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THE FORMATION AND RESEALING OF INTERCALATOR-INDUCED DNA STRAND BREAKS IN ISOLATED L1210 CELL NUCLEI

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DNA single-strand breaks and DNA-protein crosslinks induced by intercalating agents were measured in mouse leukemia L1210 cell nuclei by the alkaline elution technique. m-AMSA and 5-iminodaunorubicin produced protein-associated DNA breaks but at a lower level than that produced in whole cells. The frequency of drug-induced DNA single-strand breaks and DNA-protein crosslinks were approximately equal. The rapid formation and resealing of DNA breaks produced by m-AMSA were similar to that seen in whole cells. The DNA-protein crosslinks were also reversible after m-AMSA removal. The process by which reversible, protein-associated DNA strand breaks are produced by intercalating agents can now be studied in an isolated chemical system possessing the breaking-rejoining capacity of whole cells.

INTRODUCTION

Various DNA intercalating agents have been shown to produce reversible DNA single-strand breaks (SSB) and DNA-protein crosslinks (DPC) in whole cells (1-7). These 2 effects form and reverse stoichiometrically and cotemporally and appear to be located close to each other in the cellular DNA (3, 6). The enzymatic qualities of the DNA breaking-rejoining response produced in whole cells following intercalator treatment, has led to the hypothesis that these DNA-break-associated proteins may represent enzymes such as topoisomerases, which generate the DNA scissions (2-6).

The use of isolated nuclei, instead of whole cells, to study the DNA effects of intercalating agents would minimize drug interactions with cyto-

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Abbreviations used: SSB, single-strand breaks; DPC, DNA-protein crosslinks; m-AMSA, 4'-(9-acridinylamino)-methanesulfon-m-anisidide; 5-ID, 5-iminodaunorubicin; 2-Me-9-OH-E+, 2-methyl-9-hydroxyellipticinium.

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plasmic constituents and maximize drug access to nuclear DNA (7,8). Thus, the nature of the DNA effects produced by these agents could be characterized in an isolated chemical system. The utility of such a system would depend upon the ability to reproduce all the effects previously described in cells in the nuclei system. Filipski and Kohn (personal communication) have found that one intercalator, ellipticine, can produce SSB and DPC in isolated nuclei, but the kinetics of the formation of these effects and more critically their reversibility were not studied.

In the present study we describe the DNA effects of three chemically different intercalating drugs in isolated L1210 nuclei; the 9-anilinoacridine derivative 4'-(9-acridinylamino)-methanesulfon-m-anisidide (m-AMSA), the anthracycline derivative 5-iminodaunorubicin (5-ID), and the ellipticine derivative 2-methyl-9-hydroxyellipticinium (2-Me-9-OH-E+). These 3 agents were chosen for study as they all produce reversible, protein-associated DNA breaks in whole cells (3-5). We found that the SSB and DPC produced by m-AMSA and 5-ID in isolated nuclei were qualitatively similar to those produced in whole cells. By contrast, 2-Me-9-OH-E+ did not produce breaks in isolated nuclei. Of greatest importance, however, m-AMSA SSB and DPC were rapidly reversible in isolated nuclei suggesting both the breakage and rejoining responses elicited by these drugs can be studied in an isolated chemical system.

MATERIALS AND METHODS

Cells and radioactive labeling: L1210 mouse leukemia cells were grown in a suspension culture in RPMI 1630 medium plus 15% (v/v) fetal calf serum as previously described (3).

Isolation of L1210 cell nuclei: L1210 mouse leukemia cells were centrifuged and resuspended in an equal volume of nuclei buffer (150 mM NaCl, 1 mM KH_2PO_4 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM dithiothreitol, pH 6.4) at 4°C. These cells were again centrifuged and resuspended in 1/10 volume iced nuclei buffer. Then 9/10 volume of nuclei buffer containing 0.3% Triton X-100 was added and the mixture incubated for 10 min at 4°C. The nuclei were then pelleted by centrifugation (1200 rpm for 5 min) and resuspended in nuclei buffer at 37°. Nuclei were observed microscopically as trypan blue positive. Additionally electron microscopy of these nuclei revealed the disruption of the cell membrane, the depletion of the cytoplasmic contents and the preservation of the nuclear membrane. Membrane tags, however were still present. (Electron microscopy was performed by Dr. Lance Liotta, Laboratory of Pathology, NCI).

Drugs and drug treatment: m-AMSA base (NSC 249992), obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National

Cancer Institute, was dissolved in 100% dimethyl sulfoxide at 10 mM and stored frozen. 5-iminodaunorubicin (NSC 254681) was a gift from Dr. Robert I. Glazer, Applied Pharmacology Section, Laboratory of Medicinal Chemistry and Biology, National Cancer Institute who obtained the compound from Dr. E. Acton, Stanford Research Institute. 5-iminodaunorubicin was dissolved in glass distilled water at 1 mM and stored frozen. 2-methyl-9-hydroxyellipticinium acetate was a generous gift from Dr. J. B. LePecq, Laboratoire de Pharmacologie Moleculaire au CNRS, Institut Gustave-Roussy, Villejuif, France. Stock drug was dissolved in glass distilled water at 8.25 mM and stored frozen. All drug treatments were for 30 min at 37°C. Treatments were stopped by a 1:30 dilution of treated nuclei in drug-free nuclei buffer in the studies of disappearance of drug-induced DNA SSB and DPC.

Alkaline elution: SSB and DPC were quantified in nuclei by alkaline elution as previously described for whole cells. Drugs were removed from nuclei just prior to elution by a 1:20 dilution in iced nuclei buffer. Details of methodology and quantification of SSB and DPC using ^{3}H-DNA-labeled internal standard cells have been given in previous publications (3-7, 9).

RESULTS

DNA SSB in isolated L1210 cell nuclei: The elution rate of DNA from isolated nuclei was nearly linear. DNA from untreated and 300 R irradiated nuclei eluted slightly more rapidly than that from similarly treated whole cells (Figure 1). The process of isolating nuclei appeared to produce no more than 1 break per 10^7 nucleotides (128 $\pm$ 37 Rad-equivalents) and allowed accurate quantification of SSB and DPC produced by intercalating agents.

The frequency of SSB produced by a 30 min treatment with m-AMSA in isolated nuclei was concentration-dependent up to 2 μM thereafter becoming limited in that additional breaks were not produced at concentrations above 2 μM. The frequency of DNA breaks detected in nuclei was less than in cells (Figure 2A).

5-ID also produced DNA SSB in isolated nuclei at a lower level than in whole cells but there was no apparent saturation in the break frequency over the dose range used (Figure 2B).

While 2-Me-9-OH-E+ produced SSB in whole cells it failed to produce DNA breaks in isolated nuclei (Figure 2C) even at concentrations as high as 0.33 mM.

The SSB produced in isolated nuclei by m-AMSA and 5-ID could not be detected in the alkaline elution assay performed without the use of full deproteinizing conditions (9). This is identical to results obtained in whole cells (3-4) and indicates the close association between the drug-induced SSB and DPC.

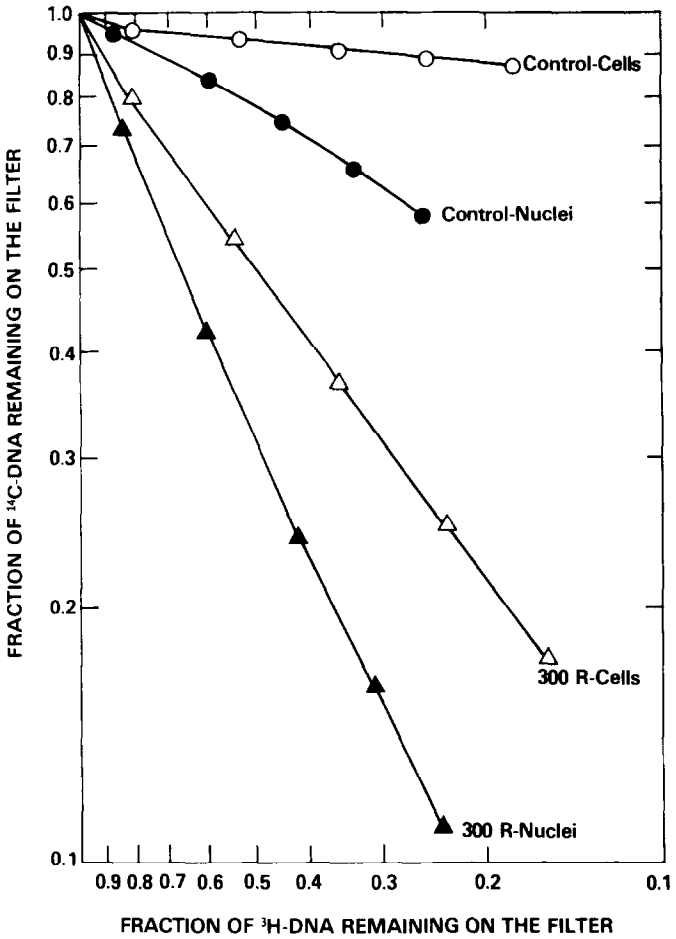

Figure 1: The elution kinetics of DNA from untreated L1210 cells (o) or cell nuclei (●) or irradiated (300 R) cells (Δ) or nuclei (▲). Elutions were performed using the proteinase-K method at a pump speed of 2 ml h^{-1} (see Materials and Methods).

DNA-protein crosslinking frequency in isolated L1210 cell nuclei: Both m-AMSA (0.5 to 3 μM) and 5-ID (1 to 10 μM) produced DPC in isolated nuclei. 2-Me-9-OH-E+ did not. m-AMSA and 5-ID-treated nuclei were concurrently assayed for SSB and DPC (Figure 3). The SSB/DPC ratio for m-AMSA was 1.30 (median of 7 determinations). All the data values were encompassed between 0.97 and 1.58. The median ratio of SSB/DPC for 5-ID (median of 9 determinations) was 1.10 (0.44-1.74). Theses ratios were the same as seen in drug-treated cells (3-5).

Kinetics of the DNA effects produced by m-AMSA in isolated nuclei: At 37°C, the SSB produced by m-AMSA (1 μM) appeared rapidly in isolated nuclei.

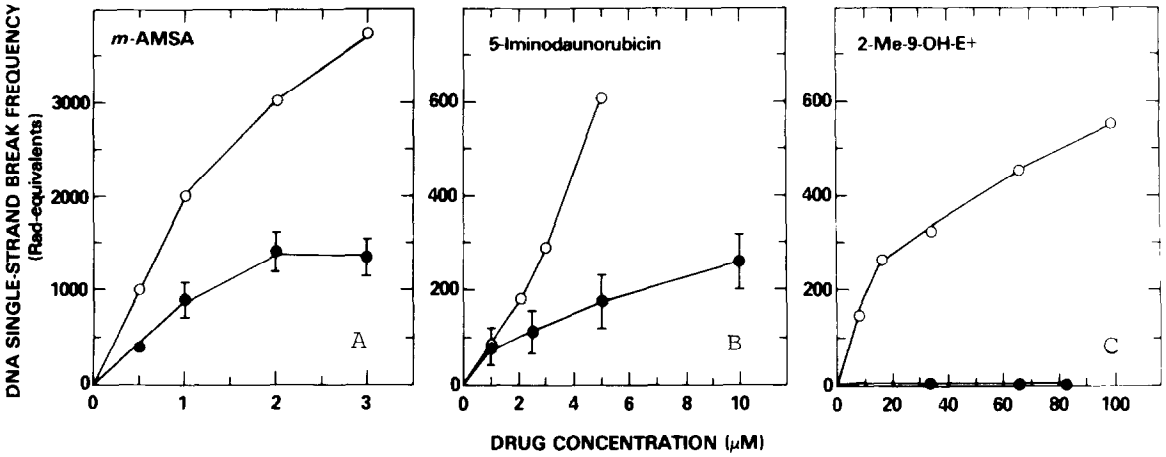

Figure 2: The concentration-dependence of intercalator-induced DNA strand break frequency in isolated nuclei (●) or whole cells (○). Nuclei were treated for 30 min at 37°C in nuclei buffer and then diluted 1:20 in iced nuclei buffer. Cells were treated for 30 min at 37°C in RPMI 1630 medium and washed twice in iced medium. Nuclei and cells were then assayed by alkaline elution using the proteinase K method at a pump speed of 8 ml h^{-1} (A) or 2 ml h^{-1} (B, C). m-AMSA (A); 5-iminodaunorubicin (B); 2-methyl-9-hydroxy-ellipticinium (C). Points are mean ± 1 S.D. of at least 3 independent experiments.

Over half of the breaks observed after a 30 minute exposure occurred within the first 10 minutes following drug treatment (Figure 4). After removal of the drug, SSB disappeared rapidly; the half-time of SSB resealing was 10 to 20 minutes. The kinetics of DPC followed a similar pattern (Figure 4). These

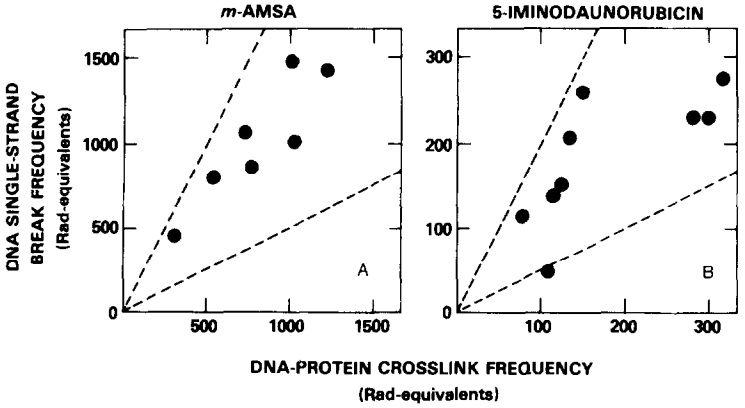

Figure 3: Relationship between intercalator-induced DNA single-strand break frequency and DNA-protein crosslink frequency in isolated nuclei. All treatments were for 30 min. SSB and DPC were assayed on aliquots of the same nuclei suspension. Broken lines encompass the area in which SSB frequency and DPC frequency approximate one another within a factor of 2. (A) m-AMSA (0.5 to 3 μM); (B) 5-iminodaunorubicin (1 to 10 μM).

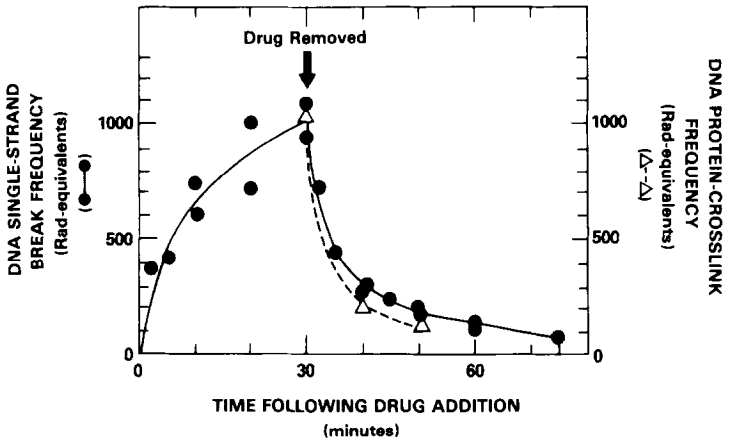

Figure 4: Kinetics of formation and disappearance of DNA single-strand breaks in isolated nuclei exposed to 1 μM m-AMSA at 37°C. Drug treatment was stopped at 30 min (arrow) by a 1:30 dilution in nuclei buffer at 37°C. Single-strand break formation and disappearance (●) and DNA-protein crosslink disappearance (Δ) were studied in 3 independent experiments.

data indicate that the rate of protein-associated DNA strand-break formation and disappearance in isolated nuclei is identical to that seen in whole cells (3).

DISCUSSION

The ability, in isolated cell nuclei, to reproduce the DNA breaking-rejoining activity stimulated by m-AMSA in whole cells leads to several critical conclusions. It suggests that the drug itself can stimulate this activity and cytoplasmic metabolism is not necessary for intercalation. It suggests that the mechanism by which the breaks are produced is localized to the cell nucleus and resists dissociation by non-ionic detergent. It suggests that the energy to break the DNA backbone and to reseal the breaks is not derived entirely from ATP which is probably removed with nuclei isolation. Finally, it suggests that at least some of the DNA breaking effect seen in cells can be reproduced in an isolated biological system without added cofactors.

Our current hypothesis, that intercalator-induced, protein-associated, DNA breaking and rejoining derives from the action of an endonuclease which is part of the cellular response to intercalator-induced DNA distortion, is supported by the results of the present work. We have postulated that such enzymes could be topoisomerases (2-6). Topoisomerases have been isolated from mammalian cell nuclei and can break and rejoin DNA without exogeneous

energy (10). The reduced DNA breaking potential seen in nuclei following treatment with these drugs when compared with effects seen in whole cells may indicate that conditions in our nuclear system have not, as yet, been optimized. This is perhaps most likely in the case of 2-Me-9-OH-E+ where no SSB were detected in nuclei despite readily discernible breakage in similarly treated whole cells that were incubated in nuclei buffer. This indicates that the nuclei buffer did not inactivate 2-Me-9-OH-E+.

Intracellular metabolism could be required to bring 2-Me-9-OH-E+ to the form in which it can produce DNA scission. Neither the metabolism nor the DNA affinity of this compound is well known (11-12). Other possibilities could be that 2-Me-9-OH-E+ break production requires a cofactor depleted during nuclei isolation, or that the enzyme responsible for 2-Me-9-OH-E+ DNA breakage itself is depleted. This drug differs from 5-ID and m-AMSA in that its breaks may be solely double-stranded (3-5) and as such may derive from a unique enzyme, gone from isolated nuclei, but present in whole cells. If the other drugs partially stimulate this enzyme in intact cells, depletion of this enzyme during nuclei isolation could also account for their inability to produce SSB to the levels expected from whole cell work.

The present system will allow study of precisely the questions raised by this initial work. What factors could be required to maximally stimulate DNA breakage? Are the processes partially energy-dependent? Finally, can the reproduction of the intercalator effect in isolated nuclei allow specific isolation of the substances responsible for this DNA scission? The reproduction of the cellular effects of intercalating agents in a system devoid of the majority of cell proteins may enhance the likelihood of success.

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