}$, $1.3 \pm 0.15 \times 10^{-6}$, $0.63 \pm 0.40 \times 10^{-6}$ respectively) at average calculated frequency of $1.4 \pm 0.3 \times 10^{-6}$. Across all 3 MAG cell lines, the stimulation was statistically significant (p value <0.00001). Comparison of stimulation of GFP+ colonies between the three MAG cell lines (1vs2 p value <0.001; 2vs3 p value <0.01; 1vs3 p value <0.001) indicate that chromatin context and location play some role in determining the potential frequency of illegitimate NHEJ to occur. The observed frequency of translocations by I-SceI here is approximately 4-fold lower than a previous model system that utilized I-SceI to induce NHEJ translocations promoted by the presence of homologous Alu cassettes (Elliott et al. 2005).

We confirmed that in our system exposure to an exogenous topoII inhibitor etoposide stimulates DNA damage and illegitimate NHEJ DNA repair between the hu*MLL* and hu*AF9* bcrs in a dose dependent manner. As expected, exposure of MAG1 cells to increasing doses of etoposide promoted increasing numbers of GFP+ fluorescent events or colonies scorable by both microscopy and by flow cytometry (Table 2).

We then tested the potential for a panel of environmental compounds that potentially act as topoII inhibitors to (1) directly promote DNA DSBs and (2) stimulate illegitimate NHEJ DNA repair between the hu*MLL* and hu*AF9* bcrs in resulting in GFP+ fluorescent colonies in a dose dependent manner. First, the potential for bioflavonoids to directly lead to DSBs was determined by the appearance of γ H2AX foci. Etoposide, quercetin, kaempferol, myricetin, genistein, and luteolin robustly induced γ H2AX foci in a dose-dependent and time-dependent manner (Figure 3 panels B,C,D,E,G, and J respectively). Fisetin, daidzein, and flavone were less potent inducers of γ H2AX foci, but still in a dose-dependent and time-dependent manner (Figure 3 panels F,H,I, and K). Other bioflavonoids biochaninA, epigallocatechin gallate, hesperitin, narigenin, as well as the phytochemical 3,3 diindolylmethane and vitamins A and B did not significantly induce detectable γ H2AX foci (Figure 3 panels I and L).

MAG1 cells were exposed for 1 hr to bioflavonoids including flavonols (fisetin, kaempferol, myricetin, quercetin), isoflavones (biochaninA, daidzein, genistein), flavones (flavone, luteolin), flavanols (epigallocatechin gallate), flavanones (flavanone, hesperidin, hesperetin, narigenin), and the phytochemical 3,3 diindolylmethane. The study also included the non-steroidal anti-inflammatory drug dipyrone and the environmental toxin benzoquinone. Dipyrone is associated with *MLL*-rearranged infant leukemia in epidemiological studies (Alexander et al. 2001). Benzoquinone is a topoII inhibitor at concentrations as low as 1–10 μ M (Lindsey et al. 2004) stimulating DNA DSBs, oxidative stress, or fragmentation (Hiraku and Kawanishi 1996) and associated with hematopoietic malignancies (Snyder 2012; Wang et al. 2012). Both compounds are believed to act by mechanisms distinct from the bioflavonoids tested here. To distinguish from the possibility that *MLL*-*AF9* bcr translocations are stimulated in response to a broad spectrum of environmental agents, MAG1 cells were exposed to vitamin A that contains ring structures similar to the bioflavonoids and vitamin B with a completely different structure. As control, cells were mock-treated with DMSO. Dose-dependent cell survival MTT assays demonstrated 25–70% cell survival within the tested ranges, and provided estimates of LD50 doses for comparison

of relative potential for each compound to induce translocations (Supplemental Figure 1). Compounds that did not induce significant amounts of cell death were exposed to a range of doses up to 200 μ M. Cell viability numbers after exposure showed that LD50 doses did not lead to long-term toxicity that would prevent detection of translocations (Supplemental Figure 2).

Following exposure, cells were cultured to allow for DNA repair by illegitimate NHEJ and a chromosomal translocation resulting in GFP+ cells (Figure 2, Table 2). By contrast, in the absence of DSBs, none of the parental cell lines generated GFP+ cells over 6 passages (Figure 3 panels A,B) indicating that the formation of stable translocations in the absence of a promoting event or factor is extremely rare, calculated at more than 2 logs lower frequency ($< 7.2 \times 10^{-9}$; Table 2). Genistein and quercetin were the most potent promoters of *MLL-AF9* translocations in this assay with calculated average frequencies at 75 μ M to be 1.2×10^{-6} and 1.1×10^{-6} , respectively (Table 2). Kaempferol and fisetin were mildly potent promoters of *MLL-AF9* translocations, while flavone and myricetin decreased in potency to weakly but consistently promoting translocations (Table 2). Daidzen and luteolin did promote *MLL-AF9* bcr translocations but required dose exposures higher than the approximate LD50 suggesting at least 40-fold lower potential to promote stable translocation events than the potent bioflavonoids (Table 2). In addition, since luteolin generated only a single GFP+ colony across multiple experiments, we cannot rule out the possibility that it was a rare spontaneous event. At doses up to 200 μ M none of the flavanones (flavanone, hesperidin, hesperetin, naringenin), biochaninA or the natural phytochemical 3,3 diindolylmethane led to observed GFP+ colonies.

Dipyrone exposure of MAG1 cells up to 100 μ M generated a single GFP+ colony in one replicate (calculated frequency of 0.03×10^{-6} ; Table 2). However, as with luteolin, we cannot rule out the possibility that it was a rare spontaneous event. Benzoquinone exposure of MAG1 cells was not sufficient to promote *MLL-AF9* translocations following exposure up to 125 μ M (frequency of $< 0.01 \times 10^{-6}$; Table 2). This contrasts from other studies in which as little as 1 μ M benzoquinone stimulated intrachromosomal homologous recombination by 2–3 fold over undamaged cells (Winn 2003) and points to the differences between intra- and inter-chromosomal DNA repair.

A one-way ANOVA comparing frequencies using Bonferroni post-hoc test demonstrated that the frequency of translocations stimulated by genistein, quercetin, kaempferol and fisetin were statistically significantly different from untreated cells (Figure 4). Further, the frequency of translocations stimulated by genistein was statistically significantly different from kaempferol, fisetin, myricetin, daidzen, flavone, luteolin, and dipyrone; the frequency of translocations stimulated by quercetin was statistically significantly different from fisetin, myricetin, daidzen, flavone, luteolin, and dipyrone; the frequency of translocations stimulated by kaempferol was statistically significantly different from myricetin, daidzen, flavone, luteolin, and dipyrone (Figure 4). Similar results were observed with MAG2 and MAG3 cells although at frequencies of $\frac{1}{2}$ log lower than MAG1 (data not shown)

We questioned whether the observed frequencies of translocations arising at the embryonic stem cell stage are comparable to hematopoietic stem cell (HSC) stage commonly presumed

to be the target of *MLL* rearrangement leukemia-initiating events. For this we utilized *in vitro* hematopoietic differentiation of the MAG1 cell line (Francis and Richardson 2007) along with exposure to the panel of potential topoII inhibitors and compounds at the calculated approximate LD50 dose. Following exposure up to the approximate LD50 dose, HSC cells were cultured to allow for DNA repair by illegitimate NHEJ and a chromosomal translocation resulting in GFP+ cells (Figure 5). Overall, the frequency of translocations induced by the more potent topoII inhibitors of the compounds at the HSC stage of differentiation was dose-dependent and not statistically different from those observed in ES cells (Supplemental Table 1). At these doses, no translocations were observed following exposure to myricetin, daidzen, or luteolin (data not shown, frequency below our limit of detection $<0.01 \times 10^{-6}$). However, we did not determine if higher doses (above LD50) would lead to extremely rare translocations as they did in ES cells. Both dipyrone and benzoquinone did induce translocations in HSC populations.

As further confirmation that GFP+ clones from both ES and HSC populations are *bona fide* translocations, we performed both spectral karyotype (SKY) analysis (Figure 6A) and PCR analysis using primers to amplify across the proposed breakpoint junctions (Figure 6B,6C). Spectral karyotype (SKY) analysis of GFP+ clones arising from each of the parental lines verified chromosomal translocations. DNA from each of the parental cell lines and GFP+ translocation clones produced PCR products with primers to amplify either GFPe1 (GFP1F-GFP2R primer pair) or GFPe2 (GFP3F-GFP4R primer pair) alone. DNA from parental cell lines did not produce PCR products across the breakpoint junctions (GFP1F-GFP4R primer pair). By contrast, DNA from GFP+ translocation clones did produce PCR products across the breakpoint junctions (GFP1F-GFP4R primer pair). DNA from GFP+ clones promoted by I-SceI DSBs produced PCR products at or near the expected 0.96 kb without end-resection prior to generation of the translocation event. DNA from GFP+ clones promoted by bioflavonoids produced PCR products that varied ranging from 0.96 kb up to several kb. Size variability is expected to be dependent on both the location of the initial breakpoints induced in *MLL* and *AF9* as well as DNA end-resection prior to generation of the translocation event.

We then questioned whether the observed frequencies of translocations in HSC induced by the potent bioflavonoids are universal to cells at all stages of differentiation. To compare the observed frequencies of translocations arising at the HSC stage with more differentiated progenitor myeloid cells, we again utilized well-characterized *in vitro* hematopoietic differentiation protocols along with exposure to potential topoII inhibitors. MAG1-derived HSC populations were differentiated into early myeloid/erythroid progenitors, exposed to compounds for 1 hr, and then seeded for CFU proliferation. The frequency of translocations induced by quercetin and genistein at the later stage of hematopoietic differentiation was dramatically reduced, and by etoposide somewhat reduced (Figure 7). The frequency of translocations induced by kaempferol, fisetin, and flavone at the later stage of myeloid differentiation was reduced so much that they did not produce detectable *MLL-AF9* translocations (frequency below our limit of detection $<0.01 \times 10^{-6}$). By contrast the number of translocations induced by dipyrone increased with myeloid differentiation further pointing a different mechanism of action by this compound.

Discussion

This reporter system demonstrated that physiologically relevant doses of bioflavonoids, particularly genistein, quercetin, kaempferol, fisetin, and flavone are potent promoters of human *MLL-AF9* bcr translocations stimulating them by at least 2 logs over undamaged cells. Bioflavonoids have pleiotropic effects in addition to directly inducing DSBs by γ H2AX induction. The *MLL-AF9* bcr translocations from the assay are likely due to a combination of both direct and indirect stimulation of NHEJ events. This system easily can be utilized at both ES and HSC stages to quantitatively assess the relative risk of a large number of compounds to induce leukemic translocations. The compounds that did not reveal translocation events have a frequency below our limit of detection ($<0.01 \times 10^{-6}$). However, in this study we did not determine if higher doses (above LD50) or increasing the number of cells treated would lead to extremely rare translocations. There was a loose correlation between compounds that promoted more DSBs and those that promoted more translocations consistent with the notion that multiple DSBs provide the opportunity for interchromosomal DNA repair (Richardson and Jasin 2000). Frequencies of translocations stimulated by genestein and quercetin are similar to those observed following endonuclease-induced I-*SceI* DSBs at both loci suggesting that these particular bioflavonoids are potent promoters of DSBs within the two bcrs even at doses that allow for significant cell survival and proliferation in culture.

The risk for certain translocations to occur most likely changes during development due to changes in gene expression, chromatin remodeling, and epigenetic factors (Gill Super 2015). Both ES and HSC harboring resultant translocations retained the capacity for significant proliferation. HSCs contain a generally more open chromatin state that may make them more sensitive to DNA damage, and prone to undergo illegitimate NHEJ repair reactions resulting in *MLL-AF9* translocations. It is also possible that stem and later progenitor cell subpopulations are equally sensitive to these agents and prone to illegitimate DNA repair leading to translocations but leukemias only arise from those rearranged cells with significant proliferative potential. These data support significantly lower relative potential of bioflavonoids forming stable translocations in more differentiated myeloid/erythroid progenitors as compared to stem cell/HSC populations by several hundred-fold. Interestingly, these data point to the differences between mechanism of action by compounds and their potential to promote translocations at different stages of hematopoietic differentiation as dipyrone became more potent with differentiation.

Importantly, these data suggest that specific *MLL* and *AF9* bcrs' motifs are the drivers of the illegitimate DNA repair reactions, are highly recombinogenic independent of their endogenous chromosomal context and in the absence of a survival advantage conferred by a functional oncogenic fusion. The development of genetic engineering technologies involving knock-in models, the use of customizable DNA nucleases, *MLL* TALENs, or the CRISPR/Cas9 system offers promising methods in which to model progression of malignancies characterized by chromosomal translocations but do not model the initial formation of these translocations, particularly in response to exposure to topoII inhibitors in a physiologically relevant manner.

The number of homologous Alu repetitive elements within the bcrs of these two regions has led to the proposal that illegitimate fusions are stimulated by local alignments of homologous sequences during the process of DNA repair (Lovett et al. 2001; Elliott et al. 2005). A previous study using a *neomycin* reporter system and Alu repetitive element cassettes within introns determined that I-*SceI* induced NHEJ-NHEJ events analogous to those observed here were promoted at a frequency of 4.2×10^{-6} (16% of all events occurring at a frequency of 2.6×10^{-5}) (Elliott et al. 2005). The frequency of NHEJ-driven translocations induced by I-*SceI* here is approximately one fourth (1.4×10^{-6}). Our lower frequency likely highlights the importance of sequence identity of Alu elements present nearest the DNA breakpoints. Alternatively it is possible that the potential in that system for other repair mechanisms (*e.g.* homologous recombination or single strand annealing) was sufficient to promote the alignment of potential junction partner DNA ends more frequently even if ultimately repair would occur via NHEJ. The *MLL* bcr is small and well-characterized such that a number of mapped infant leukemia breakpoints map to the 3' half of the bcr. Thus placement of the GFPe1 into intron 11 likely mimics the sites of breakage induced by the compounds tested. The *AF9* locus contains two bcr regions; bcr2 (10 kb) is flanked on the 5' side by a scaffold attachment region and contains multiple topoII cleavage sites (Negrini et al. 1993; Strissel et al. 2000). Thus the placement of GFPe2 into this region likely mimics the sites of breakage induced by the compounds tested. However the broader span of the *AF9* mapped bcr may have led to DNA damage and illegitimate repair events that did not reconstitute a functional GFP gene and would not be recovered here.

Our data highlight the difference between the potential to promote DNA damage and to result in a downstream chromosomal translocation analogous to those observed in leukemias. Many of the compounds we tested have been shown to inhibit topoII *in vitro* (+/- scale) and to induce *MLL* cleavage *in vivo* in hematopoietic stem cell-enriched populations (Strick et al. 2000). Our results extend those data to quantitatively assess and stratify the relative potential of each compound to induce stable *MLL-AF9* translocation events. In addition the combined data point to the multiple step pathways involved in producing stable potentially oncogenic translocations—induction of DNA damage, DNA damage response (DDR), repair pathway choice, and viability and proliferation of cells with illegitimate repair structures. Differences between damage and repair may lie in the number of DNA damage sites, the stability of the breaks incurred by these compounds, kinetics of removal of stabilized DNA-topoII complexes, or a favored mechanism of repair. Multiple bioflavonoids induce the DSB-mediated DDR as evidenced by induction of γ -H2AX foci, ATM phosphorylation, and p53 signaling (O'Prey et al. 2003; Shimada et al. 2011; Clewell et al. 2014). However, a more direct role or influence of these compounds on the repair of damage is not as clear. Interestingly, myricetin was shown to stimulate the efficiency of NHEJ in a plasmid-based annealing assay with both compatible and non-compatible DNA ends (Charles et al. 2014). This finding may in part explain our data if myricetin's stimulation of NHEJ repair promotes ligation to the nearest DNA end, thus spatially suppressing chromosome movement and illegitimate annealing to another partner. By contrast the same study showed quercetin and genistein had no apparent stimulation of NHEJ in the same assay which conversely might allow for increased frequency of illegitimate NHEJ reactions and the translocations observed here (Charles et al. 2014).

The most common sources of bioflavonoids are fruits, vegetables, soy, tea, coffee and wine. Genistein is an estrogen derivative available at health food stores as dietary and menopausal supplements, and a soy phytoestrogen present in foods, particularly soybeans, and infant soy formulas. Previous studies reported average adult soy intake to be 0.15–3.0 mg/day (Horn-Ross et al. 2001), but daily intake averages can vary and increase to 8.6mg/day in women and 7.5mg/day in men (Mulligan et al. 2007). Foods contain multiple bioflavonoids, and bioflavonoids are bio-accumulative which likely increases plasma concentrations (Ross and Kasum 2002). Study of the potential for environmental or dietary compounds to induce infant leukemias is more relevant since they cross the placental barrier as shown with the synthetic bioflavonoid EMD-49209 (Schroder-van der Elst et al. 1998), genistein (Doerge et al. 2001; Vanhees et al. 2011) quercetin (Vanhees et al. 2011), herbal medicines, dipyrone, and pesticides including mosquitocidal Baygon (Alexander et al. 2001; Pombo-de-Oliveira and Koifman 2006). Exposure *in utero* is likely more damaging due to differences in metabolic and excretion rates of mother and fetus (Todaka et al. 2005) as well as rapidly developing and proliferating fetal cells that are more sensitive to topoII inhibiting agents (Zandvliet et al. 1996).

Halogenated compounds in household and baby products are another possible risk factor for childhood leukemia to be tested. Polychlorinated biphenyls (PCBs) detectable in indoor carpets, and polybrominated diphenyl ethers (PBDEs), structurally similar to PCBs used as flame retardants, have been associated with childhood ALL (Ward et al. 2014). In support of mechanistic action, certain PCB quinone metabolites increase DNA cleavage by topoIIa *in vitro* and in cultured human cells (Bender et al. 2006).

Genetic differences among infants exposed prenatally to topoII inhibitors likely affect susceptibility to formation of chromosomal translocations. Genome-wide association studies (GWAS) have not identified common variants of susceptibility to either *de novo* or t-AML, but incorporating exposures into GWAS may reveal specific SNPs that contribute to disease (Greim et al. 2014). However, infants specifically with *MLL*-rearranged leukemias were 2.5-fold more likely to harbor NAD(P):quinone oxoreductase (NQO1) polymorphisms associated with reduced activity versus unselected cord blood (Wiemels et al. 1999). Variant alleles of DNA damage signaling or repair proteins may also promote susceptibility to flavonoid-induced translocations. Both heterozygous and homozygous mutants of the DNA damage response gene *Atm* exposed prenatally to flavonoids exhibited detectable numbers of *MLL* rearrangements (Vanhees et al. 2011). Further, inhibition or mutation of multiple DNA repair proteins potentiates cytotoxicity of topoII inhibitors, and MRE11 plays a direct mechanistic role in removal of topoII-DNA complexes in yeast and mammals (Hartsuiker et al. 2009; Lee et al. 2012). Our model cell system can readily be adapted to directly address the impact of such polymorphisms or mutations through genetic engineering of the parental cell lines or use of RNAi prior to exposures. This model system can systematically screen a large panel of compounds, even those not yet suspected to have mutagenic potential, in combination with each other to understand their additive, synergistic, or even quenching effects on the potential to promote these events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structure of GFPe1 and GFPe2 constructs in huMLL and huAF9 breakpoint cluster regions.

(A) For each construct schematic, the numbers of bases are indicated within each portion. GFPe1 includes promoter and upstream region, engineered exon 1, and adenovirus intron sequence. S indicates engineered I-SceI endonuclease site. sd indicates splice donor. GFPe2 includes adenovirus intron sequence, engineered exon 2, and polyA sequence. S indicates engineered linker containing I-SceI endonuclease site. sa indicates splice acceptor. X indicates XbaI site used for insertion Southern blot screening in C. H3 indicates HindIII site

used for insertion Southern blot screening in D. **(B)** Schematics of *MLL* bcr 8.2 kb region and *AF9* bcr 8.94 kb region and location of GFPe1 and GFPe2 insertion sites into constructs used. * indicate the strongest topoII cleavage sites known to be associated with etoposide exposure. Relative positions of BamHI and XbaI sites and relative positions of BglII and XhoI are shown that were used for Southern blotting screening of MAG cell lines. **(C)** Schematic of resultant translocation between the hu*MLL* and hu*AF9* bcrs to result in GFPe1 and GFP e2 on the same DNA duplex to allow for GFP expression. Since compounds may promote DSBs at different sites and resection of DNA at DSBs will vary, resultant length of *MLL* and *AF9* sequences between the GFP exons up to the breakpoint junctions are expected to be variable. Arrows 1F and 2R indicate PCR primers specific for amplification of GFPe1 (196 bp); arrows 3F and 4R indicate PCR primers specific for amplification of GFPe2 (661 bp); arrows 1F and 4R indicate PCR primers to confirm translocations, with expected variable distances at the molecular level dependent on *MLL* and *AF9* breakpoints and end-resection (see Figure 6B,C). **(D)** Southern blotting of GFPe1, GFPe2, MLLGFPe1, and AF9GFPe2 insertions in MAG cell lines. BamHI-XbaI generates a single fragment from the 3' end of GFPe1 to the 5' end of *MLL* bcr construct. BglII generates a single fragment from the 3' region of GFPe2 to the intron 7 region of *AF9* bcr construct. XbaI digest alone generates a single fragment from the 3' end of GFPe1 to the endogenous insertion locus. XhoI digest alone generates a single fragment from the 5' end of GFPe2 to the endogenous insertion locus.

Figure 2. Identification of GFP+ colonies resulting from illegitimate NHEJ DNA repair and a chromosomal translocation following induced DSBs, or exposure to etoposide or bioflavonoids (A-J) Stem cell GFP+ colonies were scored by inverted fluorescent microscopy. Representative phase contrast (left panels) and fluorescent (right panels) microscopy images of GFP+ colonies are shown side by side. (A,B) negative control. (C,D) I-SceI endonuclease expression to induce DSBs within the two GFP constructs in the *MLL* and *AF9* bcrs. (E,F) + etoposide. (G,H) + genistein. (I,J) + quercetin.

Figure 3. γ H2AX foci as marker of DNA damage observed 1 hr post-exposure to tested compounds at approximate calculated LD50 dose. (A) DMSO vehicle only. (B) etoposide 12.5 μ M. (C-F) flavonols—(C) quercetin 75 μ M, (D) kaempferol 100 μ M, (E) myricetin 50 μ M, (F) fisetin 25 μ M. (G-I) isoflavones—(G) genistein 75 μ M, (H) daidzein 200 μ M, (I) biochaninA 200 μ M. (J-K) flavones—(J) luteolin 200 μ M, (K) flavone 200 μ M. (L) flavanone naringenin 200 μ M.

Figure 4. Comparison of GFP+ colony numbers in stem cells resulting from illegitimate NHEJ DNA repair and a chromosomal translocation following exposure to bioflavonoids.

** GFP+ translocations stimulated by genistein, quercetin, kaempferol and fisetin were statistically significantly different from untreated cells. * GFP+ translocations stimulated by individual bioflavonoids (genistein, quercetin, or kaempferol) was statistically significantly different from other compounds as indicated. ^ GFP+ colony numbers following treatment with myricetin, daidzein or luteolin are at doses above LD50 at which GFP+ colonies were observed.

Figure 5. Cellular characterization of GFP+ HSC colonies resulting from illegitimate NHEJ DNA repair and a chromosomal translocation following exposure to etoposide or bioflavonoids. (A-H) GFP+ HSC colonies were scored by inverted fluorescent microscopy beginning 96 hrs post-exposure. Representative phase contrast (left panels) and fluorescent (right panels) microscopy images of GFP+ colonies are shown side by side. (A,B) negative control. (C,D) + etoposide. (E,F) + genistein. (G,H) + quercetin. The lack of background fluorescence in the assay is demonstrated in negative control (A) and one of the two colonies in the field following etoposide exposure (D).

Figure 6. Molecular characterization of *MLL-AF9* bcr translocations.

(A) Representative SKY image of MAG DSB-repaired translocation clone. (B) Schematic and PCR results of resultant translocations between the hu*MLL* and hu*AF9* bcrs (see Figure 1). Arrows 1F and 2R indicate PCR primers specific for amplification of GFPe1 (196 bp); arrows 3F and 4R indicate PCR primers specific for amplification of GFPe2 (661 bp); arrows 1F and 4R indicate PCR primers specific to confirm translocations, with expected variable distances at the molecular level dependent on *MLL* and *AF9* breakpoints and end-resection.

Figure 7. Comparison of frequencies illegitimate NHEJ DNA repair and chromosomal translocations by stage of hematopoietic differentiation.

White bars indicate frequencies of translocations induced in stem cells (Table 2). Grey bars indicate frequencies of translocations induced in HSC (Supplemental Table 1). Black bars indicate frequencies of translocations induced in myeloid progenitors. etop.-etoposide; querc.-quercetin; gen.-genistein; fisetin-fisetin; kaemp.-kaempferol; dip.-dipyrene.

Table 1.

Induction of interchromosomal recombination and translocations by I-SceI-induced DSBs between *MLL* and *AF9* bcrs in three independently derived ES cell lines.

Experiment/Cell Line	DNA	# of GFP+ colonies	Frequency*
MAG1	expt. 1	no DNA	0
		I-SceI	18
	expt. 2	no DNA	0
		I-SceI	22
	expt. 3	no DNA	0
		I-SceI	23
		Avg. no DNA	$< 7.2 \times 10^{-8}$
		Avg. I-SceI	$2.6 \times 10^{-6} \pm 0.23 \times 10^{-6}$
MAG2	expt. 1	no DNA	0
		I-SceI	11
	expt. 2	no DNA	0
		I-SceI	9
	expt. 3	no DNA	0
		I-SceI	10
		Avg. no DNA	$< 8.4 \times 10^{-8}$
		Avg. I-SceI	$1.3 \times 10^{-6} \pm 0.15 \times 10^{-6}$
MAG3	expt. 1	no DNA	0
		I-SceI	1
	expt. 2	no DNA	0
		I-SceI	6
	expt. 3	no DNA	0
		I-SceI	5
		Avg. no DNA	$< 8.4 \times 10^{-8}$
		Avg. I-SceI	$0.63 \times 10^{-6} \pm 0.40 \times 10^{-6}$

Three independently derived MAG cell lines were transfected by electroporation with the I-SceI endonuclease expression vector pCBASce.

2.0×10^7 cells were electroporated in each experiment. As a positive control, cells were electroporated with the GFP+ wild-type plasmid pCBAGFP (data not shown).

* The interchromosomal NHEJ repair and translocation frequency were calculated as the number of GFP+ colonies per number of cells surviving electroporation (as determined 24 hrs post-electroporation).

Table 2.

Frequency of interchromosomal NHEJ repair and translocations between hu*MLL* and hu*AF9* bcrs in MAG1 embryonic stem cells by potential inhibitors of topoisomerase II and other compounds.

Compound	Dose [#]	Scored GFP+ Events ^{&}	Average frequency
Mock treatment	n/a	0	$< 0.007 \times 10^{-6}$
Etoposide	25μM	64, 42, 35	
	50μM	80, 92, 68, 97	$8.4 \pm 1.3 \times 10^{-6}$
	75μM	180, 186, 161	
	200μM	420, 305, 389	
Genistein	75μM	15,8,14	$1.2 \pm 0.4 \times 10^{-6}$
	100μM	21, 10,31	
Quercetin	75 μM	9,14,11	$1.1 \pm 0.3 \times 10^{-6}$
	100μM	17,26,25	
Kaempferol	75μM	2,2, 1	
	100μM	6, 2, 3, 4	$0.37 \pm 0.2 \times 10^{-6}$
	200μM	7,10,9	
Fisetin	25 μM	0,0,0	
	50 μM	0,1,1	
	75 μM	4,1,2	$0.23 \pm 0.2 \times 10^{-6}$
	100 μM	3,5,4	
Flavone	75 μM	0,0,0	
	100 μM	2,2,0	$0.13 \pm 0.1 \times 10^{-6}$
	125 μM	2,1,0	
Myricetin	25μM	0, 0, 0, 0	
	50μM	0,0,0	
	75 μM	1,1,2	$0.13 \pm 0.06 \times 10^{-6}$
Daidzein	100μM	0,0,0	
	175μM	0,0,0	
	200μM	1,2,0	$0.10 \pm 0.1 \times 10^{-6}$
Luteolin	75 μM	0,0,0	
	100 μM	0,0,0	
	175 μM	0,0,0	
	200 μM	1,0,0	$0.03 \pm 0.06 \times 10^{-6}$
Dipyrene	75μM	0,0,0	
	100μM	0,1,0	$0.03 \pm 0.06 \times 10^{-6}$
	125μM	1,0,0	

MAG1 embryonic stem cells were exposed 1 hr 37°C with the indicated potential topo II inhibitors

[#] Doses and colony numbers in bold indicate the doses at the determined LD50 by MTT assays (Supplemental Figure 1). Surviving fractions of cells were calculated.

[&] GFP+ colonies were scored 96 hr later. In each row, the total number of colonies across all plates for each experiment is noted, and the total number from each experiment is separated by commas.