the latter step which appears to be most deficient in species whose adrenals are not affected by SL. Although some species differences in the rates of SL deacetylation were noted (Table 2), the differences in 7α-thio-SL metabolism were far greater. In addition, it appears to be the latter process which is organ-specific and which accounts for the tissue specificity of SL actions on cytochromes P-450 [17].

Menard *et al.* [9, 10] previously proposed that the steroid 17α-hydroxylase was involved in the activation of SL. They recognized that the destruction of adrenal cytochromes P-450 by SL occurred only in those species whose adrenals produced 17-hydroxylated steroids. Our observations lend support to their hypothesis by demonstrating that the key step in the activation process occurs in adrenals from 17-hydroxylating species (guinea pig, dog) but not in those from other rodents (rat, rabbit). Since the activity of the 17α-hydroxylase decreases as a result of SL activation [9, 10], suicide inhibition may be involved. Further studies utilizing purified 17α-hydroxylase preparations are now needed to unequivocally establish the mechanism(s) involved.

Acknowledgements—These investigations were supported in part by USPHS Grant GM-36907 awarded by the National Institute of General Medical Sciences.

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Biochemical Pharmacology, Vol. 37, No. 2, pp. 357–361, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Facile exchange of the cyano group in highly potent anticancer cyanomorpholinyl anthracyclines

(*Received* 13 *February* 1987; *accepted* 1 *July* 1987)

Recent studies have reported on an unusually potent anthracycline, 3′-deamino-3′-(3-cyano-4-morpholinyl)doxorubicin (CM-DXR), in both animal [1, 2] and human tumor [3, 4] test systems (Fig. 1). It is active in the range of 10^{-9} to 10^{-11} M against doxorubicin (DXR)-sensitive cells and only about 1.5-fold less active in P388 cells resistant to about 160-fold higher amounts of DXR [2]. An essential feature of the unique activity of CM-DXR seems to be the cyano group because the analog, morpholinyl DXR (M-DXR), is about 50-fold less active in DXR-sensitive P388 cells [2]. Previously, Ishiguro *et al.* [5] had shown that the α-cyanoamine moiety of the antibiotic, saframycin A, was essential to the interaction of this compound with calf thymus DNA; and that when [^{14}C]cyanide-labeled saframycin A was reacted with DNA, none of the radioactivity was associated with the DNA. They concluded that an iminium ion or an α-carbinolamine formed by the loss of cyanide was the actual species involved in the interaction with DNA. Lown *et al.* [6] confirmed and extended these studies with saframycin A. Thus, the uniquely high potency of CM-DXR against both DXR-sensitive and -resistant cells may be derived from the ability of CM-DXR to readily form such an alkylating species, which could explain the formation of covalent

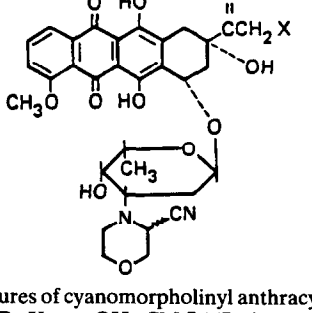

Fig. 1. Structures of cyanomorpholinyl anthracyclines (CM-DXR: X = —OH; CM-DNR: X = —H.

cross-links in DNA as reported by Begleiter and Johnston [7] in HT-29 human colon carcinoma cells, by Westendorf *et al.* [8] with L1210 mouse leukemia cells and V79 Chinese hamster fibroblasts, and by Wassermann *et al.* [9] using KBM-3 human myeloid leukemia cells. Also, cyanide-release *in situ* from CM-DXR may contribute to the cytotoxicity of CM-DXR. To examine the mobility of the cyano group of CM-DXR, we first attempted to develop procedures for cyanide detection in the 10^{-10} to 10^{-12} M range, viz. the activity range of the compound against tumor cells. Failing in this attempt, we examined the displacement of the cyano group of CM-DXR by measuring the exchange with $^{14}CN^-$ under the mild incubation conditions (37°, pH 7.3, 60 min) employed for testing the cytotoxicity of anthracyclines against P388 cells [2]. Our earliest studies on the stability of CM-DXR in phosphate buffer at pH 7 had shown <5% loss of CM-DXR during short storage times at 23° [10]. In addition, in studies performed as part of an examination of the disposition of CM-DXR by rat liver, we found that CM-DXR was stable for 3 hr at 37° in rat liver perfusage (30% whole blood) [11].

After initial findings that $^{14}CN^-$ readily exchanged with the cyano group of CM-DXR, we tested the daunorubicin (DNR) analog of CM-DXR, CM-DNR (Fig. 1), as well as the non-cyano containing anthracyclines, DXR and M-DXR.

Materials and methods

The anthracyclines, DXR, doxorubicinone (DXR-ONE), M-DXR, CM-DXR, and CM-DNR, were obtained from the sources reported previously [12]. *N*-(2-Hydroxyethyl) DXR (HE-DXR) was reported on later [10]. The KCN[^{14}C] (8.6 Ci/mol) was purchased from ICN Radiochemicals (Irvine, CA). Liquid chromatography (LC) of this material on a Nucleosil 10SB, 10 μm, 4.6 × 250 mm, strong anion exchange column (Alltech Associates, Inc., Deerfield, IL) at 30° using a mobile phase of 0.1 M sodium acetate buffer, pH 5.0, containing 0.2 M sodium perchlorate at 0.5 ml/min [13] and a radioactivity flow detector (model HP, Radiomatic Instruments & Chemical Co., Inc., Tampa, FL) yielded a principal peak at the elution position of CN^- containing 97.6% of the total radioactivity.

Separation of anthracyclines was accomplished using reverse-phase LC on a C-8 column (Ultrasphere octyl, 4.6 × 150 mm, 5 μm, Altex Scientific Operations, Berkeley, CA). The mobile phase (35% acetonitrile in 0.05 M phosphate buffer, pH 7.0) was pumped at a flow rate of 1.5 ml/min with detection and quantitation performed at 254 nm (model 1084B liquid chromatograph, Hewlett–Packard, Santa Clara, CA). The anthracyclines were resolved adequately and yielded the following sequence of retention times: DXR-ONE, 6.3 min; CM-DNR, 9.0 min (isomers not separated); diastereoisomers of CM-DXR, 10.1 and 11.1 min; HE-DXR, 15.1 min; DXR, 18.1 min; and M-DXR, 20.1 min.

Incubation conditions were those employed for tests of the activity of anthracyclines against P388 tumor cells [2], viz. 400–500 μl of 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer, pH 7.3, with the added anthracycline in a few microliters of methanol and KCN[^{14}C] or KCN in 0.01 N sodium hydroxide. The total nanomol of anthracycline or KCN[^{14}C] added are indicated in Table 1. After incubation for 1 hr at 37°, the sample was passed through a C-18 Sep-Pak cartridge (Waters Associates, Milford, MA) preconditioned with 2 ml of methanol and 5 ml water. The effluent liquid was collected and the cartridge was rinsed with 25 ml of water. The anthracyclines were stripped from the cartridge by elution with 4 ml of methanol. After evaporation of the methanol at room temperature under a stream of N_2, we dissolved the residue in 400 μl of acetonitrile. Samples of this concentrated solution were employed for LC using the UV and radiochemical detectors in series.

To obtain larger samples of the labeled anthracyclines for mass spectrometry, we incubated 1.0-ml reaction mixtures (in duplicate) containing 70 nmol of CM-DXR or CM-DNR (70 μl of 1.0 mM solution in methanol), 500 nmole of KCN[^{14}C] (50 μl of 10 mM in 0.01 N sodium hydroxide), and 880 μl of 25 mM HEPES buffer, pH 7.3, in 10-ml screw-cap glass culture tubes. After 60 min at 37°, the anthracycline was extracted into 1.0 ml of methylene dichloride, the phases were separated by centrifuging, and the duplicate organic extracts were pooled and evaporated to dryness with nitrogen. Final purification was accomplished by dissolving the residue in 100 μl of dimethyl sulfoxide and injecting 50-μl aliquots into the LC. The entire sample (two injections of 50 μl each) was chromatographed, and the anthracycline peaks were pooled in a 50-ml screw-cap tube and extracted with 5 ml of methylene dichloride. After evaporation of the organic phase to dryness, the residue was dissolved in 200 μl of methanol, the radioactive content of a 10-μl aliquot was determined in a model LS 7000 counter (Beckman Instruments, Irvine, CA), and the anthracycline level was quantitated chromatographically (10 μl) as described above. The remaining sample was subjected to mass spectroscopy. Mass spectra were determined by chemical ionization with ammonia reagent gas, using the desorption probe of a Ribermag R10-10C mass spectrometer.

Results and discussion

After several tests of the possibility of the exchange of $^{14}CN^-$ with the cyano group of CM-DXR, we performed the more deliberate, definitive studies shown in Table 1. With either a 7- or a 70-M excess of $^{14}CN^-$, we found that approximately 50% of the theoretical exchange with CM-DXR had occurred. In these test runs, we found two products of the decomposition of CM-DXR chromatographically, viz. DXR-ONE (R_f = 6.3 min) and HE-DXR (R_f = 15.1 min). The products were produced in amounts about 2-fold greater in incubation mixtures without added KCN than with added KCN or KCN[^{14}C]. Thus, it appeared that the CN^- increased the stability of CM-DXR in the incubation system. In all early tests, we established the authenticity of these peaks as well as the twin peaks of CM-DXR by co-chromatography with spiked samples. The third entry of the table is for the experiment designed to provide sufficient material for the needed test by mass spectrometry of the presence of ^{14}C in the cyano group of the recovered CM-DXR. At that time, we also tested the exchange of $^{14}CN^-$ with the cyano group of CM-DNR, the DNR analog of CM-DXR. As shown in Table 1, we calculated 63% exchange for CM-DXR and 69% exchange for CM-DNR. Tests with the non-cyano containing anthracyclines, M-DXR and DXR, were negative, indicating that substitution by $^{14}CN^-$ (in 70-fold excess) in other parts of the anthracycline molecule were not apparently occurring under the mild conditions of our tests. In these tests, nearly theoretical amounts of ^{14}C were recovered in the aqueous rinses from the Sep-Pak cartridge.

As described above, we isolated the chromatographic peaks of the [^{14}C]CM-DXR and [^{14}C]CM-DNR from the tests starting with 70 nmol of CM-DXR and CM-DNR. Figures 2 and 3 present the DCI-NH_3 spectra obtained from the reference (upper panel) and the isolated ^{14}C-labeled compounds (lower panel). The mass ions at 243 (sugar residue plus water) and 225 (sugar residue without water) from [^{14}C]CM-DXR (Fig. 2) and from [^{14}C]CM-DNR (Fig. 3) were accompanied by ions at m/z 245 and 227, respectively, which were between 14 and 20 times the natural abundance of heavy isotopes. Mass ions at 216 and 198 lacking HCN from either [^{14}C]CM-DXR (Fig. 2) or [^{14}C]CM-DNR (Fig. 3) were accompanied by ions at m/z 218 and 200, respectively, which were not different in relative intensity from those derived from the unlabeled compounds in the upper panels. These results establish

Table 1. Exchange of $^{14}CN^-$ from KCN[^{14}C] to cyanomorpholinyl anthracyclines

Compound studied*	KCN[^{14}C] added		Anthracycline (nmol)		[^{14}C]Anthracycline		% Exchange†
	nmol	nCi	Added	Recovered	nCi	nCi/nmol	
CM-DXR	50	430	7.0	2.3	9.9	4.3	50
CM-DXR	500	4300	7.0	2.4	10.3	4.3	50
CM-DXR	500	4300	70	52‡	280	5.4	63
CM-DNR	500	4300	70	39‡	230	5.9	69
M-DXR	500	4300	7.0	3.5	<0.05		
DXR	500	4300	7.0	7.0	<0.05		

* Compounds were incubated in 25 mM HEPES buffer, pH 7.2, for 60 min at 37°.
† Exchange = (specific activity of [^{14}C]anthracycline ÷ specific activity of 8.6 nCi/nmol for KCN) × 100.
‡ This product was subjected to mass spectrometry.

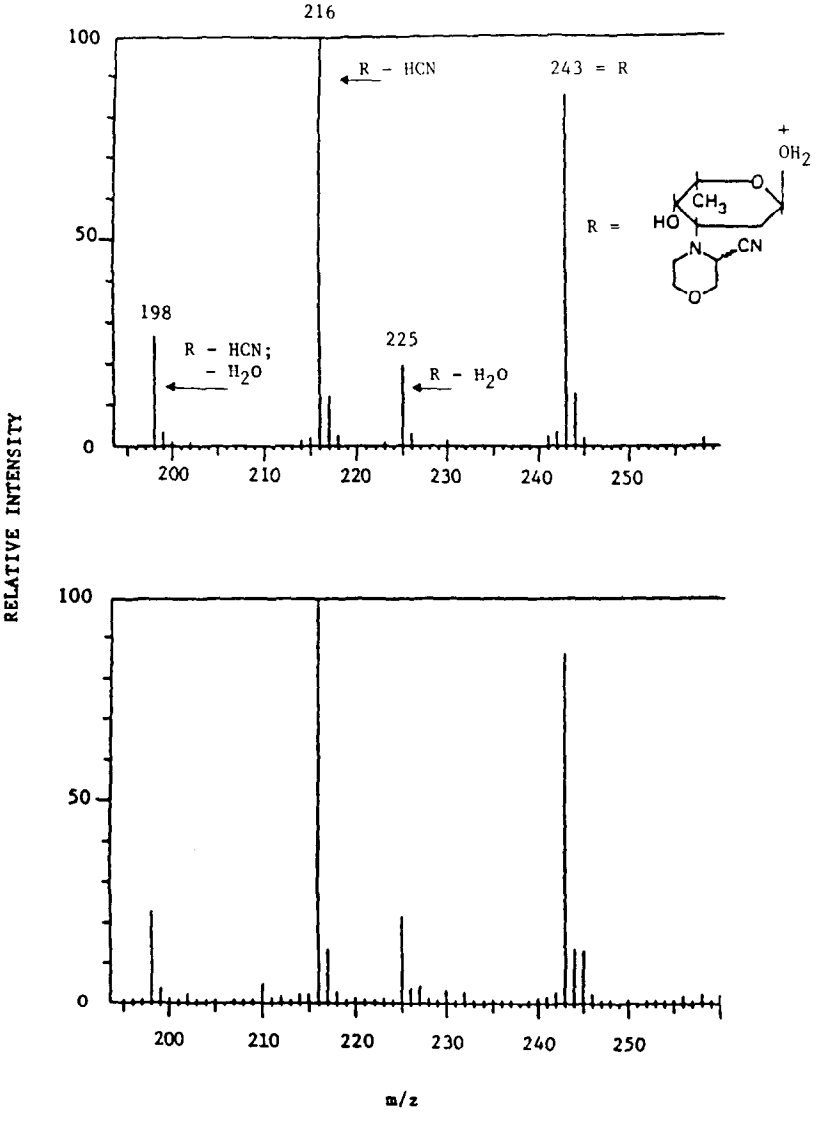

Fig. 2. DCI-NH_3 spectra of the definitive sugar-containing portion of the reference unlabeled CM-DXR (R in upper panel) and its fragments after loss of water, of HCN, and of HCN and water. Spectra of lower panel was obtained from [^{14}C]CM-DXR isolated from the incubation medium.

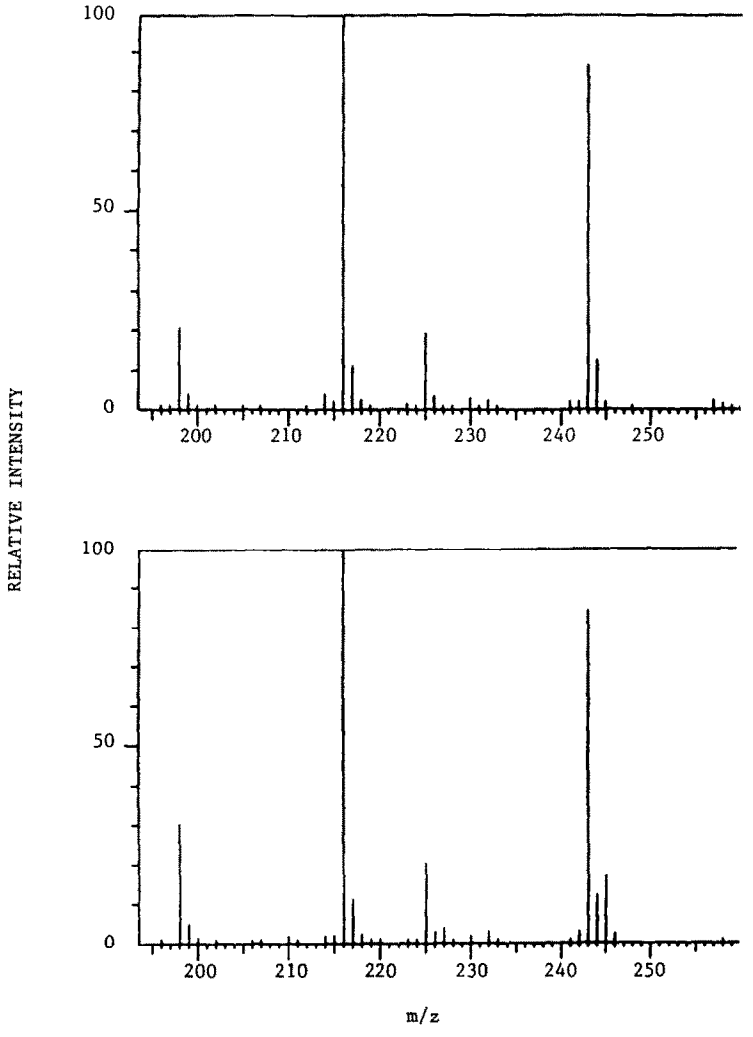

Fig. 3. DCI-NH_3 spectra of the definitive sugar-containing portion (as in Fig. 2) of the reference unlabeled CM-DNR (upper panel) and of the [^{14}C]CM-DNR (lower panel) isolated from the incubation medium (Table 1).

conclusively that the $^{14}CN^-$ from the incubation media had exchanged with the cyano groups of CM-DXR and CM-DNR. Of interest was our discovery after this work was completed of a report by Hussain *et al.* [14] wherein $^{14}CN^-$ exchange with the cyano group of mandelonitrile under very mild conditions was employed as a synthetic route to obtain ^{14}C-labeled phenylethanolamine by reduction of the [^{14}C]mandelonitrile.

Our demonstration of the facile exchange of the cyano group of CM-DXR and CM-DNR with $^{14}CN^-$ under the very mild conditions employed coupled with the demonstrations of others [7–9] that CM-DXR, and not the cyano-lacking M-DXR, produces DNA-DNA cross-links from DNA derived from a number of sources suggests that the uniquely high activity of CM-DXR is derived from an extremely reactive chemical species (iminium cation?) interacting with the DNA. Interestingly, Westendorf *et al.* [8] also reported that M-DNR as well as CM-DNR interacted ". . . covalently with DNA of primary rat hepatocytes." They concluded that M-DNR required metabolic activation to act like CM-DNR. A logical suggestion is that M-DNR was hydroxylated alpha to the morpholinyl nitrogen by the liver enzymes producing an unstable α-hydroxy analog of CM-DNR that readily decomposed to the reactive iminium cation.

Acknowledgements—Supported in part by a grant from the National Cancer Institute, CA-32215 (to J. H. P.) and SRI Research and Development funds (to M. T.).

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Biochemical Pharmacology, Vol. 37, No. 2, pp. 361–363, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Regioselective formation of a misonidazole–glutathione conjugate as a function of pH during chemical reduction

(*Received* 4 *October* 1986; *accepted* 30 *June* 1987)

Misonidazole (1-2[hydroxy-3-methoxypropyl]-2-nitro-1*H*-imidazole; Fig. 1), also known as MISO, is an experimental drug under investigation as a radiosensitizing [1, 2] and chemosensitizing [3] agent. Our previous studies concerning the metabolism of MISO by hypoxic rat livers showed that a MISO–glutathione conjugate (MISO–GSH) is formed as a major metabolite, and that high doses of MISO result in depletion of hepatic glutathione (GSH) [4, 5]. Furthermore, it was noted that formation of the MISO–GSH appears to correlate with the metabolic reduction of MISO [5]. In addition to GSH reactions, it has been demonstrated that MISO undergoes reductive metabolism in hypoxic tissue to yield reactive species which extensively alkylate tissue protein and RNA [5, 6]. Covalent binding to biological nucleophiles by reduced nitroimidazoles [7] has led to an interest in nitroimidazole reduction chemistry, and particular attention has been directed toward the characterization of the reactive intermediate. Among the reports that have appeared in the literature concerning the chemistry of 2-nitroimidazoles under reductive conditions, McClelland and Panicucci [8] have postulated a plausible mechanistic explanation for the reductive activation. McClelland and his group have proposed a mechanism where the neutral hydroxylamine (the four-electron reduction product) of a nitroimidazole derivative undergoes an acid-catalyzed and a non-catalyzed loss of the hydroxyl group, resulting in a cation that is a resonance stabilized nitrenium ion. This mechanism complements and helps to explain our observations regarding the formation of MISO–GSH under various reaction conditions.

A system developed by Varghese [9] for the chemical synthesis of MISO–GSH led to the production of a mixture of conjugates with GSH being attached to the C-4 or C-5 positions of the imidazole ring (Fig. 1). In our investigations concerned with the chemical synthesis of MISO–GSH, it was found that the selectivity for the formation of the C-4 or C-5 conjugate isomers was influenced significantly by the pH of the reaction mixture. The work described herein characterizes the influence of the reaction medium on the regioselective binding of GSH to a reductively-generated, MISO-derived electrophile. Our observations of the pH-dependent, regioselective formation of the MISO–GSH adduct have provided an opportunity to probe the apparent similarity of the reductive activation of MISO with the mechanism proposed by McClelland and Panicucci [8]. In our study, the use of tritiated MISO facilitated MISO–GSH isolation and quantitation.

Methods

Proton nuclear magnetic resonance spectra were recorded in D_2O solution on an NMC-360-MHz spectrometer

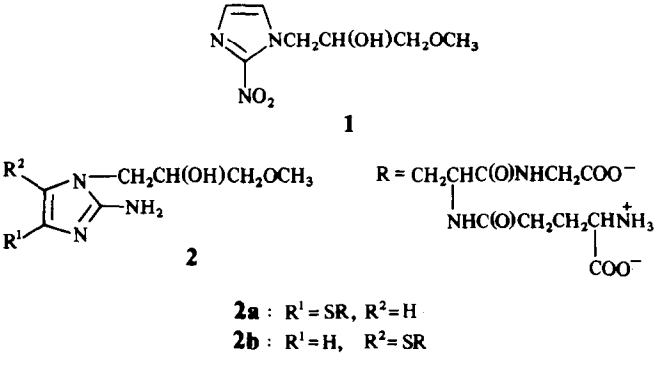

Fig. 1. Structures of MISO (1), MISO-anine (2), and glutathione.