Effects of 3'-Deamino-3'-(3-cyano-4-morpholinyl)doxorubicin and Doxorubicin on the Survival, DNA Integrity, and Nucleolar Morphology of Human Leukemia Cells in Vitro


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The potential mechanisms of the extremely potent anthracycline analogue 3'-deamino-3'-(3-cyano-4-morpholinyl)doxorubicin (MRA-CN) have been compared with those of doxorubicin (DOX) by examination of drug effects on colony formation, macromolecular synthesis, DNA integrity, and ultrastructure of human leukemia cells in vitro. Following a 1-h exposure, MRA-CN was found to be 1400-fold more cytotoxic than DOX which correlated with the drugs' inhibitory effects on DNA and total RNA synthesis. Treatment with MRA-CN resulted in a dose-dependent production of DNA interstrand cross-links as quantified by alkaline elution. One-h treatments with DOX or 3'-deamino-3'-(4-morpholinyl)doxorubicin (the non-cyano-containing analogue of MRA-CN) produced no DNA-DNA cross-links; rather they produced protein-concealed DNA single-strand breaks. After removal of MRA-CN, the DNA of KBM-3 cells displayed time-dependent fragmentation as indicated by rapid DNA filter elution during the pH 10 lys step which preceded pH 12 elution. Within 4 h of MRA-CN exposure (10 nM, 1 h), 50% of the cellular DNA was in the lysis fraction. By 24 h, all the cellular DNA was in this fraction. MRA-CN (10 nM), 3'-deamino-3'-(4-morpholinyl)doxorubicin (1 μM), and actinomycin D (1 μM), but not DOX (3 μM), each produced distinctive nucleolar macrosegregation, indicating an effect on RNA synthesis. The α-CN substituent on the morpholinyl moiety of MRA-CN appears to be responsible for the unique antitumor potency of this anthracycline. Nucleolar macrosegregation is probably associated with the morpholinyl moiety and is independent of the α-CN substituent.

INTRODUCTION

A large number of anthracyclines have been synthesized in an attempt to produce an analogue with a therapeutic ratio significantly greater than that of doxorubicin (Adriamycin). Recently, the N-alkylated derivative of DOX, MRA-CN, showed a large increase in antitumor potency over that of DOX and yet was without the potential to produce cardiotoxicity at therapeutically efficacious doses (1, 2). In addition, a human uterine sarcoma cell line resistant to DOX was not cross-resistant to MRA-CN (2). The potency of MRA-CN, and the fact that it is not cross-resistant with DOX, indicated that MRA-CN may have a mechanism(s) of antitumor action distinctly different from that of DOX itself. A structural difference between these two drugs, which could result in a mechanistic difference explaining the potency difference, is the reactive cyano-morpholinyl group, which results in covalent binding of the drug to DNA (3, 4).

MATERIALS AND METHODS

IMDM containing 1-glutamine and 25 mm 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid buffer, heat-inactivated FCS and HBSS were obtained from GIBCO Laboratories, Grand Island, NY. [2-14C]-Thymidine (50 μCi/ml), [methyl-3H]thymidine (75.5 Ci/mmol), and [G-3H]uridine (6.0 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Doxorubicin was obtained from Adria Laboratories, Columbus, OH and MRA-CN and MRA were kindly provided by SRI International, Menlo Park, CA. Actinomycin D was obtained from Merck Sharp & Dohme, West Point, PA. Drugs were kept as stock solutions in 95% ethanol (MRA, MRA-CN) or dissolved immediately prior to use in glass-distilled water (DOX, actinomycin D). Cell Culture. A myeloid leukemic cell line, KBM-3, established from a patient with acute monocytic leukemia, was used throughout the study. The in vitro growth characteristics and cytogenetic pattern of the cell line have been described in detail elsewhere (4). The KBM-3 cell line was maintained in suspension culture in a humidified atmosphere at 37°C under 95% air:5% CO2 in IMDM supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 μg/ml), l-glutamine (584 μg/ml), and sodium bicarbonate (3.024 μl/ml). The KBM-3 cell line had a colony-forming efficiency of 23 ± 2% (SD) at 103 plated cells. Cell cultures for all experiments in this study were in exponential growth phase with a doubling time of 30 to 36 h.

Leukemic Cell Colony-forming Assay. Cells were washed with PBS at room temperature and resuspended in HBSS with 5% FCS at 104 cells/ml; thereafter, either DOX or MRA-CN was added at different concentrations and the cells were incubated at 37°C for 1 h. Exposure of cells to the drug was terminated by adding 10 volumes of ice cold PBS. The cells were then centrifuged at 200 x g for 10 min at 4°C and washed twice with PBS. The cells were resuspended in HBSS with 5% FCS and assayed for surviving clonogenic cells. Untreated controls from each culture were run in parallel.

Aliquots (25 μl containing 104 cells in HBSS with 5% FCS) were added to 1 ml of a mixture of IMDM with 20% (v/v) FCS and 0.3% (v/v) agar; the mixture was plated in triplicate in 1-ml dishes containing a single layer of semisolid agar. After allowing the agar to gel at room temperature, the cell cultures were incubated for 8 days at 37°C in a fully humidified atmosphere of 5% CO2 and 12% O2 balanced with N2. At the end of the culture period, the number of colonies containing more than 50 cells were counted. The surviving fraction of clonogenic leukemic cells was calculated by comparing the number of colonies after drug exposure with their counterparts in the control plates. Survival curves were constructed as described elsewhere (5) and the IC50 was determined by linear regression analysis in each instance.

Received 1/4/86; revised 4/23/86; accepted 4/30/86.

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1 Supported in part by a National Cancer Institute Career Development Award (K04 CA01135) to R. A. N. 2 To whom requests for reprints should be addressed, at Department of Chemotherapy Research, The University of Texas M.D. Anderson Hospital and Tumor Institute, 6723 Bertner Ave., Houston, Tex. 77030.

3 The abbreviations used are: DOX, doxorubicin; MRA, 3'-deamino-3'-(4-morpholinyl)doxorubicin; MRA-CN, 3'-deamino-3'-(3-cyano-4-morpholinyl)doxorubicin; IMDM, Iscove's modified Dulbecco's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; HBSS, Hanks' calcium- and magnesium-free balanced salt solution; SD, sodium dodecyl sulfate; ICS, the concentration of drug that reduced colony formation to 50% of that for untreated cells.


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DNA and RNA Synthesis. Cells were incubated with concentrations of DOX (3 × 10⁻¹⁰ to 3 × 10⁻⁴ M) or MRA-CN (3 × 10⁻⁷ to 3 × 10⁻¹¹ M) at 37°C for 1 h. Following treatment with drug, cells were washed with HBSS and reincubated in drug-free medium. At 0, 2, 4, and 24 h after drug treatment, aliquots of cells were incubated in triplicate with either [methyl-³H]thymidine (0.1 μCi/ml) or [G-³H]uridine (0.05 μCi/ml) for 1 h. Incorporation of [³H]thymidine and [³H]uridine into DNA and RNA, respectively, was stopped by the addition of 0.9% NaCl (4°C) for centrifugation at 4°C. The supernatants were discarded and the cells were resuspended in 5 ml of an ice-cold solution of 10% trichloroacetic acid and 2% sodium pyrophosphate (w/v) in distilled water. The resulting acid-insoluble material was collected on glass microfiber filters (934-AH, Whatman Ltd., Maidstone, England). The filters were washed once with 5 ml of the trichloroacetic acid/pyrophosphate solution, twice with 5 ml of 95% ethanol, and then were air dried. Filters were placed in scintillation vials and 0.5 ml Protosol (New England Nuclear, Boston, MA) was added. Capped vials were incubated at 55°C for 30 min; 20 μl glacial acetic acid and 10 μl scintillation fluid were added, and the radioactivity was determined using a Beckman LS 5801 liquid scintillation system.

Filter Elution Assays. The elution methodology has been previously described by Kohn et al. (6) and Zwelling et al. (7). Briefly, cellular DNA was radioactively labeled in exponentially growing cells by incubation for 20 h at 37°C with [²H]thymidine (0.03 μCi/ml) or with [methyl-²H]thymidine (0.1 μCi/ml) in the presence of 10⁻⁶ M unlabeled thymidine. All drug treatments (DOX, MRA, or MRA-CN) lasted for 1 h at 37°C. At the end of the incubation period, cells were washed twice with fresh label-free medium and resuspended in fresh medium for the duration of the experiment.

Control and drug-treated cells were exposed to X-rays (300 rads) at 4°C. These cells were then combined with an equal number of [³H]thymidine-labeled L1210 murine leukemia cells that had received no drug treatment but that had been concurrently irradiated (300 rads). These combined cells were then deposited on polycarbonate filters (Millipore Corp., Bedford, MA) in a lysing solution that did not contain SDS and proteinase K (see Refs. 6 and 7). Drug induced DNA-DNA interstrand cross-links and DNA single-strand breaks are expressed as rad equivalents, the amount of X-irradiation that would produce a comparable amount of DNA break frequency (see Refs. 6 and 7).

Electron Microscopy. KBM-3 cells were treated with 10⁻⁴ M MRA-CN, 10⁻⁷ or 10⁻⁸ M MRA, 3 × 10⁻⁴ M DOX, or 10⁻⁶ M actinomycin D for 1 h at 37°C. Aliquots of drug-exposed cells were removed at 0, 2, 4, 8, 12, and 24 h in preparation for examination by electron microscopy.

The cells were rinsed in IMDM and fixed at 2.5% glutaraldehyde in 0.1 M Sorensen’s phosphate buffer. Following three rinses in Sorensen’s phosphate buffer, the cells were stained overnight in dianisobenzidine (1 mg/ml) plus 0.01% hydrogen peroxide for peroxidase cytochemistry. The cells were rinsed again in Sorensen’s phosphate buffer and postfixed in 1% osmium tetroxide for 3 h at 4°C; afterward, the samples were rinsed in distilled water and en bloc stained with 0.5% uranyl acetate overnight at 4°C. The samples were dehydrated in graded percentages of ethanol, infiltrated in graded percentages of EM bed-812 Electromicroscopy Sciences, Fort Washington, PA), and polymerized at 80°C. Thin sections were made on a LKB Ultratome III with a Dupont diamond knife. Sections were poststained with uranyl acetate and Reynolds lead citrate and examined using a JEOL 1200 EX II electron microscope.

RESULTS

Colony Formation. Survival of KBM-3 cells as a function of DOX and MRA-CN concentration is presented in Fig. 1.
MECHANISMS OF MRA-CN VERSUS DOX

Fig. 3. Effects of DOX and MRA-CN on DNA synthesis in human leukemic cells (KBM-3) following a 1-h drug treatment. Results are expressed as IC_{50} for drug-mediated inhibition of [meth-A-3H]thymidine incorporation into DNA. Experimental conditions are described under “Materials and Methods.” Data are the mean ± SD (bars) from three to five separate experiments.

Fig. 4. Effects of DOX and MRA-CN on total RNA synthesis in human leukemic cells (KBM-3) following a 1-h drug treatment. Results are expressed as IC_{50} for drug-mediated inhibition of [G-3H]uridine incorporation into total RNA. Experimental conditions are described under “Materials and Methods.” Data are the mean ± SD (bars) from three to four separate experiments.

removal of cells from exposure to drug. The difference in potency between the two drugs is readily apparent. The extent of time dependent change in DNA and RNA synthesis was drug-dependent. The ratios for the IC_{50} values of inhibitory activity on DNA synthesis at 0 and 4 h after 1 h of drug exposure were 25.1 for DOX and 2.1 for MRA-CN. The ratios for the IC_{50} values of inhibitory activity on total RNA synthesis at 0 and 4 h after 1 h of drug exposure (Fig. 4) were 1.6 for DOX and 20.0 for MRA-CN.

Drug-mediated DNA Interactions. Alkaline elution experiments were performed immediately after treatment of the cells with DOX, MRA, or MRA-CN for 1 h. DNA-DNA interstrand cross-links were produced by MRA-CN treatment (Fig. 5) but not by MRA or DOX. MRA and DOX produced protein-concealed DNA single-strand breaks. A 10-fold enhancement in the single-strand break frequency was observed, however, in the MRA-treated cells compared with the DOX-treated cells (Fig. 6).

Accurate determinations of DNA-DNA interstrand cross-links at more than 2 h after exposure to MRA-CN could not be carried out as the DNA passed through the filters with the lysis solution. In Fig. 7 the time-dependent enhancement of DNA fragmentation (DNA in lysis solution) following treat-
ment of cells with MRA-CN (10 nM, 1 h) is shown. After a 2-h incubation in drug-free medium, MRA-CN induced an increase of DNA fragmentation as assessed by an increase in cellular DNA in the pH 10 lysis fraction (see “Materials and Methods”) prior to alkaline elution. The amount of cellular DNA remaining on the filter after lysis was reduced to about 50% after 4 h, and nearly all cellular DNA was eluted with the lysis solution 24 h after the treatment.

Cell Nucleolar Morphology. Exposure of the KBM-3 cells to MRA-CN, MRA, DOX, and actinomycin D caused distinct changes in their nucleolar morphology, whereas no obvious qualitative differences were visible among other intracellular components and organelles. Nucleolar changes depicted in Figs. 8 to 12 were observed 2 h following drug removal. Exposure to 10^{-8} M MRA-CN, 10^{-6} M MRA, or 10^{-6} M actinomycin D for 1 h produced fully developed nucleolar segregation shortly after treatment (Figs. 8, 9, and 11). The “nucleolar capping” phenomenon consisted of condensation of all nucleoli within a single cell and formation of a smooth, circular outline with loss of the skein-like arrangement of the nucleolonema. In addition, the granular and fibrillar components separated to form individual portions of the nucleolar mass. At least four distinct nucleolar components became apparent: (a) aggregates of particles similar to the granular component of the normal nucleolus; (b) dense clumps of fibrillar material; (c) a light fibrillar or diffuse protein matrix; and (d) electron-dense granular components disposed adjacent to the nucleolonema, both in the nucleolar vacuoles and in the periphery of the segregated nucleoli. This fourth zone has been designated “caps” or nucleolar associated chromatin (8).

Exposure to 3 x 10^{-6} M DOX for 1 h produced nucleolar segregation (Fig. 10) as previously described (9). Nucleoli with distinct separation of the nucleolar components as seen in the MRA-CN-, MRA-, and actinomycin D-treated cells could not be observed in cells treated with DOX; furthermore, the DOX-induced changes occurred at a slower rate compared with those observed after exposure to the morpholinyl-containing compounds and actinomycin D. After treatment for 1 h with a lower dose of MRA than used previously (10^{-8} M), the nucleoli showed no obvious difference from the control (not shown). Recovery from injury induced by any of the drugs could not be observed throughout the 24-h experimental period.

DISCUSSION

The unusual potency of MRA-CN against murine leukemia cell lines was first noted by Acton et al. (1). The present data are the first to demonstrate that this same degree of antitumor potency is also present against a well-characterized human leukemia cell line, KBM-3. Using this sensitive cell line, we explored the biochemical basis for the difference between DOX and MRA-CN with respect to cell survival, inhibition of nucleic acid synthesis, DNA integrity, and nucleolar morphology. Following 1 h of drug exposure, the effects of MRA-CN and DOX on DNA and RNA synthesis were determined in log-phase KBM-3 human leukemic cells. Inhibition of DNA and total RNA synthesis by MRA-CN was 340- and a 2200-fold greater, respectively, than that by DOX (Fig. 2). These data indicate that MRA-CN shows approximately 6- to 7-fold greater specificity in inhibiting total RNA synthesis than DNA synthesis. Our observation is similar to that of Acton et al. (1) who reported that in P388 murine leukemic cells, MRA-CN.
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Fig. 9. Nucleolus 2 h following 1-h exposure to 10⁻⁶ M MRA. Nucleolar segregation is complete. × 23,438.

Fig. 10. Nucleolus 2 h following 1-h exposure to 3 × 10⁻⁶ M DOX. Partial segregation is indicated. Note that the granular component (G) remains sequestered within the fibrillar component (F). × 35,625.

Fig. 11. Nucleolus 2 h following 1-h exposure to 10⁻⁶ M actinomycin D. Fully developed segregation is shown. × 30,938.

Fig. 12. Nucleolus of non-drug-treated KBM-3 cell. Note skein-like arrangement and 3 components of nonsegregated nucleolus: granular component (G), fibrillar component (F), and nucleolar organizer region (N). × 16,500.

produced a 500- and 1000-fold greater inhibition of DNA and RNA synthesis, respectively, than did DOX. This relative selectivity by MRA-CN for inhibition of total RNA synthesis versus DNA synthesis suggested to us that this drug would share certain pharmacological characteristics with class II (nucleolar selective) anthracyclines (9, 10).

The temporal relationships of MRA-CN and DOX with respect to inhibition of nucleic acid synthesis also suggested pharmacological differences between these agents. The extent of inhibition of DNA synthesis in KBM-3 cells increased markedly over the course of 4 h after a 1-h exposure to DOX. There was very little change in MRA-CN IC₅₀ values for DNA synthesis between 1 and 4 h postdrug treatment. There was, however, a 20-fold time-dependent increase in the ratio of the MRA-CN IC₅₀ value of inhibition of total RNA synthesis (Fig. 4). These data indicate that the MRA-CN-mediated effects on total RNA synthesis are as time dependent as the DOX-mediated effect on DNA synthesis, and therefore, these two drugs may interact with nucleic acids in fundamentally different ways.

An alternate explanation for the drug-specific temporal changes observed in DNA and total RNA synthesis may relate to the difference in lipophilicity of MRA-CN and DOX. Although it has been noted that the uptake of anthracyclines is transport mediated (11), it may therefore be responsible for the rapid uptake of MRA-CN (relative to DOX) observed in colon HT-29 cells (14) and hence the rapid effect of this drug in producing inhibition of DNA synthesis. The relatively less lipophilic DOX may require a long incubation time to allow the drug to reach or react with important cellular targets. Although this explanation may account for the early effect of MRA-CN on DNA synthesis observed in KBM-3 cells, it does not adequately explain the time-dependent change in the inhibition of total RNA synthesis, which suggests that secondary drug-mediated effects on RNA synthesis may also be important.

The presence of a cyano group in MRA-CN represents an important structural difference between this compound and DOX. As described by Acton (15) MRA-CN may become "activated" to an active alkylating species by dissociation of the cyano moiety, leaving the reactive iminium ion. One consequence of the chemical reactivity of the α-cyano amine function that has recently been reported by others (3, 4) and is confirmed in the present study is the formation of drug-mediated DNA-DNA cross-links. As shown in Fig. 5, MRA-CN formed detectable DNA interstrand cross-links at concentrations as low as 5 nM. In contrast to MRA-CN, MRA produced no detectable DNA interstrand cross-links yet did result in the expected formation of protein-concealed DNA single-strand breaks qualitatively similar to those produced by DOX. It is therefore the presence of the α-cyano substituent on the morpholinyl moiety that is necessary for the formation of DNA-DNA cross-links in this test.

Extensive fragmentation of cellular DNA was observed shortly after treatment of the cells with MRA-CN, and it increased with time after removal of the drug (Fig. 7). Westendorf et al. (16) have previously noted that MRA-CN is extremely active in inducing DNA repair in primary rat hepatocyte...
cytes. This group has also suggested that quantitatively the yields of MRA-CN-mediated DNA adducts in L1210 and V79 cells are in excellent agreement with the capacity of this drug to induce DNA repair (4). Although the extent of DNA repair was not determined in our studies, the delayed DNA fragmentation in KBM-3 cells after exposure to MRA-CN may reflect increased endonuclease activity or other cellular attempts at reversal of covalent DNA interstrand cross-linking.

The relative specificity of MRA-CN inhibiting total RNA synthesis (a characteristic of class II anthracyclines) led us to examine dose- and time-dependent effects of MRA-CN, MRA, and DOX on KBM-3 nucleolar morphology. As shown in Fig. 8, MRA-CN produced distinct segregation of the nucleolar components that was remarkably similar to that produced by actinomycin D (Fig. 11). The fact that MRA itself produced nucleolar segregation (Fig. 9) similar to that produced by MRA-CN and actinomycin D strongly suggests that it is the presence of the morpholinyl group and not the cyano substituent that is important in producing this effect on total RNA synthesis. This is not unexpected as it is the presence of the two or three sugar side chains on class II anthracyclines that is believed to be responsible for making these compounds relatively nucleolar selective (10). Although reported by others (9) to have an effect on nucleolar morphology, we could not demonstrate either a time- or dose-dependent ability of DOX to produce marked nucleolar segregation similar to that produced by MRA-CN, MRA, or actinomycin D.

The similarity between the effect on nucleolar structure produced by both morpholinyl-containing agents MRA-CN and MRA and that produced by actinomycin D is striking. Actinomycin D and other compounds that induce nucleolar segregation are similar in their ability to bind to DNA with subsequent preferential blocking of the transcription of ribosomal RNA precursor. With respect to anthracyclines, Daskal et al. (9) have shown that DOX affects ribosomal RNA precursor synthesis as well as DNA synthesis and that a class II anthracycline, aclacinomycin A, selectively inhibits ribosomal RNA precursor as opposed to DNA synthesis. Preliminary studies from our laboratory using antibodies that react with human RNA polymerase I complex (17, 18), suggest that one explanation for the effect of MRA-CN on nucleolar structure is a preferential inhibition of RNA polymerase I activity; inhibition of RNA polymerase I activity was evident after treatment of HeLa cells with either MRA-CN or actinomycin D but not DOX. The roles that morpholinyl-containing compounds play with respect to inhibition of specific nucleolar protein and enzyme activities and the relationship, if any, of these interactions with respect to drug-mediated cytotoxicity are currently being explored.

The ability of MRA-CN to produce DNA interstrand cross-links and nucleolar segregation clearly makes this a multimechanistic anthracyline analogue. These biochemical pharmacological properties as well as the demonstrated lack of cardiotoxicity (1, 2) and cross-resistance in a DOX-resistant murine (19) and human (2) cell line suggest that early clinical trials of this unique antitumor agent are warranted.

ACKNOWLEDGMENTS

We wish to thank Dr. Michael Ahearn for his advice and assistance in the preparation of the electron micrographs and Lola Small for the preparation of this manuscript.

REFERENCES

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