Dissociation between Bulk Damage to DNA and the Antiproliferative Activity of Teniposide (VM-26) in the MCF-7 Breast Tumor Cell Line: Evidence for Induction of Gene-specific Damage and Alterations in Gene Expression

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ABSTRACT

In the MCF-7 breast tumor cell line, induction of bulk damage to DNA (measured either as total strand breaks or as double-strand breaks) fails to correspond with the antiproliferative activity of the demethylepipodophyllotoxin derivative, VM-26. In contrast, VM-26 produces an early (within 2–3 h) concentration-dependent reduction in c-myc expression (and of DNA synthesis) which parallels inhibition of cell growth, suggesting the possibility of effects of VM-26 at the level of genomic regions which regulate DNA replicative function. Although VM-26 also produces a reduction in c-myc expression in K562 human leukemic cells, these alterations fail to correspond with the concentration-dependent effects on cell growth in this cell line. Utilizing the newly developed alkaline unwinding/Southern blotting assay in the MCF-7 breast tumor cell line, it was determined that VM-26 induces damage within regions surrounding the c-myc gene and the β-globin gene which exceeds that induced in both α-satellite DNA and in L1 repeat sequences; damage within c-myc and β-globin also exceeds that observed throughout the genome as a whole. These findings indicate that certain genomic regions incur preferential damage in MCF-7 cells exposed to VM-26. It appears possible that damage within such genomic regions could lead to alterations in expression of select genes associated with regulation of cellular proliferation, resulting in reduced DNA synthesis, compromised cell growth, and, ultimately, cell death.

INTRODUCTION

The demethylepipodophyllotoxin derivative, VM-26, is one of a number of drugs which have been shown to interfere with the relaxation activity of DNA topoisomerase II via stabilization of the DNA-topoisomerase II complex (1–3), resulting in the production of DNA strand breaks (4, 5). Stabilization of the “cleavable complex” and the concomitant induction of DNA strand breaks have been shown to correspond closely with drug cytotoxicity and/or antiproliferative activity (6–8), suggesting that these lesions in bulk DNA mediate the antitumor effects of topoisomerase II inhibitors. In contrast, in some tumor cell lines, various topoisomerase II inhibitors have been shown to express cytotoxicity which fails to correspond with damage to bulk DNA (9–15), suggesting that bulk DNA damage is an incomplete explanation for drug action.

The present studies demonstrate that in the MCF-7 human breast tumor cell line, the induction of bulk damage to DNA by the topoisomerase II inhibitor VM-26 fails to correspond with inhibition of cell growth. In contrast, there appears to be a close correlation between growth inhibition and the inhibition of DNA synthesis, suggesting that perturbation of DNA synthesis may be an early biochemical effect of this antineoplastic drug. Consistent with these observations, VM-26 also produces an early, concentration-dependent reduction in the expression of the c-myc oncogene which corresponds with the ultimate inhibition of growth in MCF-7 cells (but not in human K562 leukemic cells).

In order to address the possibility that damage within select genomic regions may contribute to the antiproliferative activity of topoisomerase II inhibitors (16–18), an assay was developed (alkaline unwinding/Southern blotting) with the capacity to assess damage within large scale regions surrounding genes of interest (19). Using this assay, it was determined that VM-26 produces differential damage in the regions surrounding select genes in the MCF-7 breast tumor cell line. Therefore, the induction of such damage by topoisomerase II inhibitors may act as a signal leading to alterations in the expression of genes, such as c-myc, which are involved in the regulation of DNA synthesis (20, 21) and cell proliferation (22).

MATERIALS AND METHODS

Materials

DMEM (56–439) was obtained from Hazelton Research Products, Denver, PA; L-glutamine, penicillin/streptomycin (10,000 units penicillin/ml and 10 mg streptomycin/ml), and fetal bovine serum were obtained from Whittaker BioProducts, Walkersville, MD; defined bovine calf serum was obtained from Hyclone Laboratories, Logan, UT. Trypsins-EDTA (10 × 10E6 trypsins-5.3 mM EDTA) was obtained from GibCO Laboratories, Grand Island, NY. VM-26 (teniposide) was generously provided by the Bristol-Myers Co., Wallingford, CT. VM-26 was dissolved in dimethylsulfoxide (Aldrich Biochemicals, Milwaukee, WI) and maintained as a frozen stock solution for a maximum period of 2–3 weeks. Drug was diluted in incubation medium on the day of the experiment.

The radiolabeled compounds [3H]thymidine (75 Ci/μmol), [3H]uridine (27.3 Ci/μmol), and [α-32P]dCTP (3000 Ci/μmol) were obtained from ICN Radiochemicals, Irvine, CA, and DuPont NEN Research Products, Boston, MA, respectively. Nuclease S1 was obtained from Pharmacia LKB Biotechnology, Inc., Piscataway, NJ; DMSO, proteinase K, MTT, thymidine, uridine, and trichloroacetic acid and RNase A were obtained from Sigma Chemical Co., St. Louis, MO. Tetratpropyl ammonium hydroxide was obtained from Kodak Chemicals, Rochester, NY). Agarose was obtained from GibCO BRL (Gaithersburg, MD). All other chemicals were reagent or molecular grade, as appropriate.

The c-myc probe, an EcoRI/ClaI fragment of pSC41 3RC containing the third exon of the human c-myc gene, was kindly provided by Dr. Eric Westin of the Medical College of Virginia. Genomic or complementary DNA probes for glyceraldehyde-3-phosphate dehydrogenase, and β-globin (pBR.β a) were obtained from American Type Culture Collection, Rockville, MD. The 2.7kilobase human chromosome 17 α-satellite probe from plasmid p17H8 was obtained from Oncor, Gaithersburg, MD, and cloned into pUC19. The L1,
1.9-kilobase HindIII interspersed repeat was obtained from Dr. Laura Manueldidis, Yale University School of Medicine, New Haven, CT. The restriction endonucleases EcoRI and HindIII were obtained from New England Biolabs, Beverly, MA, and the nick-translation kit was obtained from GIBCO/BRL.

**Cell Lines.** The MCF-7 breast tumor cell line was kindly provided by the laboratory of Dr. Kenneth Cowan at the National Cancer Institute, Bethesda, MD, and were maintained as monolayers in DMEM supplemented with glutamine (0.292 mg/ml), penicillin/streptomycin (0.5 ml/100 ml medium), 5% fetal bovine serum, and 5% defined bovine serum. K562 human leukemic cells were obtained from the American Type Culture Collection, and were maintained as suspension cultures in DMEM supplemented with 0.292 mg/ml glutamine and 10% fetal bovine serum. All cells were cultured at 37°C in an atmosphere of 5% CO2.

**Growth Inhibition Assays for MCF-7 Cells**

**MTT Dye Assay.** The capacity of VM-26 to interfere with growth of the MCF-7 breast tumor cell line was determined using the MTT tetrazolium dye assay, as described in detail previously (10). Briefly, cells subcultured at a density of 1 x 10^4 cells/ml in 96-well microplates (Costar, Cambridge, MA) were incubated with varying concentrations of VM-26 or DMSO for 2 h. Drug was aspirated, and cells were washed with incubation medium and permitted to grow for an additional 72 h prior to determination of viable cell number.

**Cell Counting.** MCF-7 cells subcultured at a density of 1.5 x 10^5 cells/ml in 24-well plates (Costar) were incubated with varying concentrations of VM-26 for 2 h at 37°C. Drug was aspirated, and cells were washed with incubation medium and permitted to grow for an additional 72 h prior to determination of cell number. The cells were released from flasks with trypsin (0.05 mg/ml)/EDTA (0.02 mg/ml) for 5 min at 37°C, collected in ice-cold PBS, and centrifuged at 4°C. Cell pellets were then resuspended in 400 μl of ice-cold phosphate buffered saline. Thirty-μl aliquots were added to 15 ml of Hematall Isotonic Dileu (Fisher Diagnostics, Raleigh, NC) and cell number was determined by Coulter Counter (Coulter Electronics, Hialeah, FL).

**Growth Inhibition Assay for K562 Cells**

Cells were treated with various concentrations of VM-26 (0.1-10 μM) or with 0.1% DMSO for 3 h. After drug treatment, cells were washed 3 times with saline, resuspended in drug-free growth medium, and 1 x 10^5 cells were seeded in duplicate in 24-well plates (Costar). Growth was assessed 48 h after drug treatment by counting cells with a Coulter counter (Haleah, FL). The cell counting assay was substantiated using the MTT tetrazolium dye assay in 96-well U-bottom plates.

**Alkaline Unwinding (Bulk DNA Damage)**

Bulk (single-strand) damage to DNA was determined using the alkaline unwinding procedure of Kanter and Schwartz (23) as described in detail previously (15). DNA cleavage is monitored based on the differential binding of a Hoechst dye to single-stranded DNA and dsDNA. Briefly, cells in 75-cm² T flasks (Costar) were incubated with VM-26 or the vehicle control (DMSO) for 2 h, washed with PBS (pH 7.4), released from flasks by incubation with 0.05 mg/ml trypsin in 0.02 mg/ml EDTA for 5 min at 37°C, and collected in ice-cold PBS. Each condition (6 x 10^5 cells/concentration) was subdivided into three different groups: (a) dsDNA control, with no alkali-induced DNA unwinding; (b) cells treated for a 10-min alkaline unwinding period; and (c) total single-stranded DNA, where cells were sonicated before alkaline unwinding.

F-values, defined as:

\[ F = \frac{\text{alkali-treated DNA} - \text{ssDNA}}{\text{dsDNA control} - \text{ssDNA}} \]

(Ref. 23), were determined in quadruplicate, and then converted to rad equivalent based on standardization of DNA damage using a Cesium-137 irradiator to produce graded amounts of strand breakage.

**Neutral Elution Assay for Double-Strand Breaks**

Double-strand breaks in DNA were analyzed using the neutral elution assay procedure (24) as described in detail previously (25). Cells were labeled for 24 h with 1.1 μCi/ml [3H]thymidine followed by washing and incubation for an additional 24 h in thymidine-free medium. Labeled cells were incubated with drug and cells were processed as described for the alkaline unwinding assay. Approximately 5 x 10^6 cells were lysed on polycarbonate filters (Nucleopore Filtration Products, Cambridge, MA) with 2 ml of 2% SDS containing 0.5 mg/ml proteinase K (to eliminate DNA-protein cross-linking). DNA was eluted with tetrapropylammonium hydroxide at pH 9.6-10 at a flow rate of 0.8 ml/h, and fractions collected over a 17-h period were analyzed by scintillation counting. Damage was expressed as rad equivalence as determined using MCF-7 cells exposed to various doses of ionizing radiation from a cesium-137 source.

**DNA and RNA Biosynthesis**

The effect of VM-26 on the rate of DNA or RNA synthesis was determined by monitoring the rate of incorporation of [3H]thymidine or [3H]uridine into acid-precipitable material over a time course of 40 min (DNA) or 120 min (RNA); as previously described (10). MCF-7 cells in 24-well plates (Costar) were exposed to VM-26 for 2 h and washed with Hanks’ buffered salt solution (pH 7.4; Whittaker Biochemicals) at room temperature prior to analysis of [3H]thymidine or [3H]uridine incorporation into TCA-precipitable cpm. The percentage inhibition of DNA or RNA biosynthesis was calculated from the relative rates of incorporation of [3H]-labeled nucleic acid precursors in drug-treated versus untreated control cells.

**Gene Expression**

After incubation with VM-26 for appropriate times and at stated concentrations, cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (pH 7.4), and cells were lysed in 4 ml guanidine isothiocyanate and 0.5 M sodium lauryl sarcosine. RNA from MCF-7 cells was separated by ultracentrifugation through a 5.7 M cesium chloride cushion at 41,000 x g for 20 h at 20°C (15). RNA was extracted from 4 ml guanidine isothiocyanate lysates of K562 cells as described by Chomczynski and Sacchi (26) and precipitation with 70% ethanol. RNA pellets were then washed in 95% ethanol and 70% ethanol and resuspended in Milli Q water.

RNA (10 μg) was denatured in 0.02 M morpholinopropane sulfonic acid (pH 7.0), 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, and 50% formamide. The samples were separated on a 6.6% formaldehyde-agarose gel (27). Equal loading of RNA in each lane was confirmed by ethidium bromide staining. Blotting was carried out using Nytran transfer membranes (Schleicher and Schuell, Keene, NH).

Probes were radiolabeled using a nick-translation kit from GIBCO BRL and hybridized to blots in the presence of 50 mM sodium phosphate (pH 6.5), 5 x Denhardt’s solution (0.1% bovine serum albumin-0.1% Ficol-0.1% polyvinyl pyrolidine), 5 x SSC (0.75 M NaCl-0.75 M sodium citrate), 0.1% SDS, yeast RNA (250 μg/ml), 50% formamide, and 10% dextran sulfate (27). Hybridizations were for 16-20 h at 42°C. Filters were washed 3 times at 42°C for 5 min in 2 x SSC and 0.2% SDS followed by 1 wash in 2 x SSC and 0.2% SDS at 60°C and 1 wash in 0.5 x SSC and 0.2% SDS for 40 min before autoradiography.

**Alkaline Unwinding/Southern Blotting Assay for Damage within Specific Genomic Regions**

The combined alkaline unwinding/Southern blotting assay was performed as previously described (19). Briefly, MCF-7 cells in log-phase growth were labeled with [3H]thymidine (0.1 μ Ci/ml) for 24 h, followed by a chase with unlabeled medium for approximately 16 h. Cells (10-25 x 10^5 cells/concentration) were removed from the tissue culture dishes with trypsin-EDTA, resuspended in approximately 10 ml of medium, and allowed to equilibrate for 1 h at 37°C. Cells were then treated with various concentrations of VM-26 or the DMSO vehicle control for 1 h. Following treatment, cells were centrifuged at 500 x g at 0°C, resuspended in PBS, and placed on ice. Alkali-induced unwinding of the DNA was initiated with the addition of 2 ml of unwinding solution (0.044 M NaOH-1.125 M NaCl, 22°C). Unwinding was terminated with the addition of 2 ml acetic acid (0.054 M). For control groups with no unwinding, the unwinding solution and the neutralization solution were added simultaneously. Unwound single-stranded DNA was then digested with nuclease S1 (275 units), and proteins were then degraded with 0.025 ml protease K (20 mg/ml) for 4 h at 42°C.
The overall %dsDNA remaining after the unwinding was calculated by determining the relative amounts of DNA in TCA-soluble and TCA-precipitable fractions. A portion of each sample (0.1 ml) plus 0.1 ml salmon sperm DNA (100 µg/ml) were precipitated with 10% TCA for 5 min on ice. After centrifugation (2200 x g for 5 min), the supernatant was removed and added to 6 ml Safety Solve scintillation fluid (RPI Corp., Mount Prospect, IL). The precipitate was dissolved in 0.1 N NaOH for 10 min and added to scintillation fluid. The amount of labeled DNA in each fraction was determined using a Beckman LS 1801 scintillation counter. The %dsDNA was determined as the proportion of total disintegrations/min that were acid precipitable.

Following phenol/chloroform extraction of the DNA and resuspension in 10 mM Tris-1 mM EDTA, acid-precipitable disintegrations/min were again determined. These data, along with the preextraction %dsDNA, were used to calculate the amount of DNA for subsequent Southern analysis. This procedure corrects for any differences in the concentration of the samples that may have occurred during the course of the purification and ensures that the amount of acid-precipitable DNA used for electrophoresis and Southern blotting reflects the decrease in %dsDNA due to drug treatment and alkaline unwinding. Control levels of acid-precipitable DNA were determined by averaging values from two sham unwinding controls, one with and one without drug treatment.

Southern blot analysis was performed essentially as described by Sambrook et al. (28). Briefly, DNA was digested with EcoRI or HindIII overnight at 37°C, and electrophoresis was carried out in a 0.8% agarose gel. DNA was transferred to Nytran (Schleicher and Schuell) membranes and hybridized to the aforementioned probes. Blots were washed to remove background radioactivity and either directly quantitated using a Betagen Betascope or exposed to x-ray film in the absence of an intensifying screen (to increase the accuracy of the quantitation). These autoradiographic signals were then quantitated using a Shimadzu scanning densitometer (CS-9000).

RESULTS

Induction of DNA Strand Breaks/Inhibition of Growth. Exposure of MCF-7 cells to 0.1-10 µM VM-26 for 2 h inhibited growth (assessed after 72 h by the MTT reduction assay and verified by cell counting) in a dose-dependent manner (Fig. 1A). In order to examine the relationship between the induction of bulk damage to DNA and the antiproliferative activity of VM-26, single-strand breaks were determined using the alkaline unwinding assay (23), while double-strand breaks (which may be a more reliable indicator of damage to DNA induced by topoisomerase II inhibitors; Ref. 29) were monitored using neutral elution (24). Fig. 1A shows that bulk DNA damage, monitored using either assay, failed to show a consistent relationship with the concentration-dependent inhibition of growth induced by VM-26. This is shown more clearly in Fig. 1B where single-strand breaks are plotted as a function of growth inhibition. For instance, between 0.1 and 1.0 µM VM-26, growth inhibition of MCF-7 cells increased 2.5-fold (from 30 to 75%), whereas rad equivalents of single-strand DNA damage increased 5-fold (from 178 to 912 rad equivalents); in contrast, between 1.0 and 10 µM VM-26, a 6-fold increase in single-strand breaks (from 912 to 5495 rad equivalents), was accompanied by only a 10% increase in growth inhibition of these cells.

There was an excellent correspondence between the alkaline unwinding and neutral elution assays in the induction of DNA damage. Consequently, when induction of double-stranded DNA damage was plotted as a function of growth inhibition (Fig. 1B), a similar lack of correspondence was observed as for single-strand DNA damage.

The lack of correspondence between growth inhibition and either single-strand or double-strand breaks in DNA does not rule out a role for bulk DNA damage in the antiproliferative activity of VM-26 in the MCF-7 cell, since it is possible that the limited sensitivity of the DNA damage assays may also account for the apparent lack of correlation. Nevertheless, these observations do suggest that lesions other than random damage throughout the genome may contribute to the effects of VM-26 on growth of the MCF-7 breast tumor cell line.

Effects of VM-26 on DNA and RNA Biosynthesis. We next examined the relationship between VM-26 induced growth inhibition and the modulation of other biochemical functions, such as RNA and DNA biosynthesis, in the MCF-7 cell line. Fig. 2A indicates that RNA biosynthesis (measured immediately after a 2-h exposure to VM-26) failed to show a correlation with growth inhibition assessed after 72 h, e.g., minimal (<15%) inhibition of uridine incorporation occurred until cells were exposed to 5 µM VM-26 at which time growth inhibition was >75%. In contrast, Fig. 2A indicates that inhibition of DNA biosynthesis (measured immediately after a 2-h exposure to VM-26) appears to parallel growth inhibition assessed after 72 h. Indeed, inhibition of DNA synthesis plotted as a function of growth inhibition demonstrated a correlation coefficient of 0.95 (Fig. 2B). In this context, demethyllepipodophyllotoxin derivatives have previously been shown to interfere with DNA biosynthetic processes (30). This inhibition of DNA biosynthesis reflects an early biochemical response
course of 3 h, c-myc expression (as measured by densitometric scan of c-myc mRNA levels were examined by Northern blotting analysis. c-myc gene is critical for DNA replication and for growth of the cell line. Consequently, reduced c-myc expression failed to correspond with growth inhibition in this leukemic tumor cell line.

Assessment of Gene-specific DNA Damage by Alkaline Unwinding/Southern Blotting. The VM-26-induced reduction in c-myc expression in the MCF-7 cell line could represent a cellular response to generalized (bulk) DNA damage throughout the genome. It is also possible that DNA damage occurring within or near the c-myc locus compromises c-myc expression. In this context, 4′-(9-acridinylamino)-methanesulfon-m-anisidide, another topoisomerase II poison, has been shown to induce DNA damage within the c-myc locus in cell lines with amplified c-myc (16, 18). It has also been suggested that DNA damage at the level of the chromosomal loop may result in inhibition of transcription of genes positioned within or near the damaged loop (34). Studies were therefore undertaken to determine if inhibition of topoisomerase II by VM-26 resulted in preferential damage to specific large-scale chromatin domains.

The region encompassing the highly expressed c-myc oncogene was compared with inactive chromatin regions, including the β-globin and the α-satellite regions (the transcriptional state of these regions were confirmed by Northern blot analysis; data not shown). The profile of VM-26-induced damage was first examined within the chromatin region encompassing the actively transcribed c-myc oncogene. Fig. 6A represents an autoradiograph of a Southern blot hybridized with a probe for c-myc. Lanes 1 and 2 show DNA from cells treated with DMSO (vehicle control) and 10 μM VM-26, respectively, and sub-

Fig. 2. A, inhibition of growth and inhibition of DNA or RNA synthesis by VM-26 in the MCF-7 breast tumor cell line. Cells were incubated with VM-26 for 2 h prior to determination of acid-precipitable incorporation of nucleotide precursors. Values for DNA and RNA biosynthesis represent means ± SE for 3 and 4 independent experiments, respectively. B, relationship between inhibition of DNA synthesis and growth inhibition in MCF-7 cells incubated with VM-26. Data were taken from A.

and could result, in part, from “cleavable complex” blockage of replication fork movement (31) or an early delay in cell cycle progression (32).

Effects of VM-26 on Gene Expression. It is possible that the antiproliferative effect of VM-26 is mediated by the modulation of select genes which regulate DNA replication, such as c-myc. The c-myc gene is critical for DNA replication and for growth of the MCF-7 breast tumor cell line (20–22). Consequently, steady-state c-myc mRNA levels were examined by Northern blotting analysis. Fig. 3 indicates that 10 μM VM-26, which inhibits cell growth by 85%, produced a time-dependent decrease in c-myc expression. Over the course of 3 h, c-myc expression (as measured by densitometric scanning) declined by approximately 75–80% in cells exposed to 10 μM VM-26 while the DMSO vehicle control produced a variable decline that never exceeded 30%. Expression of glyceraldehyde phosphate dehydrogenase (33) remained essentially constant over the 3-h period.

The effects of various concentrations of VM-26, as well as vehicle (DMSO) controls, on expression of c-myc were determined after 3 h of drug treatment. Autoradiographs (Fig. 4A) indicated that c-myc message decreased in a dose-dependent manner (Lanes 3, 5, 7, 9, and 11), while the vehicle control produced minimal perturbations of c-myc expression (Lanes 4, 6, 8, 10, and 12). Expression of glyceraldehyde phosphate dehydrogenase was essentially unaltered by VM-26 (Fig. 4A, bottom). The concentration-dependent decline in c-myc expression induced by VM-26 (like that of DNA synthesis) closely paralleled growth inhibition (Fig. 4B). The correlation coefficient for inhibition of c-myc expression plotted as a function of growth inhibition (Fig. 4C) was approximately 0.9.

To determine if a similar association between growth inhibition and reduced c-myc was evident in other tumor cells, c-myc expression was also quantitated in K562 human leukemic cells exposed to VM-26. Fig. 5 indicates that, while c-myc expression was reduced by VM-26, these reductions (<20%) were minor at concentrations of VM-26 where growth (measured 48 h after exposure to drug) was inhibited by between 70 and 90%. The correlation coefficient of c-myc expression as a function of growth inhibition in K562 cells was only 0.4 (data not shown). Furthermore, a similar dissociation was observed when alterations in c-myc expression were compared with drug-induced inhibition of growth as measured by the MTT dye assay (data not shown). Consequently, reduced c-myc expression failed to correspond with growth inhibition in this leukemic tumor cell line.

To examine this further, growth inhibition (by 85% in both cell lines) was associated with a 70% reduction in c-myc expression in MCF-7 cells and a 60% reduction in K562 cells (data not shown). Consequently, reduced c-myc expression failed to correspond with growth inhibition in this leukemic tumor cell line.
jected to sham alkaline unwinding (essentially equivalent to standard Southern blotting). Densitometric analysis indicated that the hybridization intensity of Lane 2 was approximately 85% of Lane 1; however, overexposure of the autoradiograms did not reveal any additional smaller molecular weight bands. Furthermore, analysis of other genomic regions, including α-satellite and β-globin, indicated a similar difference in the hybridization intensities of the two lanes (data not shown). Thus, it appears that under the conditions of standard genomic southern blotting, VM-26 does not induce preferential damage within the c-myc locus when compared to either another single-copy sequence (β-globin) or an amplified region (α-satellite).

Fig. 6A, Lanes 3–5, contains DNA from cells treated with DMSO and with 1 or 10 μM VM-26, respectively, subjected to 10 min of alkaline-induced unwinding, thus permitting the examination of larger-scale chromatin regions (about 500 kilobases; Ref. 19) surrounding the c-myc locus. The marked decrease in the hybridization intensity as a function of VM-26 concentration indicates drug-induced DNA damage in this actively transcribed chromatin region. With an unwinding time of 30 min (Lanes 6–8) and with conditions similar to Lanes 3–5, the relative hybridization signals again indicate a dose-dependent increase in DNA damage; the signal intensities are reduced as compared with 10 min of unwinding, indicative of the time dependency of the alkaline-induced DNA unwinding process. Similar effects are also observed within the β-globin region (Fig. 6B).

DNA damage induced by VM-26 in c-myc, β-globin, and α-satellite DNA was quantitated from autoradiograms by either densitometric analysis or with a Betascope and bulk DNA damage as quantitated by TCA precipitation of DNA after alkaline unwinding and S1 nuclease digestion (see “Materials and Methods”). DNA damage, graphed in Fig. 6C as a function of the VM-26 concentration, is expressed as either %dsDNA (for bulk DNA) or as the Fds, the proportion of the hybridizing sequence remaining double-stranded after 10 min of alkaline unwinding (determined by Southern blotting). The data were normalized to the 10-min control unwinding (sham drug treatment) to correct for any differences that may exist in unwinding between different genomic regions. These data indicate enhanced damage to regions surrounding c-myc and β-globin, as compared to damage to α-satellite DNA and to bulk DNA (Fig. 6C). At 1 μM VM-26, the Fds of the region encompassing c-myc is about 6-fold lower than the Fds for the α-satellite region. The region encompassing β-globin, for which the Fds is only about 50% greater than that of the c-myc region, is also more sensitive to VM-26-induced damage than either the α-satellite region or the bulk of the genome. Similar effects are also observed at a higher dose of VM-26 (10 μM). We also examined VM-26-induced damage in the L1 repeat region using the AUSB assay. The 1.9-kilobase HindIII L1 repeat is thought to be associated with tissue-specific genes (most of which will be unexpressed), which are clustered in “G-dark” chromosome bands interspersed throughout the genome (35). VM-26-induced damage in this region was essen-

Fig. 4. A, Northern blot of c-myc and GAPDH expression in MCF-7 cells exposed to various concentrations of VM-26 for 3 h. Lanes 4, 6, 8, 10, and 12, denoted by D, represent volumes of the vehicle. DMSO, equivalent to volume used to dissolve corresponding concentrations of VM-26. B, quantitative representation of concentration-dependent alterations in c-myc expression in MCF-7 cells exposed to varied concentrations of VM-26 (pooled data from 3 independent experiments) and corresponding alterations in growth inhibition. C, relationship between reductions in c-myc expression and growth inhibition in cells exposed to varied concentrations of VM-26: data are taken from lì.

Fig. 5. Quantitative representation of concentration-dependent alterations in growth and c-myc expression in K562 cells exposed to varied concentrations of VM-26: data represent averages and range from 2 independent experiments.
and/or loss of viability through alterations in gene expression.

or antiproliferative activity of topoisomerase II inhibitors (9-15).

topoisomerase II complex (39, 40).

a certain degree of sequence specificity for binding of the DNA/

damage within different genomic regions, perhaps as a consequence of

consequences to the cell.

concept that these cleavable complexes and the corresponding DNA

dose of VM-26. For bulk DNA damage, which is

method for the examination of region-specific DNA lesions induced

damage within regions surrounding various genes in

plerases; the data were normalized to the sham drug

traphoresed in a 0.7% agarose gel. followed by

DNA, which was digested with HindIII, was elec-

agrose gel, followed by Southern blot analysis, in which membranes were

hybridized to a nick-translated c-myc probe. Lanes 1 and 2 represent standard Southern blotting (ab-

ence of alkaline unwinding), with DMSO vehicle

control, and 10 μM VM-26, respectively. Lanes 3-5

represent the vehicle control, 1 μM, and 10 μM

VM-26, respectively, with 10 min of alkaline

a membrane was then stripped and rehybrid-

ized to a nick-translated probe specific for β-

C, quantitative representation of damage

within regions surrounding various genes in

Heads-7 breast tumor cells treated with VM-26.

Densiometry was performed on all autoradiographs; the data were normalized to the sham drug

s, the aminoacridines, and ellipticines have been shown to stabilize a "cleavable-complex" between topoisomerase II and DNA (1-3). The

escases outside of the restriction fragment, which would there-

detect DNA damage occurring not only within the locus of the gene

(41) that the major cleavage sites induced by VM-26 occur 5' to

first exon of c-myc. It is also possible that VM-26 induces other

significant levels of DNA damage (16, 18).

topoisomerase II inhibitors, although providing a more precise local-

blotting and permits the use of clinically relevant doses of VM-26.

Ref. 19) indicate that the region encompassing c-myc sustains breaks

in this report (as well our previous report utilizing ionizing radiation;

damage produced by 1 μM VM-26 in the region surrounding c-myc: in

contrast, standard Southern blotting alone is not sufficiently sensitive

to detect damage in the c-myc locus induced by even higher concen-

trations of VM-26, i.e., 10 μM VM-26 (See Fig. 6A, Lanes 1 and 2).

This increase in sensitivity is primarily achieved by the examination of a larger "target," i.e., ~500 kilobases for the AUSB assay. This

target size more closely approaches the density of VM-26-induced DNA strand breaks than, for example, the 13-kilobase HindIII restric-

tion fragment for standard Southern blotting. Based on earlier studies

where the frequency of radiation-induced DNA strand breaks was estimated to be about 0.9 × 10⁻⁴ breaks/rad/nucleotide (24), 1 μM

VM-26, which induces about 700 rad equivalents of DNA damage, will induce 6.3 × 10⁻⁷ breaks/nucleotide, or 1.6 × 10⁶ nucleotides

between strand breaks. This represents the average distance between

breaks and does not exclude the possibility that the actual distance

between any given break is likely to vary. Indeed, the results presented

in this report (as well our previous report utilizing ionizing radiation;

Ref. 19) indicate that the region encompassing c-myc sustains breaks

at a greater frequency than that of the overall genome. The ability of

the AUSB assay to detect region-specific DNA damage at this level

represents a marked increase in sensitivity over standard Southern blotting and permits the use of clinically relevant doses of VM-26.

Previous methods utilized for the analysis of gene-specific damage by

topoisomerase II inhibitors, although providing a more precise localization of the cleavage sites, often required the use of supra-clinical concentrations of drugs and/or an amplified target in order to detect

significant levels of DNA damage (16, 18).

The AUSB assay provides an additional advantage in being able to
detect DNA damage occurring not only within the locus of the gene

but also at potentially critical lesions some distance from the gene

of interest. This may be particularly relevant to the report by Riou et al.

(41) that the major cleavage sites induced by VM-26 occur 5' to

the first exon of c-myc. It is also possible that VM-26 induces other

cleavage sites outside of the restriction fragment, which would there-

fore be undetectable using standard Southern blotting procedures.
These upstream and downstream cleavage sites, which have been shown to occur at distances up to many kilobases from a particular gene (42), may very well contribute to the mechanism of antineoplastic activity of VM-26. Studies using ionizing radiation (34) have indicated that DNA damage occurring at this level, by inducing an alteration in chromatin organization, results in inhibition of transcription of genes within the vicinity of the strand breaks, presumably due to the reduction in superhelical tension of the domain. In the present studies, it is clear that damage produced by VM-26 within regions surrounding c-myc and β-globin exceeds that within α satellite DNA, the L1 repeat sequence, and in bulk DNA. Consequently, these studies support the hypothesis that measurements of damage to bulk DNA alone may markedly underestimate damage to "critical" genomic regions. These results further suggest that both transcriptionally active (e.g., c-myc) and potentially active, or inducible, chromatin regions (e.g., β-globin) may have an increased susceptibility to DNA damaging agents. The fact that the transcriptional state of a gene does not appear to be the only factor determining susceptibility of surrounding regions to damage by VM-26 is consistent with reports by others showing topoisomerase II cleavage sites in both transcriptionally active and inactive genes (43).

Although certain transcriptionally active and inactive regions may be more susceptible to VM-26 induced damage than bulk DNA, transcriptional activity nevertheless is implicated in the susceptibility of a chromatin domain to VM-26 induced damage. This idea is supported by the observation that the inactive β-globin region was slightly less susceptible than the region encompassing the actively transcribed c-myc region. In this regard, the developmentally linked changes within the β-globin region have been examined with respect to subsequent alterations in topoisomerase II-mediated cleavage (44). In this model system inactivation of the β-globin region was not accompanied by changes in the DNase I hypersensitivity, while alterations in topoisomerase II mediated cleavage patterns were observed. Reitman and Felsenfeld (42) have also indicated a possible developmental regulation of topoisomerase II cleavage sites in the chicken β-globin locus. The results presented here may support these phenomena in that the inactive β-globin region sustains less topoisomerase II-mediated cleavage than the actively transcribed c-myc region. The precise relationship between transcriptional activity and the susceptibility of a genomic region to topoisomerase II-mediated cleavage remains to be resolved. Previous reports (41) have indicated a greater differential susceptibility to damage between the c-myc and β-globin loci, although the globin-like gene being examined was a pseudogene.

The capacity of VM-26 to produce a concentration-dependent reduction in expression of c-myc and of DNA synthesis which closely parallels and precedes growth inhibition in the MCF-7 cell line suggests that changes in c-myc expression represent an early response to topoisomerase II-mediated damage. It appears possible that, in response to either bulk DNA damage or breaks surrounding specific genes such as c-myc, there is a reduction in c-myc expression which leads to a corresponding inhibition of DNA synthesis and compromised cell growth. In this context, it has recently been reported that tumor necrosis factor produces a reduction in c-myc expression which preceded growth inhibition in human melanoma cells (45). In addition, there have been a number of reports demonstrating that various antineoplastic drugs down-modulate c-myc expression (46-51).

It is somewhat difficult to understand why the VM-26-induced alterations in DNA synthesis correlate so closely with drug-induced inhibition of DNA synthesis, since cells in S phase, actively synthesizing DNA, account for only approximately 30% of the cell population. Consequently, it is likely that VM-26 induces additional bio-

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4 Unpublished observations.

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chemical perturbations in the MCF-7 breast tumor cell which ultimately result in inhibition of cell growth. For instance, it is possible that another subpopulation of cells is blocked at mitosis, since VM-26 has been shown to inhibit G2-M transition for up to 14 h after drug treatment in (52). Another possibility is that the VM-26-induced reduction in c-myc message occurs throughout the entire cell population, regardless of their position within the cell cycle. Inhibition in DNA biosynthesis might then occur in other cells as they attempt to traverse S phase, as a consequence of the reduction in c-myc levels. Thus, although a number of VM-26-induced perturbations may ultimately lead to compromised cell growth, VM-26-induced alterations in DNA synthesis may represent an early but predictive event for VM-26-induced cytotoxicity.

Studies in the K562 human leukemic cell line demonstrate that unlike MCF-7 cells, VM-26-induced alterations in c-myc expression do not appear to correlate with growth inhibition. This observation may be related to the fact that c-myc expression in K562 cells is not closely associated with proliferative activity in this cell line (53) and suggests the possibility of differences in the mechanism of drug action, at the level of gene expression in solid versus hematopoietic derived tumors.

We would like to propose that damage within regions surrounding certain genes may provide a signal for the cell to cease growth; this signal may be mediated via reduction in the expression of specific genes, such as c-myc in MCF-7 cells. One of the goals of this laboratory will be to understand the relationships between drug-induced damage within specific genomic regions, in particular the mvc family of genes, alterations in gene expression, growth inhibition, and, ultimately, cytotoxicity.

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Dissociation between Bulk Damage to DNA and the Antiproliferative Activity of Teniposide (VM-26) in the MCF-7 Breast Tumor Cell Line: Evidence for Induction of Gene-specific Damage and Alterations in Gene Expression

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