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Comparative cytotoxicities of various morpholinyl anthracyclines

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Summary. *A series of quinone- and sugar-modified analogs of adriamycin have been tested for growth inhibition of adriamycin-sensitive (P388/S) and -resistant (P388/ADR) sublines of P388 murine leukemia cells in vitro. P388/ADR is less resistant to analogs of adriamycin containing either a 3′-deamino-3′-(4″-morpholinyl) group, MRA; or a -(3″-cyano-4″-morpholinyl) group, MRA-CN, than to adriamycin. However, MRA-CN was the most potent growth inhibitor of either subline. This potency is reduced by either modification of the quinone unit with a 5-imino substituent or restriction of the cyano-morpholinyl ring by an oxygen bridge to the daunosamine sugar. The calcium antagonist verapamil substantially increases the cytotoxicity of adriamycin to P388/ADR but has no appreciable effect on the cytotoxicity of either MRA or MRA-CN. The results suggest that increased uptake and retention by both MRA and MRA-CN may contribute to their increased cytotoxicity, but that the intense potency of the cyano-morpholinyl analogs must be due to other unique properties of these compounds.*

Introduction

The search for less toxic and more potent analogs of the antitumor antibiotic doxorubicin (adriamycin) has led to the development of analogs of doxorubicin and daunorubicin in which a morpholino ring incorporating the amino nitrogen of the daunosamine unit has been constructed. The resultant derivatives, 3′-deamino-3′-(4″-morpholinyl)doxorubicin (MRA) and 3′-deamino-3′-(4″-morpholinyl)daunorubicin (MRD) are 5- to 10-fold more cytotoxic in vitro to murine L1210 cells than are the parent compounds. In addition, MRD was 40-fold more potent than doxorubicin to P388 tumors in vivo [13]. It was subsequently found that a substituted morpholino derivative, 3′-deamino-3′-(3″-cyano-4″-morpholinyl)doxorubicin (MRA-CN) or 3′-deamino-3′-(3″-cyano-4″-morpholinyl)daunorubicin (MRD-CN) was formed as a by-product of MRA and MRD synthesis and that MRA-CN was 100–1,000 times more potent than doxorubicin against various mouse tumors in vivo [4, 5] and against human HT-29 colon carcinoma cells in vitro [10]. This marked increase in antitumor potency is not accompanied by any observed effects on myocardial lesion production in mice [5], in contrast to doxorubicin, indicating that the mechanism(s) of antitumor activity have been separated from those associated with cardiotoxicity in this compound.

The present studies were undertaken to compare the cytotoxicity of MRA-CN and a number of other closely related analogs with that of doxorubicin in doxorubicin-sensitive (P388/S) and -resistant (P388/ADR) murine cell lines in an attempt to identify chemical and biochemical properties of the molecule that are responsible for this potent antitumor activity.

Materials and methods

Chemicals. All anthracycline analogs used in these studies were synthesized and characterized by methods previously published [3, 5, 13]. Verapamil · HCl was obtained from Knoll Pharmaceutical Co., Whippany, NJ.

Cell growth inhibition. The inhibition of cell growth in cultured murine tumor cells was used as a measure of the relative in vitro potency of the test compound using an adriamycin-sensitive strain of murine P388 leukemia (P388/S) obtained from American Type Culture Collection and an adriamycin-resistant strain [7, 8, 17] of P388 (P388/ADR) obtained from the DCT tumor repository, NCI, Frederick Cancer Research Facility, Frederick, Md. Both strains are maintained in continuous culture in RPMI-1640 medium supplemented with 10% heat-inactivated calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml), and 25 m*M* HEPES buffer, pH 7.2. For drug treatment studies, 0.9-ml aliquots of culture medium containing 5×10^4 exponentially growing cells were transferred to 24-well Costar plates (Hyclone, Logan, Utah), and 0.1-ml aliquots od drug-medium dilutions were then added. Samples were run in duplicate. For continuous drug exposure studies the plates were incubated at 37° C in a humidified atmosphere for 48 h and then counted with a Coulter counter. For limited drug exposure studies, the cell-drug suspensions were incubated in 15-ml conical tubes (Falcon No. 2095) at 37° C for 1 h, followed by centrifugation at 800 *g* for 5 min to remove the drug-containing medium. The cells were washed once with 1 ml fresh medium, resuspended in 1 ml drug-free medium, and transferred to 24-well Costar plates. The plates were incubated for 48 h and counted as before. Cytotoxic potency was measured by graphically determining the IC_{50} value for each drug (concentration causing 50% inhibition of cell growth). In experiments to study the effect of verapamil on drug cytotoxicity, verapamil and the drug were added simultaneously to cells at time 0 and incubated for 48 h.

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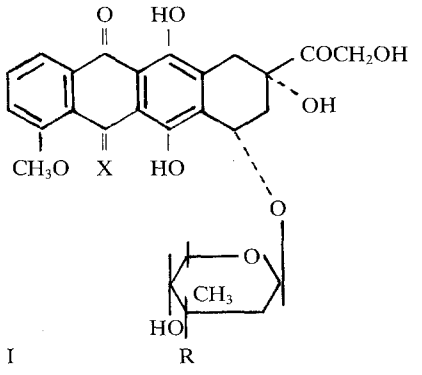
Results

The structures of the compounds tested are described in Table 1 and are of two basic types. Modifications at R involve replacement of the NH_2 group of daunosamine with a morpholino or substituted morpholino ring. The X modification involves the substitution of an imino group at C5 of the quinone ring. The latter substitution reduces the oxygen-radical-producing capacity of both doxorubicin and daunorubicin [1, 2, 6, 11] and also the cardiotoxicity of the daunorubicin derivative [1, 14]. The antitumor potency of doxorubicin is also reduced by this modification (ImA) [1, 2]. The 5-imino substitution was therefore included in the present comparisons to determine its effects in combination with the sugar-amine modifications.

Continuous exposure of P388/S to the test compounds in 48-h cultures produced a wide range of potencies, illustrated in Fig. 1. The IC_{50} for ADR is 10^{-7} *M* under these conditions, and this potency is increased ten-fold by the morpholino substitution (MRA, IC_{50} 10^{-8} *M*). The inclusion of a cyano substituent in the 3-position of the morpholino ring (MRA-CN) produced a 1,000-fold increase in potency over ADR ($IC_{50} \cong 10^{-10}$ *M*), similar to the marked cytotoxicity of this analog observed in other tumor cell assays [5, 10]. The presence of an oxygen bridge between the morpholino ring and the sugar unit, which restricts the rotation of the morpholino ring, negates the effect of the cyano substituent ($IC_{50} = 2 \times 10^{-8}$), whereas modification of the quinone moiety in MRA-CN with a 5-imino substituent (ImMRA-CN) produces a compound with potency intermediate between MRA

Table 1. Structures of doxorubicin and related anthracyclines



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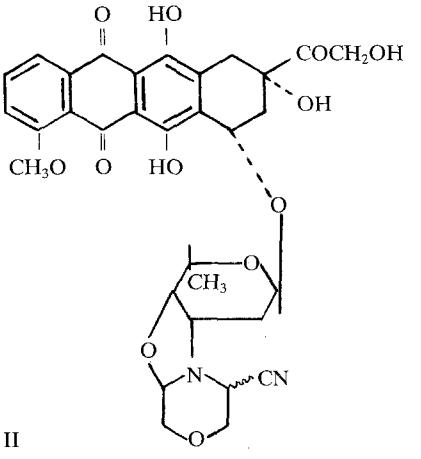
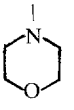
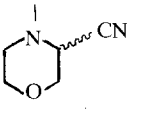
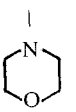
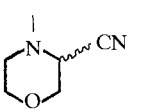


II

Compound	NSC No.	Abbreviation	X	R
Doxorubicin, Adriamycin	123127	DXR, ADR	= O	$-NH_2$
5-Iminodoxorubicin	332988	ImA	= NH	$-NH_2$
3′-Deamino-3′-(4″-morpholinyl)-doxorubicin	354646	MRA	= O	
3′-Deamino-3′-(3″-cyano-4″-morpholinyl)doxorubicin	357704	MRA-CN	= O	
4′,5″-Anhydro-3′-deamino-3′-(3″-cyano-5″-hydroxy-4″-morpholinyl)doxorubicin	373232	O-MRA-CN		See II above
5-Imino-3′-deamino-3′-(4-morpholinyl)doxorubicin	355277	ImMRA	= NH	
5-Imino-3′-deamino-3′-(3″-cyano-4″-morpholinyl)-doxorubicin	365171	ImMRA-CN	= NH	

and MRA-CN ($IC_{50} = 10^{-9}$ *M*). The 5-imino substitution has a similar effect on reducing the potency of MRA (ImMRA, $IC_{50} = 2 \times 10^{-7}$ *M*).

The cytotoxic potency of these compounds was also determined after only 1 h of exposure of cells to drugs followed by 48 h reincubation of cells in drug-free medium. From the ratios of the IC_{50} values for 1-h and 48-h exposures (Table 2), it is apparent that 1 h of exposure reduces the cytotoxic potency of the cyano-morpholino analogs MRA-CN, O-MRA-CN, and ImMRA-CN to a much lesser extent than that of the other analogs, viz. ADR, MRA, and ImMRA. This could result from increased uptake and cellular retention of the cyano-morpholino analogs, thus reducing the need for continuous drug exposure to obtain maximum cytotoxic potency.

To examine this possibility, we compared the growth inhibition of these analogs on P388/S and the doxorubicin-resistant strain of P388, P388/ADR (Table 3). The P388/ADR strain is resistant to ADR both in vitro and in vivo due to decreased drug uptake and retention resulting from either active efflux of the drug [7, 8] or a diminished cellular drug-binding capacity [17]. The effects of ADR and its analogs were compared following 48-h exposure of P388/S and P388/ADR to the drugs. The relative resistance to each drug was then determined as the ratio of the IC_{50} values for P388/ADR and P388/S (resistance index). The results (Table 3) demonstrate that of all the analogs tested, ADR is the least cytotoxic to P388/ADR and is at least 50 times less cytotoxic to this strain than to P388/S under these conditions. In contrast, the cytotoxicity of MRA-CN is reduced only five-fold in P388/ADR relative to P388/S, and it is consequently almost 10,000 times more cytotoxic to P388/ADR than ADR. In fact, all the morpholino analogs, with the possible exception of ImMRA-CN, had significantly lower resistance indices than ADR. Because the resistance of P388/ADR to ADR has been attributed to decreased drug retention, perhaps the decreased resistance of this strain to the morpholino-containing analogs, particularly MRA-CN, is the result of increased retention of these drugs.

This interpretation is supported by studies of the effects of the calcium antagonist verapamil on the cytotoxicities of these compounds. Verapamil has been shown to increase the cytotoxicity of ADR and various vinca alkaloids to P388/ADR by increasing the retention of these drugs [15, 16]. At nontoxic doses of 2.2 and 6.6 μ*M*, verapamil significantly enhanced the cytotoxicity of ADR to P388/ADR by factors of 5.0 and 17, respectively (Table 4). A slight enhancement of the cytotoxicity to P388/S was also observed at the higher verapamil concentration (2.5-fold). In contrast, only a very slight, dose-dependent effect of verapamil on MRA cytotoxicity to P388/ADR was observed, and there was virtually no effect on MRA-CN cytotoxicity to P388/ADR. It appears that the mechanism(s) of ADR resistance that operate in P388/ADR, and are partially overcome by verapamil, have little effect on either MRA or MRA-CN. If the principal mechanism of resistance indeed involves decreased drug retention, then the decreased resitance of P388/ADR to these analogs and the inability of verapamil to further enhance their cytotoxicity is most likely a result of their increased cellular retention relative to ADR.

Discussion

A previous study [5] comparing various chemical and biological properties of the analogs examined in the present study

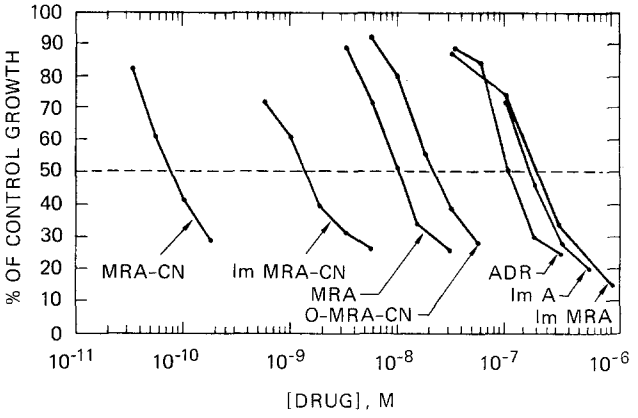


Fig. 1. Inhibition of P388/S cell growth by ADR and analogs (48 h exposure)

Table 3. Growth inhibition of P388/S and P388/ADR by ADR and analogs (48-h exposure)

Drug	IC_{50}, μ*M*		
	P388/S	P388/ADR	RI[a]
ADR	0.14	8.0	57
MRA	0.011	0.15	14
ImMRA	0.20	2.5	13
MRA-CN	0.00020	0.0010	5.0
O-MRA-CN	0.021	0.12	6.7
ImMRA-CN	0.0014	0.044	31

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

Table 2. Growth inhibition of P388/S by ADR and analogs. Effects of 1-h vs 48-h exposure

Compound	IC_{50}, μ*M*		
	1 h	48 h	1 h/48 h
ADR	1.6	0.11	15
MRA	0.10	0.011	9.1
ImMRA	3.3	0.20	17
ImMRA-CN	0.0054	0.0014	3.9
MRA-CN	0.00060	0.00017	3.5
O-MRA-CN	0.20	0.054	3.7

Table 4. Effect of verapamil on drug cytotoxicity to P388/S and P388/ADR

Drug	Cytotoxicity (IC_{50}, μ*M*)				
	P388/ADR + Ver (μ*M*)			P388/S + Ver (μ*M*)	
	0	2.2	6.6	0	6.6
ADR	11	2.2	0.64	0.13	0.052
MRA	0.10	0.090	0.060	–	–
MRA-CN	0.0015	0.0015	0.0010	0.00023	0.00022

demonstrated that the compounds containing a morpholino ring are markedly more lipophilic than the parent drugs adriamycin and daunomycin, regardless of other substituents. The differences in log p values at neutral pH between the morpholinyl and cyano-morpholinyl analogs were therefore inadequate to explain the more potent cytotoxicities of several compounds in the latter group to L1210 cells. Our studies demonstrate that the relative cytotoxicities of these compounds to P388 cells are similar to those to L1210 cells and that MRA-CN is clearly the most potent analog of the series. On the basis of studies comparing ADR and morpholinyl-daunorubicin [9], increased cellular transport appears to result from the increased lipophilicity of the morpholinyl group, and we therefore might expect increased uptake to be a property common to all the morpholinyl-anthracyclines, regardless of other substituents. This possibility is supported by the data comparing the sensitivities of P388/S and P388/ADR with these compounds (Table 3). If the observed resistance of P388/ADR to ADR is the result of decreased uptake and retention of ADR by this cell line, then the decreased resistance of P388/ADR to virtually all the morpholinyl and cyano-morpholinyl analogs tested is most likely the result of increased uptake and retention of these compounds relative to ADR. This interpretation is further substantiated by the failure of verapamil to increase the cytotoxicity of both MRA and MRA-CN to P388/ADR. If the marked increase in cytotoxicity of ADR to P388/ADR caused by verapamil (Table 4) is the result of increased uptake and retention of ADR as previously determined [16], then the lack of an effect by verapamil on the cytotoxicities of MRA and MRA-CN is probably due to their already increased uptake and retention relative to ADR.

The increased cytotoxicity of MRA-CN relative to ADR and MRA most likely results from unique properties of the compound other than merely increased cellular uptake and retention. Such properties might include alternative mechanisms of binding to DNA that are a function of the cyano substituent or even binding to alternative cellular sites (i.e., proteins, membrane lipids) that represent more lethal, less reversible cytotoxic events to the cell. This could explain the potent cytotoxicity of the cyano-morpholinyls on even brief exposure to cells (Table 2). One property of the cyano-morpholinyl analogs that distinguishes them from the corresponding morpholinyl analogs is the complete lack of basicity at the morpholino nitrogen of the cyano compounds [4]. As a result, the lipophilicity of the cyano-morpholinyls is maintained at an acid pH, whereas protonation of the morpholino nitrogen decreases the log p value in the morpholinyl analogs. Because under physiological conditions intracellular pH is lower than extracellular pH (as low as pH 4 in lysosomes), the more lipophilic nature of the cyano-morpholinyls in this circumstance could affect the subcellular distribution and retention of the cyano analogs relative to the noncyano analogs, as well as their intracellular binding. A second hypothesis that has been proposed regarding possible unique properties of the cyano-morpholinyls pertains to similarities between MRA-CN and Saframycin A [10]. Like MRA-CN, Saframycin A contains a cyano group α to a heterocyclic N, and loss of the CN can form a highly reactive cation capable of alkylating DNA or other biological nucleophiles [12], thus offering a unique binding mechanism to these types of compounds.

The effects of other modifications of MRA and MRA-CN on the in vitro cytotoxic potencies of these compounds provides further insight into the important structural parameters involved. Amination of the quinone from MRA → ImMRA and MRA-CN → ImMRA-CN significantly reduces the cytotoxic potency of both compounds, indicating that maintenance of the redox potential of the quinone ring is somehow important to the overall cytotoxicity of these compounds. The oxygen-bridged compound O-MRA-CN, with the consequent rigidity and restriction of the morpholino ring, is of particular interest for comparison with MRA-CN. The potency of this compound is comparable with that of the unsubstituted morpholinyl, MRA (Fig. 1). Yet O-MRA-CN retains other characteristics of MRA-CN, such as being nearly as potent on 1-h as on 48-h exposure to P388/S (Table 2) and exhibiting a very low resistance index for P388/ADR vs P388/S (Table 3).

These closely related analogs should provide useful tools for investigating the properties of the cyano-morpholinyl anthracyclines that contribute to their potent antitumor activity. Studies are presently under way in this laboratory to compare the uptake and retention of these compounds in P388/S and P388/ADR and their association with various subcellular fractions in these cells.

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