ROLE OF DNA-BINDING IN THE CYTOTOXICITY OF AN ANTHRACYCLINE, R20X2 AND ITS MORPHOLINO ANALOG, MX2

NOBUYASU KOMESHIMA, HIROYUKI KAWAI, SHOHACHI NAKAJIMA, MASAHIKO WATANABE, TAKASHI TSURUO[†], TOMIO TAKEUCHI[††] and NOBORU ŌTAKE[†††]

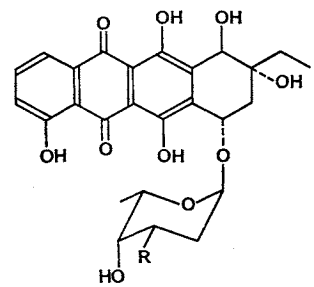
Pharmaceutical Laboratory, Kirin Brewery Co., Ltd., 3 Miyahara-cho, Takasaki, Gunma 370-12, Japan
[†]Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 1-37-1 Kamiikebukuro, Toshima-ku, Tokyo 170, Japan
[††]Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan
[†††]School of Science and Engineering, Teikyo University, 1189-4 Nishikatayama, Nagaoka-cho, Utsunomiya, Tochigi 320, Japan

(Received for publication May 1, 1989)

3′-Deamino-3′-morpholino-13-deoxo-10-hydroxycarminomycin (MX2), a morpholino anthracycline derived from 13-deoxo-10-hydroxycarminomycin (R20X2) was 16 times less cytotoxic than R20X2 against cultured P388 leukemia cells. The reduced cytotoxicity of MX2 was not explainable by intracellular or intranuclear concentration of the drug or by its DNA-intercalating activity. Binding of MX2 and R20X2 to DNA was measured, after isolating the DNA fraction from an incubation mixture of the drugs with P388 cells or with calf thymus DNA. The amount of R20X2 bound to the DNA was obviously larger than that of MX2, and was dependent on incubation time. These data suggest that the poor binding activity of MX2 to DNA contributes to its reduced cytotoxicity.

3′-Deamino-3′-morpholino-13-deoxo-10-hydroxycarminomycin (MX2) is a new morpholino anthracycline agent derived from 13-deoxo-10-hydroxycarminomycin (R20X2) by replacement of the 3′-amino group of the daunosamine residue with a morpholino group[1]. MX2 has several unique characteristics which make it attractive for clinical application. MX2 is equally cytotoxic against doxorubicin-sensitive and -resistent P388 leukemia cells[2,3], is active against intracerebrally-inoculated L1210 leukemia and is active by oral administration[2]. MX2 is less cytotoxic than its parent drug, R20X2 as judged by *in vitro* cytotoxicity and *in vivo* assays[1,2] in contrast to morpholinoadriamycin which is 2 to 3 times more cytotoxic than doxorubicin[4]. In order to obtain information on the factors which reduce the cytotoxicity of MX2, we compared MX2 with R20X2 with respect to their biological activities which were regarded as critical factors for cytotoxicity. The results suggest that a) MX2 and R20X2 bind irreversibly to DNA in P388 cells, b) this binding occurs non enzymatically, and c) the low cyto-

Fig. 1. Chemical structures of MX2 and R20X2.



toxicity of MX2 is due to its low binding activity to DNA.

Materials and Methods

MX2 and R20X2 were prepared at our laboratory. Proteinase K and calf thymus DNA (Type I) were from Sigma. For P388 cell incubation experiments, RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml benzylpenicillin, 0.1 mg/ml streptomycin and 0.05 mM 2-mercaptoethanol was used. P388 leukemia was maintained in female DBA/2 mice. P388 cells were harvested from the peritoneal cavity of tumor-bearing mice and maintained in medium at 37°C in a humidified atmosphere of 5% CO_2.

Cytotoxicity Assay

P388 cells (1×10^4 cells/ml) were treated with graded concentration of MX2 and R20X2 at 37°C for 1 hour in medium. The drug was then removed by centrifugation and washing. The cells were resuspended in fresh medium, incubated at 37°C for 3 days and counted with a Model ZBI Coulter counter.

Intracellular and Intranuclear Concentration

P388 cells (1×10^6 cells/ml) were incubated with 1 μM MX2 or R20X2 at 37°C for 1 hour in medium. The drug was removed by centrifugation and washing with HANKS' balanced salt solution (HBSS). The cells were then resuspended in 0.3 N HCl in 50% ethanol at 37°C for 30 minutes and centrifuged. The fluorescence of the supernatant was measured at an excitation of 470 nm and emission of 550 nm. Drug content was calculated by comparison with standard solutions of the drugs.

The washed cells thus obtained were solubilized with HBSS containing 0.1% Nonidet P-40 at 0°C for 5 minutes and centrifuged. The resulting nuclear pellets were extracted with 0.3 N HCl in 50% ethanol and fluorescence was measured.

DNA-intercalation

Quenching of the fluorescence of MX2 and R20X2 was measured in reaction mixtures each containing 5 μM anthracyclines and varying amounts of calf thymus DNA in a manner analogous to that of previous studies[5,6]. The reagents were dissolved in 50 mM NaCl, 1 mM EDTA and 50 mM sodium phosphate buffer (pH 7.4) and were mixed at 25°C. After 1 hour, fluorescence was measured at excitation of 470 nm and emission of 550 nm. The concentration of DNA was expressed as nucleotide concentration. The binding data were analyzed by the Scatchard method.

DNA-binding in P388 Cells

P388 cells (2×10^7 cells/5 ml) were incubated with 100 μM anthracycline at 37°C in medium. The cells were then chilled on ice and centrifuged. The resulting cell pellets were washed with 5 ml HBSS, solubilized with 3 ml HBSS containing 0.1% Nonidet P-40 for 5 minutes at 0°C and centrifuged. The resulting nuclear pellets were suspended in 2 ml of 1% sodium dodecylsulfate (SDS), 50 mM EDTA, 100 mM Tris-HCl (pH 8.5) and digested with 1 mg/ml proteinase K at 37°C for 2 to 5 hours. This was followed by extraction three times with 2 ml of water-saturated phenol - chloroform (1 : 1). The aqueous fractions were dialyzed against 0.9% NaCl for 18 hours at 4°C and added to 8 ml of 2-propanol. The precipitated DNA-drug complexes were hydrolyzed with 2 ml of 5% $HClO_4$ for 20 minutes at 70°C and centrifuged. The fluorescence of the supernatants was measured at excitation of 470 nm and emission of 550 nm. An aliquot of the supernatants was used for determination of DNA by the method of BURTON[7].

Binding to Calf Thymus DNA

Calf thymus DNA (0.25 mg/ml) was incubated with 100 μM anthracycline at 37°C in 2 ml HBSS. The incubation mixture was subsequently extracted with water-saturated phenol - chloroform (1 : 1). Finally, the DNA was isolated and the drug bound to it was determined as described above.

Enzymatically Stimulated DNA-binding

Binding of anthracyclines to nuclear DNA in the presence of an NADPH-generating system was

measured in a manner analogous to that of previous studies[8,9]. Rat hepatic nuclei (2 mg protein/ml) were incubated with 100 μM anthracyclines, 1 mg/ml NADPH, 2 mg/ml G6P and 5 U/ml G6PDH at 37°C in 2 ml of 150 mM KCl, 50 mM Tris-HCl (pH 7.4) under an air atmosphere. The nuclei were then centrifuged and washed with 150 mM KCl, 50 mM Tris-HCl (pH 7.4). The nuclei were treated with SDS, EDTA and proteinase K. The DNA was isolated and the drug bound to it was determined as described above.

Results

Cytotoxicity

MX2 and R20X2 inhibited the growth of P388 cells in a concentration dependent manner after 1 hour of exposure. The IC_{50} values for MX2 and R20X2 were 103 and 6.2 nM, respectively.

Intracellular and Intranuclear Concentrations

After 1 hour of incubation of P388 cells with 1 μM anthracyclines, the distributions of the drugs were determined. The amounts of MX2 and R20X2 incorporated into the cell fraction were 0.159 and 0.156 nmol/10^6 cells, respectively, and 36.6% of MX2 and 31.9% of R20X2 were located in the nuclear fraction (Table 1).

DNA-intercalation

The fluorescence of MX2 and R20X2 was quenched by the addition of calf thymus DNA to solutions of the drugs. The drugs afforded similar titration curves. The binding parameters of the drugs were obtained from Scatchard plots of the results of titration studies. The apparent binding constants for MX2 and R20X2 were 0.91×10^6 M^{-1} and 0.69×10^6 M^{-1}, respectively, and the apparent numbers of binding sites per nucleotide for MX2 and R20X2 were 0.11 and 0.10 respectively.

DNA-binding in P388 Cells

P388 cells were exposed to 100 μM MX2 or R20X2, and the DNA of the cells was then isolated. The drug bound to the DNA was determined by measuring the fluorescence of the hydrolysate of the DNA. The time course of DNA-binding is shown in Table 2. A zero-time value was obtained by immediate (less than 10 seconds) cooling and centrifugation after mixing the cells and the drugs. The binding of R20X2 increased with time and reached 64 pmol/mg DNA after 2 hours of exposure. The amount of MX2 (27 pmol/mg DNA) bound to the DNA after 2 hours of exposure was less than that of R20X2 and remained at the 0-time level (Table 2).

Binding to Calf Thymus DNA

Calf thymus DNA was incubated with 100 μM MX2 or R20X2, and the drug bound to the DNA was determined. The binding of R20X2 increased with time and reached 189 pmol/mg DNA after

Table 1. Subcellular locations of MX2 and R20X2 in P388 cells.

Drug	Amount of drug (nmol/10^6 cells)		N/C (%)
	Nuclei (N)	Cells (C)	
MX2	0.0583±0.0205	0.159±0.043	36.6± 8.3
R20X2	0.0497±0.0238	0.156±0.044	31.9±11.1

P388 cells were incubated with the drugs (1 μM) at 37°C for 1 hour. The drugs incorporated into the cellular and nuclear fractions were determined. Each value represents the mean±SD of 6 determinations.

Table 2. DNA-binding of MX2, R20X2 and doxorubicin.

	Time of incubation (hours)	DNA-binding (pmol/mg DNA)		
		MX2	R20X2	Doxorubicin
P388 cells	0	28.9± 7.8	14.6±0.2	
	0.5	26.3± 6.6	38.9±0.3	
	1	25.9± 3.3	52.3±0.9	
	2	26.7± 3.5	63.9±5.0	
Calf thymus DNA	0	1.7± 0.6	2.6±0.6	
	0.5	2.1± 0.3	50.1±2.0	
	1	3.0± 1.1	100.1±2.8	
	2	3.4± 1.1	188.7±2.6	
Hepatic nuclei NADPH(+)[a]	0	4.7± 0.6	28.5±1.0	305±10
	0.5	40.5± 2.9	37.6±0.9	387±29
	1	70.9±10.8	51.8±1.8	541± 4
	2	96.2± 1.8	66.8±1.6	1,030±40
NADPH(−)[b]	2	4.2± 1.0	47.4±2.2	395± 7

The drugs (100 μM) were incubated with a) P388 cells, b) calf thymus DNA or c) rat hepatic nuclei. The amounts at which they bound to the DNA were determined.
Each value represents the mean±SD of 3 determinations.
[a] With the NADPH-generating system.
[b] Without the NADPH-generating system.

2 hours of exposure. The binding of MX2 increased slowly, reaching a value of 3.4 pmol/mg DNA which was 56 times less than that of R20X2 (Table 2).

Enzymatically Stimulated DNA-binding

Rat hepatic nuclei were incubated with 100 μM MX2 or R20X2 in the presence of the NADPH-generating system, and the drug bound to the DNA was determined. The binding of the drugs increased with time, the amount of MX2 bound reached 96 pmol/mg DNA, exceeding that of R20X2 (67 pmol/mg DNA) after 2 hours of exposure. This is in a striking contrast to the data obtained with P388 cells or calf thymus DNA, Without the NADPH-generating system, the amount of MX2 bound to the DNA remained at the 0-time level, while that of R20X2 increased (Table 2).

Discussion

MX2 was 16 times less cytotoxic than R20X2, according to the IC_{50} for the growth of P388 cells. When P388 cells were incubated with MX2 or R20X2, both the intracellular concentration and distribution of MX2 were similar to those of R20X2. Analysis of intracellular drugs by HPLC showed neither MX2 nor R20X2 to be metabolized in the cells and each remained unchanged (unpublished data). Thus, the difference between the drugs in cytotoxicity against P388 cells arises not from their concentrations near target molecules but from their intrinsic activities for reacting with the molecules.

Anthracyclines have been shown to intercalate with DNA[10], and bind irreversibly to DNA[4], thereby altering the DNA template and inhibiting nucleic acid synthesis. When the DNA-intercalations of MX2 and R20X2 were measured fluorometrically, no significant difference between the drugs in binding parameters could be found.

On irreversible binding, distinct differences were noted between the drugs. After incubating P388 cells with MX2 or R20X2, the amount of drug bound to the DNA of the cells was measured by the method of WALLACE and JOHNSON[9] for demonstrating the binding of doxorubicin to hepatic nuclear DNA. The amount of MX2 bound to DNA in P388 cells was half that of R20X2 after 1 hour of incubation. The amount of R20X2 bound to DNA depended on incubation time, while this was not

the case for the amount of MX2. The higher cytotoxicity of R20X2 thus appears due to its higher DNA-binding activity. This possibility is supported by the fact that morpholino anthracyclines possessing higher/lower cytotoxicity became bound to DNA to a greater/lesser extent than the parent drugs by 1 hour of incubation with P388 cells (Table 3). The reason why a significant amount of MX2 bound to DNA at 0-time is uncertain, though possibly this may have occurred during the isolation of DNA.

SINHA *et al.*[8] consider that free radical intermediates are formed from doxorubicin during enzymatic activation, and then bind to DNA. Such a mechanism of DNA-binding is supported by the reported detection of electron spin resonance signals of free radicals in an incubation mixture of doxorubicin, hepatic nuclei and NADPH, and detection of the drug bound to DNA from this incubation mixture. When MX2 was incubated with hepatic nuclei in the presence of NADPH, the amount of MX2 bound to the DNA increased with time, and exceeded that of R20X2. This is inconsistent with the results obtained by the incubation of these drugs with P388 cells. MX2 bound to the DNA of P388 cells less than did R20X2. It would thus appear that non enzymatic mechanisms are very significantly operative in the DNA-binding of MX2 and R20X2 in P388 cells.

Possible explanations for the inconsistency of the binding of MX2 and R20X2 in the three systems include low activity of enzymes which reduce anthracyclines to free radical species in P388 cells in contrast to the hepatic nuclei and low sensitivity of MX2 and R20X2 to enzymatic reduction.

Table 3. Cytotoxicity and DNA-binding of morpholino anthracyclines and their parent drugs.

Drug	IC_{50} for P388 cell growth (nM)[a]	DNA-binding in P388 cells (pmol/mg DNA)[b]
MX2	12.2	25.9
R20X2	1.55	52.3
MX	25.0	29.0
R20X	3.53	39.5
MY5	221	196
R20Y5	16.8	372
MDXR	1.58	479
DXR	18.6	313
M13DH-DM	0.38	1,970
13DH-DM	81.3	62.1
M13DH-DXR	8.78	468
13DH-DXR	242	176

[a] P388 cells (1×10^4 cells/ml) were incubated at 37°C for 72 hours with the drugs, and counted with a coulter counter.

[b] P388 cells (2×10^7 cells/5 ml) were incubated at 37°C for 1 hour with 100 μM drug, and the drug bound to DNA of the cells was determined.

Abbreviations: MX, 3'-Deamino-3'-morpholino-13-deoxocarminomycin; R20X, 13-deoxocarminomycin; MY5, 3'-deamino-3'-morpholino-13-deoxo-11-deoxycarminomycin; R20Y5, 13-deoxo-11-deoxycarminomycin; MDXR, 3'-deamino-3'-morpholinodoxorubicin; DXR, doxorubicin; M13DH-DM, 3'-deamino-3'-morpholino-13-dihydrodaunomycin; 13DH-DM, 13-dihydrodaunomycin; M13DH-DXR, 3'-deamino-3'-morpholino-13-dihydrodoxorubicin; 13DH-DXR, 13-dihydrodoxorubicin.

Regarding the first possibility, CAPRANICO *et al.*[11] suggest that mechanisms other than free radical formation are responsible for damage to DNA by doxorubicin, since depletion of glutathione failed to increase the cytotoxicity of doxorubicin in Chinese hamster ovary cells, V-79 cells, human carcinoma cells and P388 cells. In contrast, SINHA *et al.*[12] found MCF-7 human breast cancer cells produce free radicals on exposure to doxorubicin and some radical scavengers to be capable of lessening the cytotoxicity of doxorubicin toward MCF-7 cells. Enzyme activity responsible for the formation of anthracycline radicals may possibly vary according to cell type and this would account for differences in binding.

As for the second possibility, the amount of doxorubicin (1,030 pmol/mg DNA) bound to DNA was more than 10 times that of MX2 (96 pmol/mg DNA) when they were incubated with hepatic nuclei and the NADPH generating system for 2 hours. Thus possibly MX2 and R20X2 have little sensitivity toward enzymatic reduction or radical intermediates generated from them may have little activity for binding to DNA.

The anthracycline reducing activity of tumor cells and sensitivity of anthracyclines to enzymatic reduction may act in concert to influence the DNA-binding and cytotoxicity of anthracyclines. Thus cell lines of clinical importance should be used for future study on DNA binding.

Acknowledgment

This research was supported in part by Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan.

References

1) UMEZAWA, H.; S. NAKAJIMA, H. KAWAI, N. KOMESHIMA, H. YOSHIMOTO, T. URATA, A. ODAGAWA, N. OTSUKI, K. TATSUTA, N. ŌTAKE & T. TAKEUCHI: New morpholino anthracyclines, MX, MX2, and MY5. J. Antibiotics 40: 1058~1061, 1987
2) KOMESHIMA, N.; T. TSURUO & H. UMEZAWA: Antitumor activity of new morpholino anthracyclines. J. Antibiotics 41: 548~553, 1988
3) WATANABE, M.; N. KOMESHIMA, S. NAKAJIMA & T. TSURUO: MX2, a morpholino anthracycline, as a new antitumor agent against drug-sensitive and multidrug-resistant human and murine tumor cells. Cancer Res. 48: 6653~6657, 1988
4) STREETER, D. G.; J. S. JOHL, G. R. GORDON & J. H. PETERS: Uptake and retention of morpholinyl anthracyclines by adriamycin-sensitive and -resistant P388 cells. Cancer Chemother. Pharmacol. 16: 247~252, 1986
5) DUVERNAY, V. H.; J. A. PACHTER & S. T. CROOKE: Molecular pharmacological differences between carminomycin and its analog, carminomycin-11-methyl ether, and adriamycin. Cancer Res. 40: 387~394, 1980
6) KUNIMOTO, S.; K. MIURA, Y. TAKAHASHI, T. TAKEUCHI & H. UMEZAWA: Rapid uptake by cultured tumor cells and intracellular behavior of 4′-*O*-tetrahydropyranyladriamycin. J. Antibiotics 36: 312~317, 1983
7) BURTON, K.: A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62: 315~323, 1956
8) SINHA, B. K.; M. A. TRUSH, K. A. KENNEDY & E. G. MIMNAUGH: Enzymatic activation and binding of adriamycin to nuclear DNA. Cancer Res. 44: 2892~2896, 1984
9) WALLACE, K. B. & J. A. JOHNSON: Oxigen-dependent effect of microsomes on the binding of doxorubicin to rat hepatic nuclear DNA. Mol. Pharmacol. 31: 307~311, 1987
10) PIGRAM, W. J.; W. FULLER & L. D. HAMILTON: Stereochemistry of intercalation: Interaction of daunomycin with DNA. Nature (New Biol.) 235: 17~19, 1972
11) CAPRANICO, G.; N. BABUDRI, G. CASCIARRI, L. DOLZANI, R. A. GAMBETTA, E. LONGONI, B. PANI, C. SORANZO & F. ZUNINO: Lack of effect of glutathione depletion on cytotoxicity, mutagenicity and DNA damage produced by doxorubicin in cultured cells. Chem. Biol. Interact. 57: 189~201, 1986
12) SINHA, B. K.; A. G. KATKI, G. BATIST, K. H. COWAN & C. E. MYERS: Differential formation of hydroxyl radicals by adriamycin in sensitive and resistant MCF-7 human breast tumor cells: Implications for the mechanism of action. Biochemistry 26: 3776~3781, 1987