Effects of Amsacrine and Other DNA-intercalating Drugs on Nuclear and Nucleolar Structure in Cultured V79 Chinese Hamster Cells and PtK2 Rat Kangaroo Cells

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ABSTRACT

Amsacrine (m-AMSA; 4′-(9-acridinylamino)methanesulfon-m-anisidine) is a synthetic intercalating agent with clinical utility in the treatment of acute leukemias and lymphomas. However, as with other intercalators, its mechanism of action is uncertain. We have examined structural changes induced by amsacrine and other intercalators (actinomycin D, Adriamycin, mitoxantrone, 9-aminoacridine) in cultured Chinese hamster (V79-171b) and rat kangaroo kidney epithelial (PtK2) cells, using light- and electron microscopy with simultaneous assessment of cell survival. During chronic exposure at low concentrations, amsacrine causes cell and nuclear enlargement, lobulation of the nucleus, and nucleolar segregation. Nucleolar segregation was also induced by the other four intercalators. The cytotoxic potency of these drugs, as measured by cell survival after 1-hr exposure, was compared with potency of induction of nucleolar segregation. Relative potencies in the two assays varied by more than 10-fold, with actinomycin D the most effective and amsacrine the least effective inducer of nucleolar segregation relative to cytotoxic potency. Thus, although all five intercalators induced nucleolar segregation with high specificity, this lesion does not correlate with cell killing by these drugs. However, interference with nucleolar function (i.e., ribosomal RNA synthesis) may be responsible for the reversible cytostatic effect observed on chronic exposure to some intercalators (actinomycin D, 9-aminoacridine) at low concentrations.

INTRODUCTION

A number of drugs which bind reversibly to DNA by intercalation are now in clinical use as antitumor agents. Included in this important group are several antitumor antibiotics with established clinical utility in the treatment of leukemias (daunorubicin) or solid tumors (ADRIA, ACT-D), as well as newer synthetic intercalating agents such as the acridine derivative m-AMSA. The latter agent has high activity against acute leukemias and offers an alternative to daunorubicin in combination chemotherapy protocols (1, 11). In addition, of other intercalating agents are under consideration as experimental antitumor agents or under evaluation of cytotoxicity using clonogenicity as an end point (reviewed in Refs. 2 and 26). This ultrastructural lesion, which is believed to reflect interference with nucleolar rRNA synthesis (26), has recently been observed following exposure to other intercalating agents such as acridine orange and proflavine (19, 25), quinacrine (10), ADRIA and other anthracyclines (6), MITO (32), ethidium (8), triostin C (7), and nitracrine (9). Other nuclear changes such as alterations in chromatin structure (5, 9, 10, 25) and increased nuclear pleomorphism (31) have been observed following exposure to other intercalating agents such as acridine orange and proflavine (19, 25). In addition, other intercalating agents are under consideration as experimental antitumor agents or have recently entered clinical trials (MITO, ametantrone, bisantrone) with the hope of finding drugs which retain the broad spectrum antitumor activity of ADRIA without its dose-limiting cardiotoxicity. MITO in particular appears to have promise for the treatment of acute leukemia and lymphoma (4), and advanced breast (29) and prostate (14) cancer.

The mode of action of the intercalating agents is unclear. Earlier studies have focused on the inhibition of nucleic acid biosynthesis associated with intercalative binding to DNA (13, 34). In the case of ACT-D, a potent and selective inhibition of rRNA synthesis has been demonstrated at low concentration (15, 16), although proof is lacking that this inhibition is the cause of cell killing. The significance of inhibition of DNA-dependent polymerase activity by other intercalating agents is even less certain, since such inhibition is typically observed only at concentrations high enough to produce other detectable effects as well. Of particular interest among such effects is the induction of DNA breaks, especially the protein-masked single-strand breaks which appear to be induced only by DNA intercalators (21, 22). Evidence has emerged recently to suggest that m-AMSA may induce such breaks by poisoning DNA topoisomerases (12, 17). However, the significance of these lesions has been called into question by the failure to demonstrate a correlation between induction of protein-masked DNA breaks and cytotoxicity (39). Alternative hypotheses for the mechanism of action of m-AMSA focus on the formation of chemically reactive drug metabolites (24) or oxygen free radicals (38).

In an attempt to provide new insight into possible mechanisms of cytotoxicity, we have investigated the effects of m-AMSA and other representative intercalators on the ultrastructure of cultured mammalian cells. To our knowledge, no electron-microscopic investigations on the structural changes produced by m-AMSA have been reported. However, the effects of other intercalators have been examined extensively with the electron microscope. Numerous studies have shown that ACT-D readily induces nucleolar segregation both in vivo and in vitro (reviewed in Refs. 2 and 26). This ultrastructural lesion, which is believed to reflect interference with nucleolar rRNA synthesis (26), has also been observed following exposure to other intercalating agents such as acridine orange and proflavine (19, 25), quinacrine (10), ADRIA and other anthracyclines (6), MITO (32), ethidium (8), triostin C (7), and nitracrine (9). Other nuclear changes such as alterations in chromatin structure (5, 9, 10, 25) and increased nuclear pleomorphism (31) have been reported for some of these agents. Cytoplasmic alterations such as increase in the number of lysosomes (19), the formation of helical polymers (9), and mitochondrial swelling (8, 32) have occasionally been noted. However, these studies have, in general, been qualitative rather than quantitative, and have not included evaluation of cytotoxicity using clonogenicity as an end point. Therefore, the significance of these ultrastructural lesions to cell killing is unknown.
In the present study, we have attempted to relate structural changes to cytotoxicity by simultaneously assessing clonogenicity and cell structure as determined with the light- and electron microscope in a comparative study of 1-hr exposures to 4 representative intercalating agents with clinical antitumor activity: m-AMSA, ACT-D, ADRIA, and MITO. In addition, we have investigated a simple analogue of m-AMSA, 9-AA, which also binds to DNA by intercalation (33, 36) but lacks antitumor activity. We have also examined structural changes produced by m-AMSA following chronic exposure at low concentrations.

MATERIALS AND METHODS

Cell Culture. Transformed Chinese hamster fibroblasts (V79-171b cell line) were obtained from Dr. W. R. Inch, London, Ontario, Canada, and were maintained in exponential growth in an atmosphere of 5% CO₂ in air by subculture to 10⁴ cells/25-sq cm T-flask (Falcon) twice weekly. Cells were harvested for subculture or determination of cell density by treatment with 0.07% trypsin in citrate:saline (0.015 M trisodium citrate:0.134 M KCl, pH 7.2) for 10 min at room temperature. Cell clumps were then dispersed by pipetting, and cell density was determined using an electronic particle counter (Coulter Electronics). The V79 cells were demonstrated to be tumorigenic during the course of this study by s.c. inoculation of 10⁶ cells in immune suppressed mice prepared using a modification of the method described by Steele et al. (28). PtK₂ (rat kangaroo kidney epithelium) cells were obtained from Dr. C. R. Riedel, Albany, NY, and maintained by weekly subculture to 2 x 10⁵ cells/25-sq cm T-flask. Single cell suspensions were prepared by treatment with 0.25% trypsin in citrate:saline for 10 min at 37° followed by passage 6 times through a 26-gauge needle. The culture medium for both cell lines was α-minimum essential medium (27) containing 10% (v/v) heat-inactivated (56°, 40 min) fetal calf serum without antibiotics. Both cell lines were demonstrated to be free of Mycoplasma by cytochemical staining (3). Cultures were reestablished from a frozen reference stock after not more than 20 passages. Doubling times in exponential phase were approximately 9 hr for V79 cells and 25 to 30 hr for PtK₂ cells.

Drug Treatment. The isethionate salt of m-AMSA (a gift from Dr. B. C. Baguley, Cancer Research Laboratory, University of Auckland), mitoxantrone dihydrochloride (a gift from the Warner-Lambert Co., Ann Arbor, MI), Adriamycin hydrochloride (Farmitalia, Milan, Italy), and 9-aminoacridine dihydrochloride (Sigma Chemical Co., St. Louis, MO) were dissolved in dimethyl sulfoxide at 1 mM. Drug solutions were stored at 4° and diluted with medium before exposure to cells.

For evaluation of the effects of chronic exposure to drugs, V79 cultures were initiated at 10⁴ cells/ml in Nuncel (Nunc, Roskilde, Denmark) multwell dishes (0.5 ml/well), or 60-mm diameter Permanox (Lux Scientific Corp., Newbury Park, CA), or Nuncel Petri dishes (3 ml/dish) and incubated for 24 hr before addition of drugs in an equal volume of culture medium. After incubation for a further 45 hr, cultures were trypsinized to determine cell density or were fixed for electron microscopy. The cells grew equally well on Nuncel and Lux dishes. Analogous experiments were performed with PtK₂ cells by initiating cultures at 10⁶ cells/ml (5 ml/60-mm dish) and adding drugs in 1 ml of culture medium 48 hr later. Cultures were trypsinized to determine cell densities or were fixed for microscopy after a further 75-hr incubation. The IC₅₀ was defined as the drug concentration which decreased the cell density to 50% of the concurrent controls without drug.

For 1-hr drug exposures, V79 cell cultures were initiated at 10⁶ cells in 9 ml/60-mm Petri dish. After 48 hr, drugs were added in an equal volume of growth medium containing 40 mg 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, on a warmed aluminum block, and immediately transferred to a submerged grill in a 39° water bath. During these manipulations, the temperature in the culture did not drop below 35°. Cultures were covered with a Lucite dome and flushed with a humidified stream of 5% CO₂ in air to maintain the pH at 7.2 to 7.4. After 1 hr, the temperature in the cultures was 37.0–37.5°.

Determination of Clonogenicity. Drug treatments were terminated by washing V79 cultures 3 times with PBS at room temperature, and single-cell suspensions were prepared by trypsinization. Up to 10⁶ cells were plated in 60-mm Petri dishes in medium (5 ml) containing 15% (v/v) neonatal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). After incubation for 8 days, plates were stained with 0.5% methylene blue in ethanol, and colonies containing more than 100 cells were counted. The surviving fraction was expressed as the plating efficiency of treated cells relative to the control plating efficiency which ranged from 69 to 83%.

Electron Microscopy. Cell monolayers growing in Permanox dishes were rinsed twice at 37° with PBS and fixed at 37° for 45 min in 3% glutaraldehyde buffered to pH 7.2 in PBS. After 5 rinses in PBS, the cells were postfixed at room temperature for 45 min in 1% osmium tetroxide in the same buffer, dehydrated in graded ethanols, and flat-embedded in the dishes in Epon. Selected cells were serially thin-sectioned (70 to 90 nm thick) parallel with the growth surface on an LKB V ultramicrotome with a diamond knife. Sections were stained with 1.75% uranyl acetate in 70% ethanol and with lead citrate (18). Photographs were taken with a Philips EM 300 electron microscope.

Determination of Frequency of Nucleolar Lesions. For each drug concentration, a single large thin section (approximately 0.5 mm square) which was parallel to the growth surface and passed through the midregion of up to several hundred cells was scanned systematically, and the morphologies of 100 nucleoli, each from a separate cell, were assessed. The criterion used for determining the presence of nucleolar lesions in a cell was whether the nucleolus was irregular (i.e., normal; see Figs. 1D and 2A) or roughly spherical (i.e., lesion present; see Figs. 2B and 3, A to C) in sectional outline. Therefore, all stages of nucleolar segregation [as described by Simard et al. (26) and shown in Fig. 3, A to C] were classified as nucleolar lesions. This was found to be the most objective and consistent morphological criterion which could be used. Results of 2 independent experiments with each drug (Table 2), or scoring of different sections from the same experiment, by 3 different investigators, were all in good agreement. Untreated cells scored in the same way for the presence of rounded nucleoli in 5 groups of 100 gave values of 0 to 1%.

Light Microscopy. Four blocks of m-AMSA-treated Epon-embedded cells from each Petri dish, all of which had been fixed at the same time, were randomly selected for analysis. The cells were sectioned 1-μm thick, parallel with the growth surface on an LKB V ultramicrotome with a glass knife, and sections through the midregion of the cells were mounted on glass slides and stained with 1% aqueous toluidine blue. To ensure that each cell would be measured only once, only one section per block was used for the analysis. The cells were photographed with a Zeiss Photoscope with a ×40 objective and printed at final magnifications of ×2000 to 2500. A stage micrometer was photographed for calibration. Micrographs were made from different areas of the same section, giving a total of 14 micrographs, each containing 7 to 20 complete cell profiles for analysis. The nuclear and cytoplasmic perimeters and areas for each complete cell profile were measured on the photographic prints using a digitizing tablet (Fig. 8). The lobulation index (ratio of the measured nuclear perimeter to the circumference of the circle of equivalent area) was used to quantify the deviation of the nuclear contour from circularity. This measure was chosen in preference to the perimeter:area ratio as a means of quantitating changes in nuclear shape, since the latter will also reflect changes...
in nuclear size. Cells from each dish were also sectioned perpendicular to the growth surface and analyzed as described above.

**RESULTS**

**Growth Inhibition and Cell Killing**

The cytotoxic potencies of m-AMSA, ACT-D, ADRIA, MITO, and 9-AA were compared under conditions of chronic (45-hr) or acute (1-hr) exposure of log-phase V79 cells in culture. Determination of total cell density after 45 hr (Chart 1) indicated a wide range of growth-inhibitory potencies, with IC_{50} ranges from 1.1 nM for ACT-D to 4020 nM for 9-AA (Table 1). For a wide range of growth-inhibitory potencies, with IC_{50}s ranging from 1.1 nM for ACT-D to 4020 nM for 9-AA (Table 1). For m-AMSA, ADRIA, and MITO, significant loss of clonogenicity was observed at all concentrations which caused growth inhibition, indicating that for these 3 drugs, growth inhibition was accompanied by cell killing and was probably primarily a consequence of it. In contrast, growth inhibition by low concentrations of ACT-D and 9-AA (at or below the IC_{50}) was not accompanied by significant cell killing (Chart 1). This distinction between the 2 groups of intercalators was observed consistently in repeat experiments (Table 1).

Growth inhibition by m-AMSA was also assessed using the PtK_2 cell line, which was less sensitive than the V79 line with an IC_{50} of 200 ± 23 (S.D.) nM (3 experiments) following 75-hr exposure. The contribution of cell killing to this growth inhibition was not determined, since PtK_2 does not form colonies at low-density in α-minimum essential medium.

When plating efficiency of V79 cells was determined immediately after a 1-hr drug exposure at 37°C (Chart 2; Table 2), survival curves for ADRIA, m-AMSA, and MITO were close to exponential with a slight upward concavity for m-AMSA and MITO and a small shoulder (n = 2.6) for ADRIA. The ACT-D survival curve displayed a larger shoulder (n = 3.8) with a biphasic exponential indicative of a resistant subpopulation comprising approximately 10% of the total. When assessed on this basis (acute cell killing) rather than chronic growth inhibition, the potency of ACT-D was greatly reduced relative to the other agents. The survival curve for 9-AA differed from the other agents in its large shoulder and pronounced curvature at low survival (Chart 2, inset).

**Light- and Electron Microscopic Observations**

**Chronic Exposures.** When V79 or PtK_2 cells were exposed to m-AMSA at concentrations in the vicinity of the IC_{50}, conspicuous cell enlargement was evident by light- or electron microscopy (Fig. 1, A to E). Morphometric analysis of light micrographs of V79 cells treated with 12.5 or 25 nM m-AMSA for 45 hr and sectioned parallel with the growth surface indicated pronounced increases in both nuclear and total cellular cross-sectional area, such increases being greater at the higher drug dose (Table 3). m-AMSA-treated cells sectioned perpendicular to the growth surface also possessed greater sectional areas (mean, 371 sq μm) than untreated cells (mean, 118 sq μm). However, the most striking change in m-AMSA-treated V79 cells was the marked irregularity of the nuclear contour, with pronounced lobulation evident by light- or electron microscopy (Fig. 1, B and D). The extent of nuclear lobulation evident by light microscopy of thick

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**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC_{50} (nM)</th>
<th>Surviving fraction</th>
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<tr>
<td>m-AMSA</td>
<td>16.8 ± 1.5 (6)</td>
<td>0.21 ± 0.04 (6)</td>
</tr>
<tr>
<td>ADRIA</td>
<td>14.8 ± 1.2 (6)</td>
<td>0.074 ± 0.016 (3)</td>
</tr>
<tr>
<td>MITO</td>
<td>3.0 ± 0.3 (7)</td>
<td>0.14 ± 0.06 (2)</td>
</tr>
<tr>
<td>ACT-D</td>
<td>1.1 ± 0.2 (8)</td>
<td>0.95 ± 0.10 (3)</td>
</tr>
<tr>
<td>9-AA</td>
<td>4020 ± 330 (8)</td>
<td>0.85 ± 0.08 (4)</td>
</tr>
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* Concentration of drug which reduced the total cell density to 50% of that in control cultures.

† Plating efficiency after 45-hr exposure to drugs at the concentration corresponding to the IC_{50}, as a fraction of the control plating efficiency.

‡ Mean ± S.E.

Numbers in parentheses, number of experiments.
NUCLEOLAR SEGREGATION BY INTERCALATORS

Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of assays</th>
<th>n*</th>
<th>D&lt;sub&gt;40&lt;/sub&gt; (nm)</th>
<th>N&lt;sub&gt;50&lt;/sub&gt; (nm)</th>
<th>D&lt;sub&gt;40&lt;/sub&gt;N&lt;sub&gt;50&lt;/sub&gt;</th>
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<tr>
<td>m-AMSA</td>
<td>5</td>
<td>1.0</td>
<td>455 ± 30</td>
<td>30,500 ± 500</td>
<td>0.015 ± 0.001</td>
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<tr>
<td>ADRIA</td>
<td>3</td>
<td>2.6 ± 0.9</td>
<td>247 ± 12</td>
<td>760 ± 60</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>MITO</td>
<td>2</td>
<td>1.0</td>
<td>49 ± 10</td>
<td>855 ± 55</td>
<td>0.057 ± 0.012</td>
</tr>
<tr>
<td>ACT-D</td>
<td>2</td>
<td>3.8 ± 0.4</td>
<td>290 ± 30</td>
<td>1.7 ± 0.2</td>
<td>171 ± 48</td>
</tr>
<tr>
<td>9-AA</td>
<td>4</td>
<td>65 ± 13</td>
<td>72,000 ± 2,000</td>
<td>14,800 ± 2,800</td>
<td>5.1 ± 1.0</td>
</tr>
</tbody>
</table>

* Extrapolation number (intercept on the ordinate obtained by back-extrapolation of the exponential region of the survival curve).

Table 3

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>No. of cells</th>
<th>Total area (sq μm)</th>
<th>Nucleus area of total cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>76</td>
<td>176 ± 40</td>
<td>73 ± 18</td>
</tr>
<tr>
<td>12.5</td>
<td>50</td>
<td>302 ± 144</td>
<td>111 ± 51</td>
</tr>
<tr>
<td>25</td>
<td>84</td>
<td>499 ± 252</td>
<td>193 ± 88</td>
</tr>
</tbody>
</table>

* See "Materials and Methods." 

sections is quantitated as the lobulation index (see "Materials and Methods") in Table 3, which indicates a significant and progressive increase in the deviation of the nuclear contour from circularity after treatment with 12.5 or 25 nM m-AMSA. Cells sectioned perpendicular to the growth surface showed similar lobulation indices (1.08 for untreated and 1.25 for 25 nM).

No ultrastructural changes in the cytoplasm or nucleoplasm of V79 or PtK2 cells were detected after m-AMSA treatments in the vicinity of the IC<sub>50</sub>, but changes to nucleolar structure were observed. The nucleoli of untreated V79 and PtK2 cells were observed in sectional outline and consisted of intermixed granular and fibrillar elements (Figs. 1D and 2A). The nucleoli of V79 cells exposed to 12.5 nM m-AMSA resemble those of control cells. However, approximately 45% of the cells exposed to 25 nM m-AMSA for 45 hr contained nucleoli which showed the early stages of nucleolar segregation, i.e., they were roughly spherical in sectional outline (Fig. 2B). In contrast, the nucleoli of PtK2 cells exposed to m-AMSA at a concentration causing similar growth inhibition (250 nM for 75 hr) showed complete segregation into separate granular and fibrillar masses (Fig. 2C).

ADRIA and MITO resembled m-AMSA in causing conspicuous nuclear lobulation and rounding of the nucleoli in V79 cells exposed for 45 hr to concentrations in the vicinity of the IC<sub>50</sub>. In contrast, the nucleoli of V79 cells exposed to 6.25 to 25 nM ACT-D for 45 hr were not lobulated. However, the nucleoli of all ACT-D-treated cells were completely segregated (Fig. 2D).

Effects of Short-Term Exposures to Intercalators. To investigate further the possible importance of nucleolar damage in the action of m-AMSA and other intercalators, V79 cells treated at a range of concentrations for 1 hr were examined by electron microscopy. The frequency of cells with nucleolar alterations and the severity of ultrastructural changes increased with m-AMSA concentration, demonstrating the characteristic stages of nucleolar segregation described for other drugs and cell types (26). At concentrations ranging from 20 to 50 μM, a decreasing proportion of the nucleoli was normal in appearance (as in Figs. 1D and 2A). At low concentrations (20 to 35 μM), some nucleoli appeared rounded, presenting roughly circular outlines, but were otherwise normal with intermixed fibrillar and granular components (Fig. 3A). Cells exposed to higher concentrations (50 μM) of m-AMSA for 1 hr possessed some nucleoli in which the granular and fibrillar components showed initial stages of segregation (Fig. 3B), while others were completely segregated (Fig. 3C). This pattern of advanced segregation was similar to the familiar pattern produced by ACT-D (Fig. 3D) with its characteristic "bull's-eye" appearance (2).

To obtain a quantitative dose-response relationship for the induction of nucleolar changes, we scored nucleoli as aberrant if they showed a rounded outline (as seen in Fig. 3A), this being the most sensitive and objective indicator of the onset of nucleolar segregation. This approach provided a comparison of the 5 intercalating agents, which differed markedly in their abilities to induce nucleolar changes. ACT-D was the most potent of the agents tested, and m-AMSA and 9-AA the least, with MITO and ADRIA demonstrating intermediate potency (Chart 3). N<sub>50</sub> concentrations for 1 hr exposures were estimated from data such as shown in Chart 3. N<sub>50</sub> values determined from 2 separate experiments with each agent were in good agreement (Table 2). For all drugs, complete nucleolar segregation (separation of fibrillar and granular components, as in Fig. 3, C and D) was evident at the doses causing a high frequency of rounding of nucleoli, with no qualitative differences in the nature of the nucleolar aberrations being observed for the different intercalators.
found that in PtK2 cells nuclear tabulation and induction of either nuclear or cytoplasmic (e.g., cytoskeletal) changes. The changes in nuclear morphology appear to be quite different than the normal G2-phase volume has been observed in other cell lines by Coulter pulse-height analysis (35), and is presumably a reflection of continued RNA and protein synthesis during the G2 block induced by this drug (30). Such "unbalanced growth" is well known as a general feature of many cytotoxic drugs (20).

DISCUSSION

Under conditions of prolonged exposure to m-AMSA at concentrations which cause growth inhibition and cell killing, 4 morphological changes have been observed in V79 cells: total cell (and nuclear) enlargement; nuclear lobulation; clumping of chromatin; and nuclear changes. We have confirmed that the apparent cell enlargement is not a consequence of flattening of cells after treatment, since sections cut perpendicular to the growth surface also demonstrate increased sectional areas. Enlargement of m-AMSA-treated cells to considerably greater than the normal G2-phase volume has been observed in other cell lines by Coulter pulse-height analysis (35), and is presumably a reflection of continued RNA and protein synthesis during the G2 block induced by this drug (30). Such "unbalanced growth" is well known as a general feature of many cytotoxic drugs (20).

Nuclear lobulation, such as that induced by m-AMSA (Table 3) is a more unusual finding. Such lobulation, which has also been observed in myocardial cells exposed to ADRIA (31), was very pronounced in some V79 cells after 45-hr exposure, resulting in bizarre nuclear sections under the electron microscope (Fig. 1E). The changes in nuclear morphology appear to be quite different from those due to apoptosis or coagulative necrosis (23). The mechanism of the observed lobulation is unclear and could reflect either nuclear or cytoplasmic (e.g., cytoskeletal) changes. The lobulation appears to develop slowly, since no such effects were observed after 1-hr exposures, even at concentrations 3000-fold higher than used in chronic exposures. In fact, we have recently found that in Ptk2 cells nuclear lobulation and induction of micronuclei both increase to a maximum at a concentration of approximately 250 nM and decrease at higher concentrations.4

At these higher concentrations, cell division is almost completely inhibited. This observation raises the possibility that development of lobulation, like induction of micronuclei, may be dependent on progression of cells through mitosis. This could suggest that postmitotic repackaging of m-AMSA-damaged chromatin, or some other aspect of nuclear reformation, is aberrant. Whatever the mechanism, the significance of nuclear lobulation deserves further investigation, since the present study suggests a possible relationship between cytotoxicity and the induction of lobulation by intercalators. Thus, this phenomenon was observed with those intercalators (m-AMSA, ADRIA, MITO) which caused cell killing during chronic exposure, but not with ACT-D which caused only reversible growth inhibition.

Conspicuous changes in chromatin structure were seen after treatment with m-AMSA only at very high concentrations (200 μM for 1 hr) when an increase in heterochromatin was observed, primarily at the nuclear periphery. Some tendency towards aggregation of chromatin was also noted with ACT-D, ADRIA, and MITO, but these effects were not always consistent in different experiments and were not investigated further.

The only other morphological alteration observed in cells exposed to m-AMSA was that of the nucleolus. The rounding of the normally irregular nucleolus of V79 cells after chronic m-AMSA exposure at 25 nM (Fig. 2B) is suggestive of the early changes seen during nucleolar segregation (25, 26). In Ptk2 cells, completely segregated nucleoli were seen at m-AMSA concentrations giving comparable growth effects (Fig. 2C). These observations suggested to us that nucleolar damage could be a significant component of the cytotoxicity of m-AMSA. We therefore investigated nucleolar segregation further as a possible ultrastructural correlate of the cytotoxicity of m-AMSA and other intercalating agents. These comparisons were most conveniently made using 1-hr drug exposures, since readily interpretable survival curves could then be generated for all agents.

The intercalating agents studied demonstrate a wide range of cytotoxic potencies, as measured by the D10 (drug concentration reducing cell survival to 10% of controls) for 1-hr exposure, and a wide range of potencies as inducers of nucleolar segregation, as measured by the N50 (Table 2). However, these 2 parameters of drug action show no correlation, with marked variation in the ratio D10/N50 for different intercalators (Table 2). This ratio varies from a high value of 190 for ACT-D (indicating marked nucleolar segregation relative to toxicity) to a low value of 0.015 for m-AMSA. Thus, relative to its cytotoxic activity, ACT-D is over 10-fold more effective than is m-AMSA in inducing nucleolar segregation. Of the other intercalators, 9-AA shows the greatest effect on nucleolar structure, while ADRIA is less active relative to its cytotoxic potency, and MITO shows very low activity (Table 2).

Previous studies have shown that nucleolar segregation is induced by many intercalators (2, 26). The present study confirms the specificity with which a structurally unrelated group of intercalators causes nucleolar segregation in the absence of other acute ultrastructural changes, but clearly excludes this phenomenon as a unitary mechanism of cytotoxicity for these drugs.

Our data would allow that ACT-D could exert its cytotoxic action through interference with rRNA synthesis in the nucleus. However, since cell killing requires ACT-D concentrations much higher than those inducing nucleolar segregation (Table 2), it appears probable that the latter lesion is readily reversible. At

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4 W. R. Wilson, unpublished observations.
very high concentrations, it is conceivable that inhibition of rRNA synthesis is of sufficient duration to cause cell killing, but other possible mechanisms of cell killing must clearly be considered, even for this intercalator. Our recent demonstration that the relationship between micromolecular induction and cell killing is similar for ACT-D, m-AMSA, and ADRIA (37) suggests that induction of chromosome breakage may be a more plausible cause of lethality for ACT-D and other antitumor intercalators.

It is of interest that ACT-D and 9-AA, the 2 intercalators capable of reversibly inhibiting V79 cell growth (with little cell killing) during prolonged exposure (Chart 1; Table 1), have the greatest effects on nucleolar structure relative to their toxicity (Table 2). This suggests that the reversible cytostatic effect of intercalators like ACT-D and 9-AA could be mediated through inhibition of rRNA synthesis, with chromosome breakage representing the toxic lesion at higher concentrations. This hypothesis is being evaluated further by comparing DNA breakage and inhibition of macromolecular biosynthesis by this panel of intercalating agents.

ACKNOWLEDGMENTS

The authors would like to thank A. Fenn and S. Tapp for technical assistance, A. Ellis and I. MacDonald for help in producing the illustrations, J. Westbrooke for typing the manuscript, and Dr. J. Probert for his support and interest.

REFERENCES

Fig. 1. Effect of chronic exposure of V79 cells to m-AMSA. A, light micrograph of untreated cells. × 1,540. B, light micrograph of cells following 45-hr exposure to 25 nM m-AMSA. × 1,540. C, electron micrograph of untreated cell; nuclear profile (arrows) is relatively smooth; N, nucleolus. × 5,650. D, electron micrograph of untreated cell; N, nucleolus. × 10,400. E, electron micrograph of cell following 45-hr exposure to 25 nM m-AMSA; nuclear profile (arrows) is tortuous; N, nucleolus. × 5,650.
Fig. 2. Effects on the nucleolus of chronic exposure to DNA-intercalating drugs. A, the nucleolus of an untreated V79 cell is irregular in outline (arrows) and consists of intermixed granular (G) and fibrillar (F) components and less-dense nucleolar vacuoles (V). × 20,900. B, some nucleoli of V79 cells following 45-hr exposure to 25 nM m-AMSA are roughly circular in outline (arrows). Intermixed granular (G) and fibrillar (F) components and nucleolar vacuoles (V) are present. × 37,780. C, the nucleolus of a PtK2 cell following 75-hr exposure to 250 nM m-AMSA. The granular (G) and fibrillar (F) components are fully segregated. × 56,400. D, V79 cell following 45-hr exposure to 2 nM ACT-D; the nuclear profile (arrows) is relatively smooth, and nucleoli (N) are fully segregated. × 5,100.
NUCLEOLAR SEGREGATION BY INTERCALATORS

Fig. 3. Effects on the V79 nucleolus of short-term (1-hr) exposure to DNA-intercalating agents. A, 35 μM m-AMSA; this nucleolus is roughly spherical in outline and intermixed granular (G) and fibrillar (F) components, and nucleolar vacuoles (V) are present. × 33,200. B, 50 μM m-AMSA; this nucleolus shows the initial stages of segregation into separate granular (G) and fibrillar (F) components and smaller nucleolar vacuoles (arrows). × 37,800. C, 50 μM m-AMSA; this nucleolus is fully segregated into discrete granular (G) and fibrillar (F) components. × 41,360. D, 0.4 μM ACT-D; the characteristic complete segregation of the nucleolus into discrete granular (G) and fibrillar (F) components. × 25,270.
Effects of Amsacrine and Other DNA-intercalating Drugs on Nuclear and Nucleolar Structure in Cultured V79 Chinese Hamster Cells and PtK₂ Rat Kangaroo Cells

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