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Uptake and retention of morpholinyl anthracyclines by adriamycin-sensitive and -resistant P388 cells

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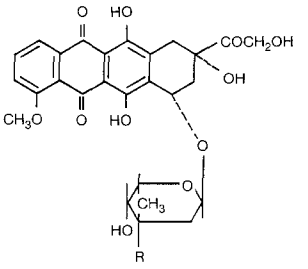
Summary. 3'-Deamino-3'-(4-morpholinyl)adriamycin (MRA) and 3'-deamino-3'(3-cyano-4-morpholinyl)adriamycin (MRA-CN) were compared with adriamycin (ADR) in ADR-sensitive (P388/S) and -resistant (P388/ADR) murine leukemia cell lines with respect to cytotoxicity and cellular accumulation. MRA is only two- to threefold more cytotoxic to P388/S in culture than ADR, whereas MRA-CN is 500-fold more cytotoxic than ADR to this cell line. Yet both MRA and MRA-CN retain their potency against P388/ADR in spite of a 150-fold decrease in potency for ADR. The observed noncross-resistance of both MRA and MRA-CN in P388/ADR correlates with their increased cellular uptake and retention relative to ADR and the inability of P388/ADR to exclude these analogs as readily as it does ADR. The decreased uptake of MRA and MRA-CN in P388/ADR relative to P388/S (1.5 to 2.0-fold), the increased efflux, and the ability of verapamil to enhance cellular uptake of these analogs in P388/ADR, as it does with ADR, all indicate that the mechanism of ADR-resistance effects ADR and the morpholino analogs in a similar manner but to far different extents. The potent cytotoxicity of MRA-CN appears to be related to strong cellular interactions of the drug with macromolecules that are characterized by its nonextraction from cells by chloroform: methanol or 10 *M* urea and may therefore represent covalent binding.

Introduction

The anthracycline antibiotic doxorubicin (Adriamycin, ADR) continues to be an important agent in the chemotherapy of cancer because of its broad spectrum of activity against human tumors [4, 24]. However, its utility also continues to be limited by toxicities, the most prevalent of which is an irreversible, dose-related cardiotoxicity [4, 24], and by the development of an acquired resistance to this agent, a resistance that extends to other types of antitumor agents as well, thus posing a severe problem in the application of alternative therapies [12].

A host of analogs of ADR have been synthesized in attempts to structurally distinguish the desired antitumor effects of ADR from its undesirable properties and to determine the underlying biochemical mechanisms involved [1]. Analogs of ADR and daunorubicin [2, 11], in which a morpholinyl ring was constructed to incorporate the 3'-amino nitrogen of the daunosamine unit (Fig. 1), resulted in highly lipophilic compounds that are more rapidly transported by tumor cells [7] and demonstrate increased antitumor potency both in vitro and in vivo against murine leukemias [2, 11]. The most promising analog of this series, 3'-deamino-3'-(3-cyano-4-morpholinyl)ADR (MRA-CN, Fig. 1) is 100 to 1000 times more potent than ADR against a variety of murine tumors [2, 19], including an ADR-resistant strain of P388. Similar potency is exhibited against various human tumor cell lines in culture [8, 16], including an ADR-resistant human sarcoma. Furthermore, the marked increase in antitumor potency of MRA-CN is unaccompanied by an increase in cardiotoxic potency in murine models [2, 16], indicating a clear separation of cardiotoxic and antitumor actions in this compound, in addition to the observed noncross-resistance to ADR-resistant tumors.



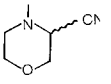
COMPOUND	NSC No.	ABBREVIATION	R
Doxorubicin, Adriamycin	123127	DXR, ADR	$-NH_2$
3'-Deamino-3'-(4 -morpholinyl)-doxorubicin	354646	MRA	
3'-Deamino-3'-(3 -cyano-4 -morpholinyl)doxorubicin	357704	MRA-CN	

Fig. 1. Structures of ADR, MRA, and MRA-CN

In the present studies, the cellular uptake and retention of MRA and MRA-CN are compared with those of ADR in ADR-sensitive (P388/S) and -resistant (P388/ADR)

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cells. We found that increased uptake and retention of both analogs may account for the noncross-resistance of either MRA or MRA-CN in P388/ADR, but that the intense potency of MRA-CN may result from unusual cellular-binding properties of the drug that are characterized by resistance to certain cell extraction methods. Preliminary reports of these findings have been presented [17, 18].

Materials and methods

Chemicals. MRA and MRA-CN were the generous gift of Dr. E. M. Acton (SRI International). Adriamycin·HCl was obtained from Aldrich Chemical, Milwaukee, Wis. Verapamil·HCl was obtained from Knoll Pharmaceutical, Whippany, NJ.

Cell growth studies. Inhibition of cell growth in cultured murine tumor cells by test compounds was carried out as previously described [19]. P388/ADR was obtained from the DCT tumor repository, NCI, Frederick Cancer Research Facility, Frederick, Md. P388/ADR is derived from the ADR-sensitive P388 line (P388/S) and its drug responsiveness has been described [5, 6]. Both strains are maintained in continuous culture in RPMI-1640 supplemented with 10% heat-inactivated calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml), and 25 mM HEPES buffer (pH 7.2). P388/ADR has been maintained in this manner for up to 5 months without loss of ADR resistance.

Drug uptake and efflux. The uptake of drugs was determined in cell cultures containing 10^6 cells/ml and 1 μ*M* drug incubated at 37 °C for up to 4 h. At various times, 2×10^6 cells were harvested from duplicate cultures by centrifugation and the cells were washed free of drug-containing media by resuspending them three times in phosphate-buffered saline (PBS). The washed cell pellets were stored in ice prior to extraction. For drug efflux determinations, cells incubated with 1 μ*M* drug for 4 h were washed as described above, then resuspended in drug-free media at 10^6 cells/ml and reincubated. Aliquots of 2×10^6 cells were harvested at 0, 1, 2, 4 and 20 h postincubation and washed in PBS. Cell extractions were performed by one of three methods: with either chloroform:methanol (3:1, v/v) or 0.3 N HCl:50% ethanol with 3 ml of solvent at 0° or 37 °C for the periods of time indicated. The cell suspensions were centrifuged to remove cell debris and the supernates were retained for fluorescence analysis. Extraction with 10 *M* urea was performed by solubilizing the cell pellet in 0.2 ml of the reagent and incubating for 1 h at 37 °C. Following the incubation period, the urea solution was extracted twice with 0.5 ml chloroform, the phases were separated by centrifugation at 12,000 *g* for 1 min and the chloroform extracts combined. Insoluble cell debris accumulated at the phase interface and was re-extracted with 3 ml of 0.3 N HCl:50% ethanol for 30 min at 37 °C to recover any fluorescent material not extracted by the urea treatment. The chloroform extract was divided into two equal aliquots and evaporated to dryness. One aliquot was dissolved in 3 ml of 0.3 N HCl:50% ethanol for total fluorescence analysis and the second was dissolved in 0.1 ml of acetonitrile for HPLC analysis. Total recovery of drug from the urea incubations by chloroform extraction was 75% for MRA-CN and 85% for MRA.

Total fluorescence of cell extracts was assessed using an Aminco-Bowman spectrophotofluorometer at an excitation wavelength of 470 nm and an emission wavelength of 580 nm. Recoveries are expressed as molar equivalents of the parent drug and represent the means of duplicate determinations, with a coefficient of variation of $< 10\%$. HPLC analysis was performed using a 4.6×150 mm column of Altex Ultrasphere octyl [13]. The mobile phase was 60% methanol:40% 0.05 *M* citrate buffer (pH 4.0):2.5% tetrahydrofuran at a flow rate of 15 ml/min. Fluorometric detection was performed using an Aminco-Bowman spectrophotofluorometer at the wavelengths described above.

Results

Cell growth inhibition

The relative effectiveness of ADR, MRA, and MRA-CN for inhibiting cell growth of P388/S and P388/ADR are compared in Table 1. MRA is only about 2 to 3 times more cytotoxic to P388/S than ADR, but MRA-CN is about 500 times more cytotoxic to this strain than ADR. However, both morpholinyl analogs retain their potency against P388/ADR, in contrast to the 158-fold decrease in potency for ADR. The resistance of this strain to ADR is greater than we had observed with an earlier passage of P388/ADR (RI = 58; see [19]) and probably accounts for differences in the observed cytotoxicities of MRA and MRA-CN in the two studies.

Drug uptake and efflux

Figures 2 and 3 depict the uptake and efflux of the three compounds from both P388/S and P388/ADR cells extracted with 0.3 N HCl:50% ethanol. It is apparent in Fig. 2 that both MRA and MRA-CN are more rapidly and extensively transported by the sensitive strain than is ADR. In particular, MRA attains a maximal level (500 pmol/10^6 cells) of cell-associated drug that represents 50% of the available drug. There is also a very immediate and appreciable (~ 120 pmol/10^6 cells) cell-drug association observed with MRA at the zero sampling time, prior to incubation, that is not apparent with ADR or MRA-CN. The maximal level attained by MRA at 2 h decreases rapidly to about the same level attained by MRA-CN at 4 h. Following removal of drug from the media, both MRA and MRA-CN are equally well retained by P388/S, at least up to 4 h after drug removal, whereas ADR decreases by about 50% during that time. However, extensive loss of drug-derived fluorescence takes place in both MRA- and MRA-CN-treated cells during the subsequent

Table 1. Growth inhibition of P388/S and P388/ADR by ADR, MRA, and MRA-CN

Drug	IC_{50}, μM		
	P388/S	P388/ADR	RI[a]
ADR	0.076	12	158
MRA	0.031	0.047	1.5
MRA-CN	0.00015	0.00023	1.5

[a] Resistance index $= \frac{\text{P388/ADR}}{\text{P388/S}}$

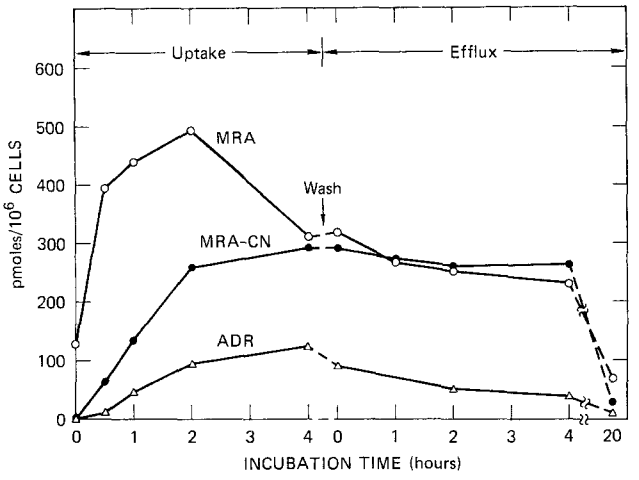

Fig. 2. Uptake and efflux of ADR, MRA, and MRA-CN in P388/S. Cells were extracted with 0.3 N HCl:50% ethanol for 30 min at 37 °C

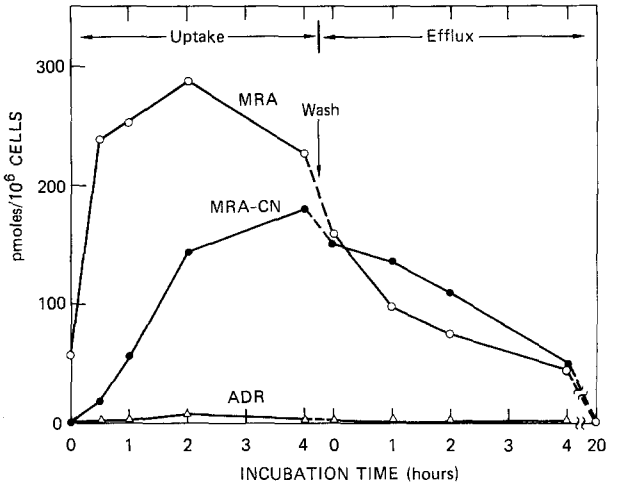

Fig. 3. Uptake and efflux of ADR, MRA, and MRA-CN in P388/ADR. Cells were extracted with 0.3 N HCl:50% ethanol for 30 min at 37 °C

Table 2. Effect of verapamil on drug uptake in P388/ADR

Drug	Drug uptake (pmol/10^6 cells)[a] Verapamil conc. (μM)		
	0	2.2	6.6
ADR	9	21 (2.3)[b]	36 (4.0)
MRA	331	527 (1.6)	619 (1.9)
MRA-CN	228	349 (1.5)	353 (1.5)

[a] Treated with 1 μ*M* drug for 4 h
[b] Factor of increase over control value

16 h; MRA fluorescence decreased to 75 pmol/10^6 cells and MRA-CN fluorescence decreased to 30 pmol/10^6 cells. This decrease is not due to cell destruction because the cells are nearly 100% viable at this point, as judged by trypan blue dye exclusion.

As shown in Fig. 3, the uptake of ADR by P388/ADR is less than 10% of that attained in P388/S, but the rate and extent of MRA and MRA-CN uptake are still at least 50% of those observed in P388/S. The initial association of MRA with P388/ADR at the zero time sampling (66 pmol/10^6 cells) is also noticeably less than that observed with P388/S, suggesting that it represents a true cell-drug association rather than nonspecific background fluorescence. The rate of efflux of both MRA and MRA-CN is noticeably greater than was observed in P388/S. Intracellular levels of both drugs decrease about threefold in the initial 4 h of the efflux phase – from 150 to 50 pmol/10^6 cells. Cell-associated drug fluorescence continues to fall – to undetectable levels at 20 h in both MRA- and MRA-CN-treated cells. However, this may be the result of cell destruction because these cells are < 10% viable at this time.

The effects of verapamil on drug uptake are summarized in Table 2. Verapamil, a calcium antagonist that increases the cytotoxicity of ADR to P388/ADR, reportedly by increasing cellular retention of the drug [5, 6], stimulated ADR uptake fourfold at a concentration of 6.6 μ*M*. The uptake of MRA and MRA-CN are also increased by verapamil, to levels at least equivalent to those attained in

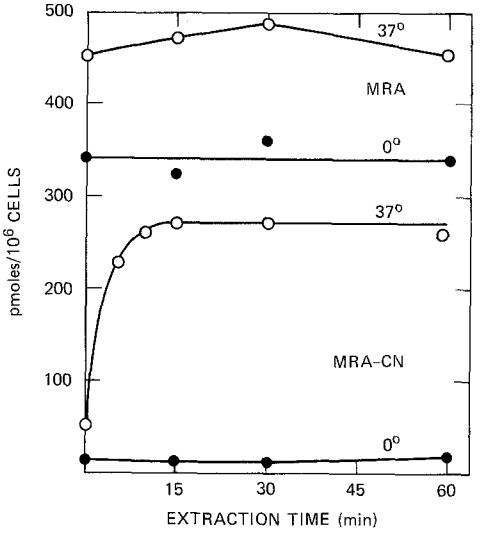

Fig. 4. Extraction of MRA and MRA-CN from P388/S by 0.3 N HCl:50% ethanol. Cells were incubated with 1 μ*M* drug for 2 h

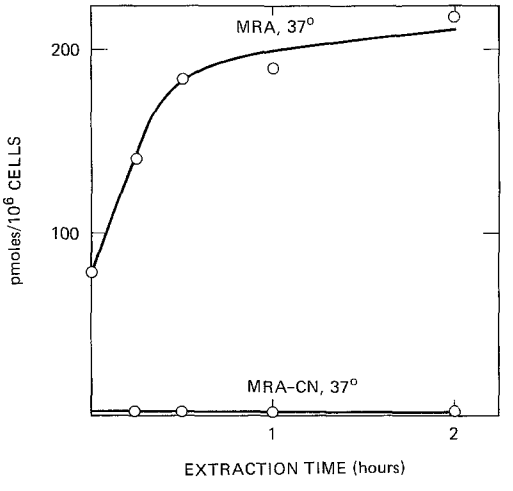

Fig. 5. Extraction of MRA and MRA-CN from P388/S by chloroform:methanol (3:1, v/v). Cells were incubated with 1 μ*M* drug for 2 h

Table 3. Extraction of drug-treated P388/S cells[a] by HCl:Ethanol vs chloroform:methanol

Drug	pmoles/10^6 cells			
	HCl/ Ethanol	$CHCl_3$/ Methanol[b]	$CHCl_3$/ Methanol resistant	(%)
ADR	41	33	8	(20)
MRA	356	261	95	(27)
MRA-CN	220	<10	>210	(>95)

[a] Treated with 1 μ*M* drug for 2 h
[b] Cells extracted for 1 h at 37°C
[c] Percent of HCl:ethanol-extractable material resistant to chloroform:methanol extraction

P388/S (Fig. 2). However, the factors of increase are less than that for ADR, being 1.9-fold for MRA and 1.5-fold for MRA-CN.

Cellular binding properties

MRA and ADR are readily extracted from drug-treated cells by 0.3 N HCl:50% ethanol (Figs. 2 and 3), a standard extraction method for the fluorometric analysis of anthracyclines [3, 7]. Figure 4 illustrates that the recovery of MRA from P388/S cells is somewhat temperature-dependent, being 25%–30% less at 0° than at 37 °C, but is independent of incubation time at either temperature. In contrast, the recovery of fluorescence from MRA-CN-treated cells is both time- and temperature-dependent. The recovery at 0 °C is negligible up to at least 60-min incubation, but the recovery at 37 °C is maximal after only about 15 min of incubation.

The difference in extractability of fluorescence from MRA vs MRA-CN-treated cells is also apparent when chloroform:methanol (3:1, v/v) is employed as the extraction solvent (Fig. 5). Extraction of MRA by this method is time-dependent at 37 °C but is nearly maximal by 30 min. MRA-CN-treated cells exhibit no recoverable fluorescence under these conditions, even up to 2 h of incubation.

The relative extractability of ADR, MRA, and MRA-CN by the two solvent extraction methods is shown in Table 3. Identical cell samples from drug-treated cultures were extracted by each of the two solvent mixtures under the same incubation conditions. For ADR and MRA, only 20% and 27%, respectively, of the fluorescence equivalents recoverable by HCl:ethanol extraction are not recoverable by chloroform:methanol extraction (chloroform:methanol-resistant). The recovery of ADR is consistent with the report of Bachur et al [3] that chloroform:methanol is less effective than HCl:ethanol in the extraction of daunomycin from tissue homogenates, as determined by total fluorescence. However, nearly all of the HCl:ethanol-recoverable drug-fluorescence from MRA-CN-treated P388/S cells is resistant to chloroform:methanol extraction. Drug fluorescence from MRA-CN-treated P388/ADR cells is also nonextractable by chloroform:methanol (data not shown).

MRA-CN-associated drug fluorescence is also resistant to extraction from drug-treated cells by a more stringent method – treatment of the cells with 10 *M* urea at 37 °C for 1 h (Table 4). Although MRA-asociated drug fluorescence

Table 4. Extraction of drug-treated p388/S cells[a] by 10 *M* urea vs HCl:ethanol

Compound	pmoles/10^6 cells[b]		
	HCl:ethanol	10 *M* Urea	HCl:ethanol following 10 *M* urea
MRA	337	327	<10
MRA-CN	204	<50	170

[a] Treated with 1 μ*M* drug for 2 h
[b] Cells extracted for 1 h at 37°C

recoverable by this treatment is about equal to that with HCl:ethanol extraction, <25% of the HCl:ethanol-extractable fluorescence in MRA-CN-treated cells is recoverable by 10 *M* urea treatment. The urea-extractable fluorescence from MRA-treated cells was totally recovered as the parent compound by HPLC analysis, and no aglycone products were observed. No fluorescent components were detectable in the urea extracts from MRA-CN-treated cells. Re-extraction of the insoluble cell debris from MRA-CN-treated cells by HCl:ethanol *following* 10 *M* urea treatment established that at least 83% of the HCl:ethanol extractable fluorescence remains associated with the cell debris following the urea treatment.

Discussion

In a previous study [7] comparing the cellular pharmacology of various morpholinyl and methoxypiperidinyl derivatives of daunorubicin in human colon carcinoma cells in vitro, a correlation was obtained between cellular accumulation and the lipid solubilities of various analogs. No correlation was observed, however, between cellular accumulation and the cytotoxicities of the compounds. The present studies support these findings to the extent that the highly lipophilic morpholinyl analogs, MRA and MRA-CN [2], are both more rapidly transported and more effectively retained by P388 cells than is ADR, most notably in P388/ADR. Despite this fact, cellular accumulation alone does not explain the differences in cytotoxicity of these compounds. Although the initial uptake of MRA is ~5 times that of ADR in P388/S and the subsequent retention is likewise greater, the cytotoxicity of MRA to P388/S is not significantly different from that of ADR (two- to threefold). The lack of correlation between drug uptake and cytotoxicity is more evident when MRA and MRA-CN are compared. The maximal uptake of MRA in P388/S cells is nearly twice that of MRA-CN and the compounds are equally well retained, but the cytotoxicity of MRA is nearly 200-fold less than that of MRA-CN. Cellular accumulation could not be a significant factor in determining relative cytotoxicities when the effective drug concentrations differ by so large a factor. The time of drug exposure is also not a significant determinant of cytotoxicity with either of these compounds because the potency of both MRA and MRA-CN is reduced <10-fold by reducing drug exposure time from 48 h to 1 h [19].

The differences in cytotoxic potencies of the three compounds is most evident in P388/ADR where ADR potency is reduced 158-fold whereas that of MRA and MRA-CN remains relatively unchanged. The differences

in uptake of MRA and MRA-CN compared with ADR are also more extreme in P388/ADR, where ADR uptake is reduced to < 10 pmol/10^6 cells, but MRA and MRA-CN attain levels of 285 and 175 pmol/10^6 cells, respectively (Fig. 3). The resistance of this cell line to ADR has been attributed to the decreased accumulation of the drug resulting from either "active efflux" of the drug [5, 6] or a diminished cellular drug-binding capacity [23]. The decreased accumulation of MRA and MRA-CN in P388/ADR (Fig. 3) relative to P388/S (Fig. 2) is apparent from the reduced levels attained during the uptake phase and the increased rate of elimination during the efflux phase. Thus, the resistance mechanism that operates in these cells to reduce cellular ADR to ineffective levels also affects MRA and MRA-CN accumulation, although to a much lesser extent. The ability of verapamil to block this mechanism and thereby enhance the cellular accumulation of ADR in P388/ADR [14, 20] was also observed in these studies; 6.6 μM verapamil produced a fourfold enhancement of ADR accumulation in P388/ADR (Table 2). MRA and MRA-CN uptake was also somewhat enhanced by verapamil (1.5- to 1.9-fold) to levels at least equivalent to those attained in P388/S (Fig. 2). We had previously observed, however [19], that verapamil does not significantly enhance the cytotoxicity of MRA or MRA-CN in P388/ADR as it does that of ADR. The modest effects of verapamil on both the uptake and cytotoxicity of the morpholinyl analogs in P388/ADR compared with the more pronounced effects on ADR reflect the lack of cross-resistance of MRA and MRA-CN in P388/ADR and suggest the possibility that the uptake of these compounds is already sufficient to saturate cellular targets for growth inhibition in both strains to the extent that further enhancement of drug uptake by verapamil does not significantly enhance cytotoxicity. It must be considered, however, that drug transport and the effects of verapamil on this process may be different at concentrations where MRA-CN is cytotoxic (0.1 to 1.0 nM), compared with the effects of verapamil observed in the present studies performed with 1 μM drug.

In any case, it is apparent that the intense potency of MRA-CN relative to ADR or MRA in either cell line must result from unique properties of the compound in addition to increased cellular retention. One property of MRA-CN that distinguishes it from ADR or MRA is the complete lack of basicity at the morpholinyl nitrogen [2]. The presence of an ionizable amino nitrogen in the daunosamine moiety of anthracyclines may be an important factor for cellular retention and binding to intracellular sites [15]. This may be the case for MRA, a compound in which a high degree of lipophilicity favorable to membrane transport is combined with a basic morpholinyl nitrogen that may become protonated under the slightly acidic conditions of intracellular pH and subsequently bind to intracellular sites. MRA-CN, however, retains its neutrality and hence its lipopohilicity, regardless of pH [2]. This may affect the subcellular distribution of MRA-CN relative to MRA and hence the sites of cellular interaction. The inability of MRA-CN to ionize to a charged species also reduces the likelihood that simple electrostatic interactions are involved in the intracellular binding of the drug and that other forms of interaction must occur. The possibility that MRA-CN may interact covalently with biological molecules is indicated by the observation that MRA-CN binds irreversibly to isolated calf thymus DNA [10] and by the suggestion that induction of unscheduled DNA synthesis (UDS) in rat hepatocytes [22] and the promotion of DNA-DNA cross-links in L1210 and V79 cells [21] by MRA-CN and MRD-CN indicate covalent interactions with DNA. MRD was active only in the UDS assay, indicating a need for metabolic activation with this compound.

Our finding that MRA-CN (or its metabolites) is resistant to extraction from P388 cells under conditions which ADR and MRA are readily extractable indicates that MRA-CN is bound in the cell in a different manner from these agents. In particular, the nonextractability of MRA-CN by 10 M urea strongly suggests that the interaction is covalent in nature. The binding appears to be reversible, however, judging from the eventual decrease in cell-associated drug fluorescence after 20 h in drug-free media (Fig. 2 and 3). The ability of MRA-CN, but not MRA, to form covalent adducts in P388 cells correlates with the ability of MRA-CN and MRD-CN, but not MRD, to promote DNA-DNA cross-links in L1210 and V79 cells [21] and suggests a possible relationship between the two phenomena. One hypothesis that has been advanced regarding possible covalent interactions of MRA-CN pertains to similarities between it and the antitumor antibiotic saframycin A [8]. Like MRA-CN, saframycin A contains a cyano group α to a heterocyclic N. Loss of the CN group results in the formation of a highly reactive cation capable of alkylating DNA or other biological nucleophiles [9]. The observed binding of saframycin to DNA was reversible by heat and lower pH conditions, which is consistent with the temperature-dependent, acid extractability of MRA-CN observed in these studies.

Studies are in progress to improve detectablity of MRA-CN and its possible metabolites so that the biochemical pharmacology of this unique compound can be studied at levels more compatible with cytotoxic drug levels, thus permitting determination of the important cytological effects of the drug.

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