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SHORT COMMUNICATIONS

Enhancement of intercalator-induced deoxyribonucleic acid scission and cytotoxicity in murine leukemia cells treated with 5-azacytidine

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The major modified base in mammalian DNA is 5-methylcytosine, usually in the sequence m^5CpG [1, 2]. Alterations in the pattern of cytosine methylation have been hypothesized to be causally related to gene expression and cell differentiation [3–8]. For example, hypomethylation of cytosine at gene sites may signal transcription. How changes in the modification of specific DNA bases lead to the regulation of transcription or differentiation is not known; it is likely that such small alterations must, in some way, change chromatin conformation to produce the global effects observed. The nuclease hypersensitivity of actively transcribing chromatin supports the concept that active chromatin exists in a conformation that is different from that of inactive chromatin [9].

5-Azacytidine (5-aza-CR) is a pyrimidine analog which is incorporated into DNA and RNA and blocks cytosine methylation in newly replicated DNA [10–13] by inhibiting DNA methyltransferase [14]. 5-Azacytidine treatment can induce cellular differentiation and gene expression, the latter even in humans with genetic defects in globin synthesis [15].

DNA intercalating agents (drugs which interdigitate between adjacent DNA bases) produce a novel type of DNA scission whose properties suggest that its formation results from interference (by intercalation) with ongoing topological alteration of DNA by breaking–rejoining enzymes, and that its disappearance results from reversal of the interference [16–20]. DNA breaks formed in this way have been detected in a variety of mammalian cells as well as in isolated cell nuclei [21, 22]. Substances which alter the degree of chromatin compaction alter the quantity of DNA breaks produced by intercalators, suggesting that changing the conformation of the DNA target quantitatively changes subsequent DNA strand scission [23]. Further, estradiol, a hormone known to induce gene transcription in human breast cancer cells, enhances intercalator-induced scission in these estrogen-responsive cells [24]. We now report the effects of 5-azacytidine treatment upon the DNA breakage produced by subsequent intercalator exposure in murine leukemia L1210 cells. 5-aza-CR was chosen because it inhibits DNA methylation at concentrations which still allow cell growth and colony formation, thus permitting us to test the hypothesis that hypomethylation could lead to an altered susceptibility of cellular DNA to intercalator-induced breakage, presumably by altering chromatin structure.

The data in Table 1 were generated as follows. Mouse leukemia L1210 cells were grown and DNA was radiolabeled as reported in a previous publication [18]. Colony formation was performed in soft agar as previously reported [18]; 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (m-AMSA) treatments were 1 μM for 30 min at 37° in these assays. DNA single-strand breakage was quantified by alkaline elution with proteinase [18]; m-AMSA treatments were 0.1 μM for 30 min at 37° in these assays. The small quantity of DNA scission produced by 5-aza-CR was subtracted from that produced by 5-aza-CR plus m-AMSA prior to calculating the mean shown in Table 1. Cellular uptake of m-AMSA was quantified as previously described using [^{14}C]-m-AMSA [25]; [^{14}C]m-AMSA treatment was 1 μM for 30 min at 37° [11]. This was performed in triplicate. Results are the mean ± 1 S.D. The rate of DNA synthesis was measured by the incorporation of acid precipitable radioactivity over 60 min by 5×10^5 cells exposed to 1 μCi/ml of [^{3}H]-thymidine. This was performed in triplicate. Results are means of replicates from 5-aza-CR-treated cells divided by results from means of replicates from untreated control cells. Flow microfluorometry was performed on 5-aza-CR-treated and untreated cells, and the fraction of cells in S-phase was calculated as previously described [26, 27]. DNA methylation was quantified in 5-aza-CR-treated and untreated cells by exposing the cells to 1 μCi/ml of [^{3}H-*methyl*]-L-methionine in methionine-depleted culture medium during the 18-hr 5-aza-CR treatment. The quantity of 5-methylcytidine (^{3}H-dpm/nmole m^5CdR) was then quantified by high pressure liquid chromatography as previously described [28]. Corrections for drug-induced alterations in the rate of DNA synthesis could be made as the DNA of the cells was radiolabeled with [^{14}C]deoxycytidine prior to 5-aza-CR treatment and the [^{14}C]TdR (^{14}C-dpm/nmole dTR) incorporated into cellular DNA was also quantified by high pressure liquid chromatography methods [28]. Therefore, the relative specific activities of the cellular DNA for [^{14}C]TdR in 5-aza-CR-treated versus untreated cells reflect drug effects on the rate of DNA synthesis (per cent of control DNA synthesis). Each value for percent of control methylation was then divided by the percent of control DNA synthesis, thus separating effects on a post-synthetic modification of DNA which result from a lower DNA synthesis rate from effects which more specifically reflect altered methylase activity. These corrections were always less than 20%.

5-aza-CR, 0.5 μM for 18 hr, produced a reduction in the rates of cell growth and DNA synthesis, while also reducing survival in colony formation assays (Table 1). Immediately following the 18-hr 5-aza-CR treatment, the frequency of DNA single-strand breaks (SSB) produced by the intercalator m-AMSA (quantified by alkaline elution with proteinase [18]) was similar in 5-aza-CR-treated cells and in untreated controls ($P > 0.1$). However, 24 hr after 5-aza-CR removal, m-AMSA-induced DNA scission was enhanced significantly ($P < 0.001$). The DNA breaks produced by m-AMSA in 5-aza-CR-treated cells were not detected by alkaline elution performed without proteinase. Intercalator-induced DNA–protein crosslinking was also enhanced in 5-aza-CR-treated cells, and, within a factor of 2, equalled the DNA SSB frequency produced. In addition to the DNA effects of m-AMSA, the DNA scission produced by the anthracyclines adriamycin and 5-iminodaunorubicin and the ellipticine analog 2-methyl-9-hydroxyellipticinium was also increased by pretreatment of cells with 5-aza-CR for 18 hr followed by 24 hr in drug-free medium. Thus, the DNA effects of intercalators were quantitatively, but not qualitatively, different in 5-aza-CR-treated cells compared with untreated cells [16–20]. This enhanced susceptibility to m-AMSA-induced DNA scission produced by 5-aza-CR was similar in the DNA of cells that were labeled with [^{14}C]thymidine for 20 hr *prior to* 5-aza-CR treatment (222.9 rad-equivalents without 5-aza-CR vs 498.2 with 5-aza-CR) and in the DNA of these same cells

Table 1. Effects of 5-azacytidine treatment (0.5 μM, 18 hr) on various cellular functions and on m-AMSA-induced DNA scission and cytotoxicity in exponentially growing mouse leukemia L1210 cells*

		Cell growth [fraction of control growth ± 1 S.D. (N)]	Colony formation [survival fraction ± 1 S.D. (N)]		DNA SSB [rad-equivalents ± 1 S.D. (N)]		Uptake molar concentration ± 1 S.D.	[^{3}H]-Thymidine uptake (% of control)	Fraction of cells in S-Phase [± 1 S.D. (N)]	DNA methylation (% of control)
			No AMSA	+AMSA	No AMSA	+AMSA				
Immediately following 5-aza-CR treatment	Control			0.21 ± 0.03 (4)		267 ± 37 (3)			0.35 ± 0.02 (5)	
	5-aza-CR	0.68 ± 0.04 (14)	0.34 ± 0.23 (4)	0.13 ± 0.02 (4)	0 (3)	215 ± 61 (3)		73	0.35 ± 0.03 (3)	31, 52
24-Hr following 5-aza-CR treatment	Control			0.33 ± 0.16 (4)		222 ± 35 (8)	2.8 ± 0.2		0.35 ± 0.02 (5)	
	5-aza-CR	0.51 ± 0.25 (13)	0.49 ± 0.21 (5)	0.06 ± 0.04 (5)	19 ± 22 (8)	381 ± 67 (8)	2.7 ± 0.5	73	0.36, 0.32	65

* m-AMSA treatments were for 30 min at 37° either immediately following the 18-hr treatments with 5-aza-CR or 24 hr following removal. (N) equals the number of experiments. Experimental details are given in the body of the text and in cited references.

labeled with [^{3}H]thymidine *during* the 18-hr 5-aza-CR treatment (208.7 rad-equivalents without 5-aza-CR vs 587.4 with 5-aza-CR). Thus, the potentiation of the effects of m-AMSA was on both the DNA synthesized prior to, as well as on that synthesized during, 5-aza-CR treatment.

This enhancement could not be explained by an increase in the cellular uptake of m-AMSA (quantified directly with [^{14}C]-m-AMSA) [25] or by an effect on the rate of DNA synthesis (which was only minimally reduced and unchanged from the 27% inhibition level observed immediately following 5-aza-CR treatment). m-AMSA-induced SSB frequency was also quantified in nuclei isolated from cells treated with 5-aza-CR and incubated for 24 hr without drug and compared with that produced by m-AMSA in nuclei isolated from untreated control cells [21, 22]. This frequency was enhanced in the nuclei isolated from 5-aza-CR-treated cells as well as in the cells themselves, suggesting that the mechanism of enhancement results from an alteration of nuclear chromatin rather than from an effect on the cytoplasmic component of the cell. We have shown recently that an 18-hr treatment of L1210 cells with subtoxic concentrations of arabinosyl cytosine or hydroxyurea increases the percentage of cells in S-phase, the susceptibility of the DNA of these cells to intercalator-induced scission, and the susceptibility of the cells to intercalator-induced cytotoxicity [26, 27]. However, flow microfluorometry of 5-aza-CR-treated cells did not reveal a drug-induced alteration in the fraction of cells in S-phase. It is therefore unlikely that 5-aza-CR produced its DNA effects through a redistribution of cells within the cell cycle. 5-aza-CR did produce an enhancement in the cytotoxic potency of m-AMSA and the anticipated decrease in cytosine methylation [28].

We have hypothesized that the protein-associated DNA scission produced by intercalator treatment of mammalian cells results from interference by the intercalated drug with the ongoing breaking–rejoining cycle of DNA topoisomerases, enzymes which would be normally responsible for negatively supercoiling DNA [29]. The effects of m-AMSA on cellular DNA are similar to those of oxolinic acid, detergents and prokaryotic DNA gyrase on isolated DNA [30, 31]. Although the ability of 5-aza-CR to enhance this scission could result from effects on the quantity or DNA affinity of topoisomerase, it is more likely that 5-aza-CR could alter chromatin structure through drug incorporation into DNA, or through alterations in chromatin structure which result from the production of fully unmethylated DNA sites.

It is known that cytosine methylation is a post-synthetic DNA modification which occurs at hemimethylated DNA sites, i.e. a methylated parent strand cytosine (within a G-C pair) is detected by the enzyme, which then methylates the newly-synthesized daughter strand cytosine [1, 2]. To change a fully methylated DNA site to a fully unmethylated one thus necessitates the pharmacologic block of daughter strand methylation plus an additional round of DNA synthesis using the newly-synthesized, unmethylated daughter strand as template. This model could explain why the enhancement in DNA scission is not seen immediately following, but only 24 hr after, 5-aza-CR removal [32]. We speculate that the fully unmethylated sites produce an alteration in DNA conformation (such as a Z- to B-DNA transition [33, 34]) or an altered association between DNA and nucleoproteins [35] which could lead to a new DNA conformation that is a better target for intercalation, particularly if chromatin structure is altered in regions of DNA specifically accessible to intercalation [33, 34, 36–38].

It should be noted, however, that the reduction in DNA methylation produced by 5-aza-CR in L1210 cells was transient as it began to return to basal levels following the removal of the drug (Table 1) (see also Ref. 14). Although fully unmethylated sites require time to form, if during that time 5-aza-CR is removed, thus removing the methylase inhibition, the total number of methylated bases will begin to increase. In agreement with this model, the potentiation of m-AMSA-induced SSB is somewhat less pronounced as the time following 5-aza-CR treatment extends beyond 24 hr (data not shown). Further, the DNA of L1210 cells treated with 5-aza-CR and then grown in drug-free medium for several weeks did not exhibit a permanent alteration in susceptibility to intercalator-induced scission. Retreating these cells with 5-aza-CR followed by 24 hr in drug-free medium again resulted in an enhanced susceptibility of cellular DNA to intercalator-induced scission. Thus, alterations in DNA produced by 5-aza-CR in L1210 cells did not appear to be permanent.

Possible consequences of such alterations in DNA structure are effects on gene expression or differentiation [36]. The enhanced intercalator-induced scission may be a sign that such a structural alteration in chromatin has occurred and may serve to (1) predict the occurrence of biological events such as transcription or differentiation, (2) connect these cellular events with biochemical ones such as topoisomerase function, and (3) localize the critical sites for the initiation of these events within cells.

The work with 5-aza-CR provides further evidence that the magnitude of intercalator-induced DNA scission produced by a given drug dose can be affected by changes within the cell nucleus, probably within the chromatin structure. Although these results might not be a function of pharmacologic manipulation of DNA methylation, this possibility exists and substantiates our previous work which suggested that altered chromatin configuration may be produced pharmacologically, result in an enhanced susceptibility to intercalator-induced scission, and possibly enhance intercalator-induced cytotoxicity [23, 27]. Future work will attempt to ascertain the precise mechanism of the synergistic effects of antimetabolites and intercalators and potentially extend these observations to new strategies in the treatment of human malignancies.

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Altered *in vitro* uptake of norepinephrine by cardiovascular tissues of young spontaneously hypertensive rats*

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A number of different observations indicate that the sympathetic nervous system is involved in the development of spontaneous hypertension in the Okamoto strain [1–5], and the young spontaneously hypertensive rat (SHR) is thought to be a suitable model for the study of sympathetic activity at a prehypertensive stage.

Prior to 3 weeks of age, systemic arterial pressure of SHR is reported to be not much different from that of control rats [6]. Since the rapid rise of blood pressure is detected normally at 4 weeks [3, 6–8], the period of initiation should be considered to precede 4 weeks of age [6].

Plasma norepinephrine (NE) levels are higher in SHR at 3–4 weeks of age than in age-matched WKY controls [2, 4, 7, 9], but no significant difference is present between the two strains by 12 weeks of age [2]. Dopamine β-hydroxylase (DBH) is secreted by the process of exocytosis from sympathetic nerve endings with NE [10]. Serum DBH activity in SHR after 6 weeks of age and also at 16 weeks is not significantly different from that of WKY [11, 12]; however, at 3 weeks of age, both mesenteric vessel and serum DBH activities of SHR are about two times higher than those of WKY [7, 12, 13].

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Cardiac NE turnover, which may reflect sympathetic activity, is increased in SHR from 30 to 60 days after birth [14]. The turnover of NE in the kidney and skeletal muscles of SHR, compared to WKY, is also significantly higher at 5 weeks of age but it is no longer elevated at 9–14 weeks when hypertension is stabilized [5, 14]. No such changes are seen in these organs of WKY or Wistar rats when turnover of NE is compared at 5–9 weeks [5].

We have previously reported neuronal incorporation of [^{3}H]NE by isolated storage vesicular fractions of mesenteric arteries [15] and portal-mesenteric (P-M) veins and atria [16] of adult SHR and age-matched WKY controls. [^{3}H]-NE, measured in storage vesicular fraction, was found to be significantly greater in the mesenteric arteries but those of atria and P-M veins were reduced significantly [16]. In the present study, we investigated [^{3}H]NE uptake by mesenteric artery and atria of SHR and their age-matched WKY at 3 and 4 weeks of age. Our previous reports of adult rats excluded cytoplasm from the storage vesicle pellet and, therefore, dealt with [^{3}H]NE uptake and retention by storage vesicles and not total [^{3}H]NE uptake into nerve terminals. The present study, on the other hand, represents the total [^{3}H]NE uptake by the synaptosomal fraction including uptake of NE both from synaptic cleft to neuronal cytoplasm and from cytoplasm to the interior of storage