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In situ stimulation of topoisomerase II-induced cleavage sites in the c-*myc* protooncogene by antitumor agent pMC540 is associated with gene expression

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The antitumor activity of pMC540 has been shown to be mediated via its interaction with topoisomerase (Topo) II eventually leading cells into apoptosis. This agent was also found to down regulate the expression of the c-*myc* oncogene in L1210 leukemia cells. To investigate the possibility that damage within select genomic regions may contribute to the antiproliferative activity of pMC540, differential damage in regions surrounding the c-*myc* locus as well as other select genes was determined. Southern blot hybridization experiments show that pMC540 treatment induces *in situ* DNA cleavage products in the 5′ end of the c-*myc* oncogene of L1210 leukemia cells. In cells pre-treated with 50 μM ethidium bromide, an inhibitor of the Topo II-dependent DNA cleavage, a subsequent treatment with pMC540 failed to induce DNA cleavage, suggesting that the cleavage activity of pMC540 was Topo II dependent. pMC540-induced cleavage does not appear to correlate with the over-expression of the c-*myc* oncogene in these cells as another over-expressed gene c-*myb* was not affected. Thus, it is proposed that the c-*myc* gene may be a preferred target for pMC540 may mediated antiproliferative activity.

Key words: c-*myc*, gene cleavage, leukemia, preactivated merocyanine 540, topoisomerase II.

Introduction

In recent years, a number of investigations have demonstrated a strong association between expression of protooncogenes, cellular growth factors and growth control of normal and neoplastic cells.[1] It also has been demonstrated that perturbations in normal cellular protooncogenes can transform them into activated oncogenes. These changes may occur by a variety of mechanisms (e.g. amplification, translocation, point mutation) and typically result in aberrant gene expression or an altered gene product.[1–3] Once activated, oncogenes appear to play a critical role in the tumorigenic phenotype.[2,4]

A paucity of data exists regarding the effects of clinically efficacious antitumor agents on oncogene expression. It is conceivable, however, that different antitumor agents may selectively inhibit the expression of specific oncogenes. For example, a preliminary report indicated that exposure to mechlorethamine suppressed c-*myc* expression but did not affect *ras* expression in the Burkitt lymphoma Daudi cell line.[5] Another report examined the effect of a variety of clinical antileukemic chemotherapeutic regimens on c-*myc* expression in patients with leukemia.[6] Because amplification of genes such as *myc* and Her 2/neu has been correlated with poor prognosis,[1,2] it could be inferred that over-expression of such genes contributes to tumor cell growth or their survival. If over-expression of each of these genes contributes to tumorigenicity, strategies to decrease their expression may retard tumor growth.

We have previously reported that L1210 cells are susceptible to the cytotoxic action of the photoproducts in preactivated merocyanine 540 (pMC540). This compound also has been shown to spare a majority of the normal cells and tissues.[7,8] Preactivation is a novel process in which the pharmacologically inactive photoactive compounds are illuminated under controlled conditions before they are used in biological systems. This process results in the formation of previously unknown compounds that are no longer dependent on light and hence can be used systemically. We have previously reported the antitumor and antiviral activities of pMC540.[7–10] The mechanism of photoproduct formation during the process of preactivation involves the attack of singlet oxygen generated at excited states (conventional photodynamic effect) on the dye molecule itself.[9]

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Our more recent data suggest that pMC540 blocks the rejoining reaction of DNA topoisomerase (Topo) II by stabilizing a reversible enzyme DNA complex, termed the cleavable complex.[11,12] Protein denaturant treatment of this cleavable complex leads to a double-chained DNA break. Therefore, it has been proposed that the cytotoxic action of this antitumor compound is due to the formation of the cleavable complex.

The role of Topo II in cell proliferation has been emphasized in part: levels of Topo II activity are increased in regenerating hepatocytes and in tumor cells.[13,14] Several lines of evidence have also assessed the requirement of Topo II for transcriptional activity of genes.[15] The enzyme could affect the changes in chromatin structure accompanying gene activation or inactivation.[16] Our other data suggest that the treatment of L1210 mouse leukemia cells with pMC540 results in the down regulation of c-*myc* in a dose-dependent manner and concomitant enhanced survival of BDA/2 mice bearing pMC540-treated cells (unpublished data). However, pMC540 has been shown to display a high degree of preference for certain types of tumor cells while sparing a majority of the normal cells. These results prompted us to investigate the effects of pMC540, a compound that mediates its cytotoxic effect in part via the interaction with Topo II, on c-*myc* oncogene. This oncogene, known to be involved in cell proliferation processes, has been shown to be in a permanent state of transcriptional activity.[17] For the studies of the effect of pMC540 on c-*myc* oncogene, we have used mouse leukemia L1210 cells that contain amplified and over-expressed c-*myc* gene. The rationale for this approach is based upon the fact that a Topo II-mediated drug effect would be more pronounced as compared to cells containing a single copy of the gene.

In this paper we report that some of the cleavage sites induced by pMC540 in the L1210 cell c-*myc* locus are located in the 5′ end of the c-*myc*, close to some DNase I hypersensitive sites that are assumed to reflect the activity of the gene. These data suggest that Topo II-dependent drug activity of pMC540 correlates with the gene activity. Since c-*myc* overexpression is frequently found to be related to human cancer progression, we suggest that this gene could be an important target for Topo II-dependent antitumor drugs.

Methods

Mouse leukemia L1210 cell line was purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% horse serum, 0.25 m mol/l L-glutamine and 100 units penicillin/100 μg streptomycin (Gibco, Grand Island, NY) at 37°C in 5% CO_2/95% air. The cell concentration was kept between 0.5 and 5 × 10^5 cells/ml with fresh medium every 2–3 days.

Metrocyanine 540 was purchased from Eastman Fine Chemicals, Eastman Kodak (Rochester, NY). To prepare pMC540, merocyanine 540 (1 mg/ml) in 70% aqueous ethanol was preactivated by exposure to a bank of fluorescent lamps (cool white 40 W; GE Cleveland, OH) for 18 h.[8] After preactivation, ethanol was removed by rota evaporation and the final concentration was adjusted to 50 mg/ml in 2.5% ethanol:phosphate buffered saline, pH 7.2.

Cells in exponential growth (1–2 days old culture) *phase* were exposed to pMC540 (70.2 and 140.4 μmol) for 8 and 12 h at 37°C in fresh serum-free growth medium. Cells were then washed with 50 mM Tris, pH 7.9, 1 mol EDTA and immediately lysed with 2% SDS, 50 mM Tris, pH 7.9, 20 mM EDTA. Proteinase K was added to a final concentration of 1 mg/ml for 4 h at 50°C. Cell lysate was then treated twice with phenol and DNA extracted with ether, precipitated with ethanol, dried and resuspended in 10 mM Tris, pH 7.9, 0.1 mM EDTA as already described.[18] Cell nuclei were isolated and treated with pancreatic DNase I as described in detail by Dyson *et al.*[19] Samples of cell DNA (10 μg) were digested to completion by *Hin*dIII restriction endonuclease and electrophoresed n horizontal 1.2% agarose gels as previously described.[18]

RNA preparation and gene expression

Total RNA was extracted from untreated and treated cells by using 4 M guanidine isothiocyanate lysates of L1210 cells as described by Chomezynski and Sacchi[20] and precipitated with 95% ethanol. RNA pellets were then washed in 70% ethanol and resuspended in MiliQ water.

RNA (10 μg) was denatured in 0.02 M morpholino propane sulfonic acid (pH 7.0), 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde and 50% formamide. Samples were separated on 6.6% formaldehyde–1% agarose gels.[21] Equal washing of RNA in each lane was confirmed by ethidum bromide (EthBr) staining. RNA blots were prepared by using nitrocellulose membraines and hybridized with the human c-*myc* probe 1.4 kb (Oncor, Gaitherburg, MD) third exon. The probe was labeled with [^{32}P]dCTP to a specific activity of

$2–4 \times 10^8$ c.p.m./μg using the nick translation technique.[18] Hybridizations were performed under stringent conditions[22] and hybrids were revealed by autoradiography (Kodak XAR5 films). Phage λ–*Hin*dIII and φX174–*Hae*III DNA fragments were used as gel calibration markers.

Results

L1210 cells in exponential phase of growth were treated with 70.2 and 140.4 μM pMC540 for 8 and 12 h. These doses have been shown to induce double-stranded DNA breaks *in vitro*.[11] DNA preparations from pMC540-treated and untreated cells were digested to completion with *Hin*dIII restriction endonuclease and analyzed by Southern blot hybridization using human c-*myc* probe (third exon). In results presented in Figure 1(A), genomic DNA from untreated L1210 cells revealed the presence on autoradiograms of a germ line 6.5 kb DNA (lane 1). In addition to this band, genomic DNA from drug-treated cells presented several hybridization bands of smaller size (lanes 3–6) that could be related to the *in situ* c-*myc* gene cleavage products generated by the drug. The intensity of bands increased with increasing drug concentration, indicating that cleavage sites were not generated at random. In contrast to pMC540, the native merocyanine 540, a biologically inactive compound, did not induce cleavage bands (lane 2).

In order to demonstrate whether another intercalative drug with a high affinity for DNA was able to stimulate *in situ* double-stranded DNA cleavage of the c-*myc* gene, EthBr was used under conditions identical to those used for pMC540. Results from these experiments show that EthBr (50 μM) did not induce formation of cleavage bands (lane 7). Furthermore, when L1210 cells were pretreated for 30 min with 50 μM EthBr and then incubated with 70.2 and 140.4 μM of pMC540 for 3 h, a complete inhibition of c-*myc* gene cleavage was observed as determinekd by comparing the patterns of lanes 8–10 and lanes 3–6 (Figure 1).

Cleavage sites generated by pMC540 were mapped in the c-*myc* locus and do not appear to be arranged at random. Sites of preferential cleavage (generating the most intense band) was located in the 5′ part of the the c-*myc* locus (arrow 5) and in the introns (arrows 1–4).

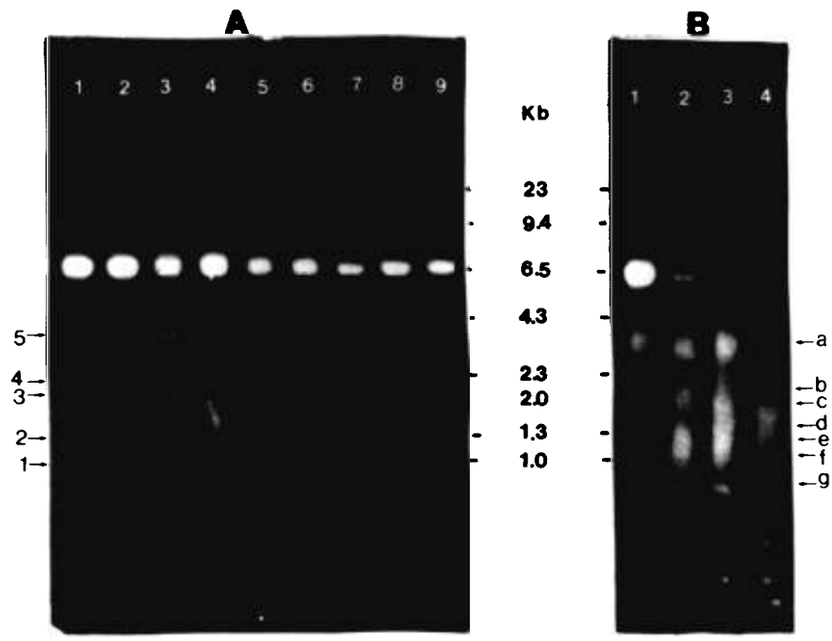

Figure 1. (A) Cleavage sites induced *in vivo* by antitumor drug in the amplified c-*myc* gene of L1210 cells. DNA preparations (10 μg) from drug-treated L1210 cells (see Methods) were digested with *Hin*dIII and analyzed by Southern blot hybridization using a ^{32}P-labeled c-*myc* probe (third exon). 1, Untreated cells; 2, treated with MC540 (100 μM); 3 and 4, pMC540 treated (70.2 μM, 8 and 12 h); 5 and 6, pMC540 treated (140.4 μM, 8 and 12 h); 7, EthBr (50 μM); 8, EthBr (50 μM) + pMC540 (70.2 μM); 9, EthBr (50 μM) + pMC540 (140.4 μM). (B) DNase I hypersensitive sites determined in the amplified c-*myc* of L1210 isolated nuclei. DNA preparations (10 μg) were digested with *Hin*dIII and analyzed by Southern blot hybridization using c-*myc* (third exon). 1, Control nuclei; 2–4, nuclei treated for 5 min at 37°C with 0.5, 1 and 2 μg/ml of DNase I. The blots were exposed to Kodak XARS film to 3 days. DNA length fragments are given in kb pair.

DNase I hypersensitive sites in the c-*myc* gene were determined on L1210 isolated nuclei.[19] Drug-stimulated Topo II cleavage sites and DNase I hypersensitive sites clearly appear to map within the same region of the c-*myc* 5′ end (Figure 1B). In contrast, the two DNase I hypersensitive sites (arrows c and d) have no Topo II cleavage sites equivalents.

It is possible that the antiproliferative effects of pMC540 are 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 L1210 cells. Thus, L1210 cells were treated with pMC540 and their steady state c-*myc* mRNA levels were examined by Northern blot analysis. This treatment produced an initially detectable decrease in c-*myc* transcripts after 4 h of drug exposure. At concentrations of 70.2 and 140.4 μmol pMC540, initial reductions of 1.8 and 4- to 5-fold, respectively, in c-*myc* transcripts were observed after a 1 and 12 h of treatment (Table I). Treatment of L1210 cells with 140.4 μmol pMC540 resulted in approximately 28 and 59% cell kill at 1 h and 12 h, respectively. A comparison of the alterations in c-*myc* expression with the drug induced inhibition of L1210 cell growth, measured by the MTT assay, did not reveal any correlation between these two parameters. Thus, reduced expression of c-*myc* in response to pMC540 treatment could not be attributed to cell death. Further analysis of L1210 cells revealed that c-Ha-*ras* and c-*myb* genes were also not cleaved by the treatment with pMC540. It is interesting to note that the c-*myb* gene, like the c-*myc* gene, is also amplified and over-expressed in L1210 cells, yet it remained unaffected by treatment with pMC540 (data not shown). These results suggest that c-*myc* may be more susceptible to the action of pMC540. Our findings also indicate that cell lines like L1210 with c-*myc* RNA steady state levels are highly susceptible for more intense cleavage induced by pMC540 and it is not associated with gene amplification. Since c-*myc* cleavage increases with expression, this could be associated with the proliferative state of the target cell.

Table 1. Variations of c-*myc* mRNA levels in pMC540-treated L1210 cells

Duration of treatment (h)	Percent of c-*myc* mRNA levels at	
	70.2 μM pMC540	140.4 μM pMC540
0	100[a]	100[a]
1	53.8	56.92
2	45.36	42.04
4	29.9	25.65
8	24.9	37.34
12	23.51	18.9
24	12.1	8.8

Total RNA was prepared from exponentially growing L1210 cells treated with various concentrations of pMC540 for various periods of time and analyzed by Northern blot hybridization (see Materials). c-*myc* mRNA levels were measured by densitometer scanning of autoradiograms values normalized against 28S tRNA values obtained by scanning the UV photograph from EthBr colored gels.

[a] c-*myc* RNA in the absence of pMC540 treatment.

Discussion

In this report, we have shown that the Topo II-dependent antitumor compound pMC540 induces *in situ* DNA breaks in the c-*myc* oncogene of treated leukemia cells. However, native merocyanine 540, which does not depend or stimulate Topo II activity,[11] failed to induce DNA cleavage. In order to verify that the observed DNA breaks in the c-*myc* oncogene are due to drug-induced stimulation of Topo II EthBr was used. EthBr has been shown to inhibit the formation of the Topo II–DNA cleavable complex *in vivo* and *in vitro*.[23] Results from these experiments show that when cells were exposed to EthBr prior to pMC540 treatment, DNA breakage was inhibited, confirming that pMC540 mediates DNA cleavage activity via stimulation of Topo II enzyme activity. Southern blot analysis of DNA from treated cells revealed discrete bands. The intensity of these bands increased with increasing concentration of pMC540 used. Thus, these breaks were not randomly generated. In random cleavage patterns, an increase in drug concentration would have generated a wide spectrum of heterogeneously sized fragments.

It has been reported that the drug induced preferential cleavage sites maps within a region located in the first c-*myc* exon that is implicated in the control of c-*myc* expression. Other minor sites are located in introns and exons of the gene. Whatever the biological significance of these sites, their presence could be correlated with Topo II recognition consensus sequences.[24] Some Topo II cleavage sites (in Figure 1A) are clearly within DNase hypersensitive sites or very close to these sites. Similar observations have been already reported *in vivo*.[25,26] This situation argues in favor of the common targets for DNase I and Topo II or is likely to be related to an open structure of chromatin[16] in transcriptionally active genes (or parts of genes), since such a structure would be more accessible to both enzymes. The precise relationship between transcriptional

activity and susceptibility of a genomic region to Topo II-mediated cleavage remains to be resolved.

The capacity of pMC540 to produce a dose-dependent reduction in expression of c-*myc* and growth inhibition in the L1210 cells suggests that changes in c-*myc* expression represent an early response to Topo II-mediated damage. It appears that in response to either DNA damage or breaks surrounding specific genes such as c-*myc*, there is a reduction in c-*myc* expression which leads to inhibition of 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.[27] In addition, a number of reports have demonstrated that various antineoplastic drugs down-modulate c-*myc* expression.[28–33]

Variations in growth conditions are known to modulate Topo II activity[34] and c-*myc* expression.[35] Moreover, experiments of sensitivity to DNase I, performed on the c-*myc* chromatin in quiescent (under the conditions of serum deprivation) and growing L1210 cells, have shown that hypersensitive site patterns were identical (data not shown). These results suggest that in the presence of pMC540 only a fraction of Topo II is capable of reacting with the c-*myc* chromatin, independently of the variation of Topo II activity.

Another hypothesis is that the c-*myc* gene cleavage observed *in situ* depends on the chromatin accessibility to nuclear proteins associated with the transcriptional activity of the gene, explaining the relationship between c-*myc* transcription and cleavage activity induced by pMC540. It is quite intriguing that only the c-*myc* gene was found to be cleaved because cleavage bands in several other highly amplified and over-expressed genes such as the c-*myb* and c-Ha-*ras* could not be detected.

The importance of the c-*myc* protein in the DNA replication process has been emphasized.[36,37] Activated c-*myc* gene (amplified or overexpressed) is often associated with cellular proliferation and cancer progression.[38,39] In view of these observations, our data suggests that c-*myc* might be a prefered target for pMC540. This observation would be consistent with several other antitumor drugs such as anthracyclines, epipodophyllotoxins and ellipticines in relation to their common mechanism of action with Topo II. Experiments are underway to enhance our understanding of the relationships between drug-induced damage within specific genomic regions, in particular the c-*myc* family of genes, alterations in gene expression, growth inhibition and, ultimately, cytotoxicity of various photoproducts in pMC540.

Conclusion

Treatment of L1210 leukemia cells with pMC540 induces *in situ* DNA cleavage at the 5′ end of the c-*myc* oncogene. The cleavage of c-*myc* was inhibited in cells pre-treated with EthBr, an inhibitor of the Topo II-dependent DNA cleavage, suggesting that the cleavage activity of pMC540 was Topo II dependent. The observed pMC540-induced cleavage does not appear to correlate with over-expressin of the c-*myc* oncogene in these cells as another over-expressed gene, c-*myb*, was not affected. These data suggest that c-*myc* may be a prefered target for pMC540-mediated antiproliferative activity.

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