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The mechanism for anthracycline-induced inhibition of collagen biosynthesis

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Abstract

One of the recognized side effects accompanying anti-neoplastic anthracyclines administration is poor wound healing resulting from impairment of collagen biosynthesis. However, the precise mechanism of anthracyclines-induced inhibition of collagen synthesis has not been established. We have suggested that prolidase, an enzyme involved in collagen metabolism, may be one of the targets for anthracyclines-induced inhibition of synthesis of this protein. Prolidase [EC 3.4.13.9] cleaves imidodipeptides containing C-terminal proline, providing large amount of proline for collagen synthesis. Therefore, we compared the effect of daunorubicin and doxorubicin on prolidase activity and collagen biosynthesis in confluent cultured human skin fibroblasts. We have found that daunorubicin and doxorubicin coordinately induced the inhibition of prolidase activity ($IC_{50} = 0.3$ and 10 μM, respectively) and collagen biosynthesis ($IC_{50} = 1$ and 15 μM, respectively) in cultured human skin fibroblasts. The inhibitory effect of daunorubicin or doxorubicin on prolidase activity and collagen biosynthesis was not due to anti-proliferative activity of these drugs as shown by cell viability tetrazoline test. The decrease in prolidase activity due to the treatment of confluent cells with the anthracyclines was not accompanied by any difference in the amount of enzyme protein recovered from these cells as shown by Western immunoblot analysis. It may be suggested that the inhibition is a post-translational event. Since prolidase is metalloprotease, requiring manganese for catalytic activity, and anthracyclines are known as chelators of divalent cations, we considered that the chelating ability of anthracyclines might be an underlying mechanism for the anthracyclines-induced inhibition of prolidase activity. In order to determine the ability of daunorubicin or doxorubicin to form complexes with manganese (II), potentiometric method was employed based on the measurement of protonation constant by pH-metric titrated assay. We have found that both anthracyclines form stable complexes with manganese (II). The composition of the daunorubicin–Mn(II) complex was calculated as 3:1 while that of doxorubicin–Mn(II) complex was 2:1. The constant stability value for the investigated complexes were calculated as $\beta_{av} = (1.74 \pm 0.01) \times 10^{23}$ for daunorubicin, and $\beta_{av} = (1.99 \pm 0.025) \times 10^{11}$ for doxorubicin. The higher ability of daunorubicin vs. doxorubicin to chelate manganese and inhibit prolidase activity may explain the potential mechanism for its greater potency to inhibit collagen biosynthesis.

Keywords: Collagen; Daunorubicyn; Doxorubicin; Fibroblast; Prolidase

1. Introduction

Daunorubicin and doxorubicin are anthracyclines, antitumor agents with a wide spectrum of activity in treating malignancies (Casazza, 1986). The use of anthracyclines is accompanied by several untoward side effects (Calabresi and Parks, 1980). One of the recognized side effects accompanying the administration of these drugs is poor wound healing. It has been suggested that deficient collagen formation may contribute to the reduced wound healing among patients treated with these drugs (Devereux et al., 1979). Moreover, the anti-proliferative effect of daunorubicin and doxorubicin may contribute to the reduced wound healing (Bland et al., 1984). However, the precise mechanism of this anthracycline-induced reduction of collagen production is not well understood.

One of the enzymes involved in collagen biosynthesis is prolidase [EC 3.4.13.9]. This enzyme is a cytosolic exopeptidase that cleaves imidodipeptides with C-terminal proline (Endo et al., 1989; Phang and Scriver, 1989). The primary biological function of the enzyme involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen resyn-

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thesis (Chamson et al., 1989). The efficiency of proline recycling from imidodipeptides was found to be about 90% (Jackson et al., 1975). Therefore, prolidase activity (despite the collagen gene expression) may be a rate-limiting factor in the regulation of collagen biosynthesis.

Prolidase is a metalloprotease. It has a specific requirement for manganese and the ion (Mn^{2+}) activates the enzyme (Myara et al., 1985). Anthracyclines are known to form stable complexes with divalent cations (Powis, 1994). For instance, doxorubicin, daunorubicin, carminomycin and 5-imino-daunorubicin form very strong complexes with Fe(II), Fe(III), Zn(II), Mg(II), Ca(II), Yb(III), Cu(II) and Pd(II). Therefore, it seems possible that the inhibition of prolidase by anthracyclines involves the chelation of manganese metal required for the enzyme activity.

In this study, we have compared the ability of daunorubicin and doxorubicin to form complexes with manganese, the stability constant of such complexes and their effects on prolidase activity and collagen biosynthesis in cultured human skin fibroblasts.

2. Materials and methods

2.1. Materials

Glycyl-proline (Gly-Pro), bacterial collagenase (type VII), trypsin, bovine serum albumin (BSA), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), doxorubicin and manganese chloride were purchased from Sigma (USA) as were most other chemicals used. Dulbecco's minimal essential medium (DMEM) and foetal bovine serum (FBS) used in cell culture were obtained from Gibco (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals (USA). Polyclonal anti-human prolidase antibody was a gift from Dr. C.R. Scriver (Montreal Children's Hospital, Montreal, Quebec, Canada). Nitrocellulose membrane (0.2 μm) and sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were procured from Bio-Rad Laboratories (USA). Horseradish peroxidase labeled anti-rabbit immunoglobulin G antibody was purchased from Promega (USA). L-5[^{3}H]Proline (28 Ci/mmol) and ECL Western detection system were received from Amersham (UK). Daunorubicin was a gift from Prof. G. Grynkiewicz (Farmaceutical Institut, Warsaw). [^{3}H]Thymidine (6.7 Ci/mmol) came from NEN (USA).

2.2. Fibroblast cultures

Normal human skin fibroblasts, obtained by punch biopsy from 11-year-old male donors were maintained in DMEM supplemented with 10% FBS, 2 mmol/l glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin at 37°C in a 5% CO_2 in an incubator. The cells were used between the 12th and 14th passages. The fibroblasts were subcultivated by trypsinization. Subconfluent cells from Costar flasks were detached with 0.05% trypsin, 0.02% ethylene-diaminetetraacetic acid (EDTA) in the calcium-free phosphate-buffered saline (PBS). For the prolidase assay, cells were cultured in 6-well plates (Costar). For these experiments, the cells were counted in hemocytometers and inoculated at 1×10^5 cells per well in 2 ml of growth medium. Cells reached confluency at day 6 after inoculation and in most cases such cells were used for the experiments. Confluent cells were treated for 24 h with the studied drug in growth medium.

2.3. Collagen production

The incorporation of radioactive precursor into protein was measured after labeling confluent cells in serum-free medium with varying concentrations of daunorubicin or doxorubicin for 24 h with 5[^{3}H]proline (5 and 28 Ci/mmol) as described previously (Oyamada et al., 1990). Incorporation into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase according to the method of Peterkofsky et al. (1982). Results are shown as combined values for cell plus medium fractions.

2.4. Determination of prolidase and prolinase activity

The activity of prolidase was determined according to the method of Myara et al. (1982), which is based on the measurement of proline by Chinard's reagent (Chinard, 1952). Briefly, the monolayer was washed three times with 0.15 mol/l NaCl. Cells were collected by scraping and suspended in 0.15 mol/l NaCl, centrifuged at low speed ($200 \times g$) and the supernatant was discarded. The cell pellet (from two wells) was suspended in 0.3 ml of 0.05 mol/l Tris–HCl, pH 7.8, and sonicated three times for 10 s at 0°C. Samples were then centrifuged ($18{,}000 \times g$, 30 min) at 4°C. The supernatant was used for protein determination and then for prolidase activity assay. The activation of prolidase requires preincubation with manganese, therefore 0.1 ml of supernatant was incubated with 0.1 ml of 0.05 mol/l Tris–HCl, pH 7.8 containing 2 mmol/l $MnCl_2$ for 24 h at 37°C. After preincubation, the prolidase reaction was initiated by adding 0.1 ml of the preincubated mixture to 0.1 ml of 94 mmol/l glicyl-proline (Gly-Pro) to a final concentration of 47 mmol/l. After additional incubation for 1 h at 37°C, the reaction was terminated with 1 ml of 0.45 mol/l trichloroacetic acid. In the parallel tubes reaction was terminated at time "zero" (without incubation). The released proline was determined by adding 0.5 ml of the trichloroacetic acid supernatant to 2 ml of a 1:1 mixture of glacial acetic acid: Chinard's reagent (25 g of ninhydrin dissolved at 70°C in 600 ml of glacial acetic acid and 400 ml of 6 mol/l orthophosphoric acid) and incubated for 10 min at 90°C. The amount of proline released was determined colorimetrically by reading an

absorbance at 515 nm and calculated by using the proline standards. Protein concentration was measured by the method of Lowry et al. (1951). Enzyme activity was calculated as nanomoles of released proline per minute per milligram of supernatant protein. Prolinase activity assay was performed in the same way, except preincubation with manganese was omitted and Pro-Gly was used as a substrate.

2.5. SDS-PAGE

Slab SDS-PAGE was used, according to the method of Laemmli (1970). Samples of cell supernatants (50–100 μg of protein) were incubated for 5 min at 100°C in 62.5 mmol/l Tris–HCl, pH 6.8, containing 2.0% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.001% bromophenol blue. Samples containing 20 μg of protein were subjected to electrophoresis on a 0.1% SDS-polyacrylamide slab gel (composed of 4% stacking gel and a 7.5% separating gel) at 50 V per gel for 1.5 h at room temperature in a pH 8.3 running buffer containing 25 mmol/l Tris, 192 mmol/l glycine and 0.1% SDS. The following Bio-Rad's unstained high molecular weight standards were used: myosin (200 kDa), galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa).

2.6. Western blot analysis

After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris, 0.2 mol/l glycine in 20% (v/v) methanol. The protein was transferred to 0.2 μm pore-sized nitrocellulose at 100 mA for 1 h by using an LKB 2117 Multiphor II electrophoresis unit according to the method described in the manual accompanying the unit. Nitrocellulose containing molecular weight standards was stained for 1 min with 0.2% Panceau S, positions of standards were marked with S and S NC marker (Schleicher and Schuell, Germany) and destained in TBS-T solution (20 mmol/l Tris–HCl buffer, pH 7.4, containing 150 mmol/l NaCl and 0.05% Tween 20). Nitrocellulose was blocked with 5% dried milk in TBS-T for 1 h in room temperature, with slow shaking. Then the nitrocellulose was incubating with polyclonal antibody against human prolidase at a concentration of 1:3000 in 5% dried milk in TBS-T for 1 h as previously. After the incubation, nitrocellulose was washed with TBS-T (1×15 and 2×10 min) with slow shaking. In order to analyse prolidase, second antibody, horseradish peroxidase labeled antibody against rabbit's Fc IgG was added at a concentration of 1:5000 in TBS-T and incubated for 30 min with slow shaking. Then, nitrocellulose was extensively washed with TBS-T (5×10 min) and submitted to ECL Western blotting detection system (Amersham) for 1 min The nitrocellulose was covered with Saran Wrap and exposed to film for 30 s to 1 min.

2.7. Cell viability assay

The assay was performed according to the method of Carmichael et al. (1987) using MTT. Confluent cells, cultured for 24 h with various concentrations of daunorubicin or doxorubicin in 6-well plates were washed three times with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37°C in 5% CO_2 in an incubator. The medium was removed and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm with background subtraction at 650 nm. The cell viability of fibroblasts cultured in the presence of daunorubicin or doxorubicin was calculated as percent of control cells.

2.8. Mitogenic assay

To examine the effect of daunorubicin and doxorubicin on fibroblast proliferation, the cells were seeded in 24-well tissue culture dishes at 1×10^5 cells/well with 1 ml of growth medium. After 48 h ($1.6 \pm 0.1 \times 10^5$ cells/well), plates were incubated with varying concentrations of studied anthracyclines and 0.5 μCi of [^{3}H]thymidine for 24 h at 37°C. Cells were rinsed three times with PBS, solubilized with 1 ml of 0.1 mol/l sodium hydroxide containing 1% SDS, scintillation fluid (9 ml) was added and radioactivity incorporation into DNA was measured in scintillation counter.

2.9. Daunorubicin–Mn(II) and doxorubicin–Mn(II) stability constants determination

In order to prepare the daunorubicin–manganese (II) and doxorubicin–manganese (II) complexes, the solutions of the respective anthracycline (10 ml of 0.005 mol/l, water solution) and manganese chloride (10 ml of 0.0017 mol/l, water solution) were mixed and incubated for 2 h at room temperature.

To determine the ability of daunorubicin or doxorubicin to form complexes with manganese (II) as well as the stability of the complexes, the pH-metrical titrated assay was performed (Inczedy, 1979). Since during complex formation between metal ion (M) and ligand (L), protons (H^+) are released:

$$M + HL \rightarrow ML + H^+$$

the equilibrium ratio of this process was determined by measuring the concentration of hydrogen ion (pH).

The complexing agent—daunorubicin as well as doxorubicin (both at concentration 0.005 mol/l, water solutions), which are weak acid s(proton base), were titrated potentiometrically with sodium hydroxide (0.1 mol/l) for 3 h in the presence of neutral salt (0.2 mol/l KNO_3) in order to obtain the constant ionic strength of the solution ($I = 0.056$). The pH changes of the solution during titra-

tion were measured by CP-315 microcomputer pH-meter ELMETRON (Zabrze, Poland) The accuracy of the readout was ± 0.01 pH. Based on the titrating data and equation: $pH = f(a)$, where a is the titrating fraction, the titrating curve was made. Then, similarly, the anthracycline solution of the same composition, but with manganese ions (II), was titrated and the titrating curve was made. In the range between pH 3 and 10, and in conditions where the concentration of ligand C_L [daunorubicin] or [doxorubicin] was not much higher than the concentration of metal C_M [Mn(II)], the complex formation, which was reflected by the difference between two titrating curves, was observed.

The composition of the complex was calculated based on the concentration values of the total and free ligand, total concentration of Mn(II) and the average ligand number, considering the side reactions (protonation).

2.10. Statistical analysis

In all experiments, the mean values for six independent experiments $\pm$ standard deviations (S.D.) were calculated unless otherwise indicated.

3. Results

Confluent human skin fibroblasts were used to test the effect of daunorubicin and doxorubicin on prolidase activity and collagen biosynthesis. The rationale for the use of confluent cells in the experiments was that prolidase activity (Myara et al., 1985) and collagen biosynthesis (Makela et al., 1990) are dependent on cell density and rise when cell density increases. Prolidase activity and collagen biosynthesis were measured in fibroblasts treated for 24 h with different concentrations of daunorubicin or doxorubicin. It has been found that both anthracyclines induced a decrease in fibroblasts' prolidase activity in a dose-dependent manner (Fig. 1). The concentrations of drugs required for 50% inhibition (IC_{50}) of fibroblasts' prolidase activity were found to be about 0.3 ± 0.1 μM for daunorubicin and about 10 ± 3 μM for doxorubicin. Similar effects of the anthracyclines on collagen biosynthesis were found. The drugs induced a decrease in collagen biosynthesis in confluent human skin fibroblasts in a dose-dependent manner (Fig. 2). IC_{50} for collagen biosynthesis was found at about 1 ± 0.2 μM for daunorubicin and 15 ± 3 μM for doxorubicin. In both experiments, IC_{50} values were calculated on the basis of the anthracycline concentration in medium of cultured cells.

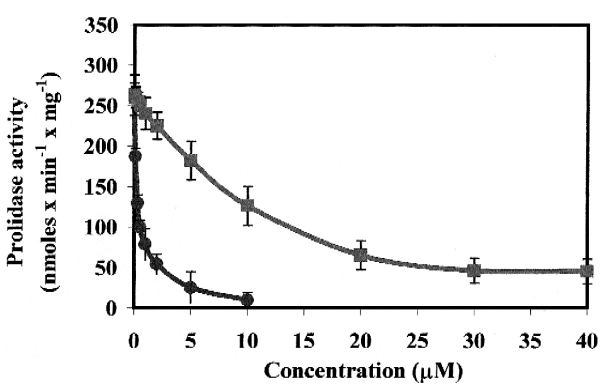

Fig. 1. The activity of prolidase in confluent human skin fibroblasts cultured for 24 h with different concentrations of daunorubicin (●) and doxorubicin (■). The enzyme activity was calculated as nanomoles of proline released from substrate during 1 min per milligram of supernatant protein.

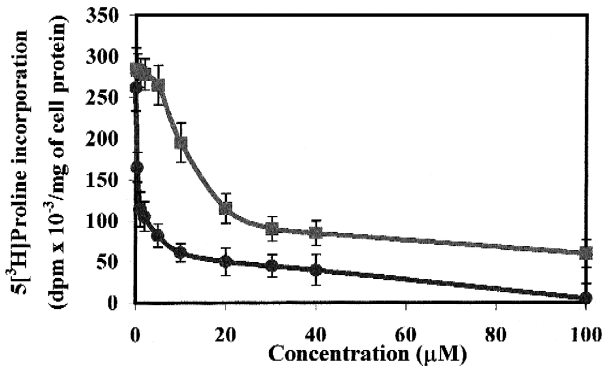

Fig. 2. Effects of daunorubicin (●) and doxorubicin (■) on 5[^{3}H]proline incorporation into protein susceptible to the action of bacterial collagenase in confluent human skin fibroblasts. Collagen synthesis is reported as dpm of radioactive proline incorporated into proteins susceptible to the action of bacterial collagenase per milligram of cell protein.

The decrease in prolidase activity due to the treatment of confluent cells with daunorubicin and doxorubicin was not accompanied by any difference in the amount of enzyme protein recovered from these cells as shown by Western immunoblot analysis (Fig. 3). It may be suggested that the inhibitory effect of daunorubicin and doxorubicin on prolidase activity is a post-translational event.

Considering the possibility that the effects of the anthracyclines on prolidase activity and collagen biosynthesis

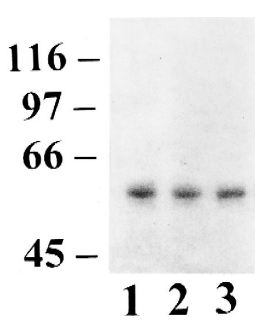

Fig. 3. Western immunoblot analysis of prolidase from control fibroblasts (lane 1) and fibroblasts treated for 24 h with 10 μM of daunorubicin (lane 2) and with 40 μM of doxorubicin (lane 3). The same amount of supernatant protein (20 μg) was run in each lane.

Table 1
Viability of confluent human skin fibroblasts treated for 24 h with different concentrations of daunorubicin (A) and doxorubicin (B)

Concentration (μM)	Cell viability (% of control)
A. Daunorubicin	
0	100
0.1	98.9 ± 1.1
1.0	98.3 ± 1.3
10.0	91.3 ± 3.6
B: Doxorubicin	
0	100
25	96 ± 2
50	93 ± 3
100	87 ± 2
125	76 ± 4
150	78 ± 4

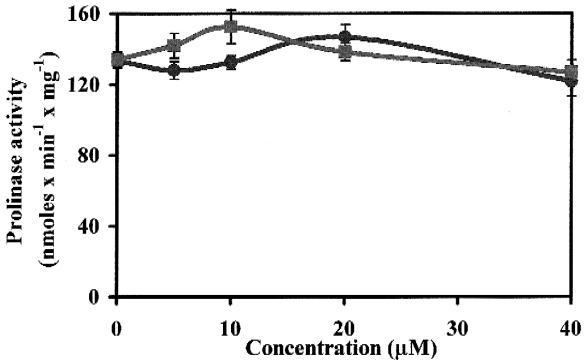

Fig. 5. The activity of prolinase in confluent human skin fibroblasts cultured for 24 h with different concentrations of daunorubicin (●) and doxorubicin (■). The enzyme activity was calculated as nanomoles of proline released during 1 min per milligram of supernatant protein.

may be a result of the anti-proliferative action of the drug, the assay for cell viability with different concentrations of daunorubicin and doxorubicin were performed. Cell viability was measured by the method of Carmichael et al. (1987) using tetrazolinum salt. Considering the possibility of daunorubicin and doxorubicin interaction with tetrazolinum salt, the control test (non-specific reaction) was performed with both reagents incubated for 24 h in cell-free system. Values were corrected for non-specific reaction. The viability of cells incubated for 24 h with indicated concentrations of daunorubicin or doxorubicin are presented in Table 1. As can be seen, daunorubicin at about 1 μM and doxorubicin at about 25 μM (the concentrations at which prolidase activity and collagen biosynthesis are strongly inhibited) did not significantly affect the confluent human skin fibroblasts. An increase in the daunorubicin concentration up to 10 μM resulted in about 10% reduction of viability of these cells. For doxorubicin, about 10% reduction of viability of the cells was observed at a concentration of about 100 μM. It suggests that the inhibitory effects of daunorubicin and doxorubicin (at concentration up to 10 and 100 μM, respectively) on prolidase activity and collagen biosynthesis are not due to the cytotoxicity of these drugs.

On the other hand, DNA synthesis (measured by radioactive thymidine incorporation assay) was inhibited at IC_{50} of 0.3 μM for daunorubicin and 0.6 μM for doxorubicin (Fig. 4).

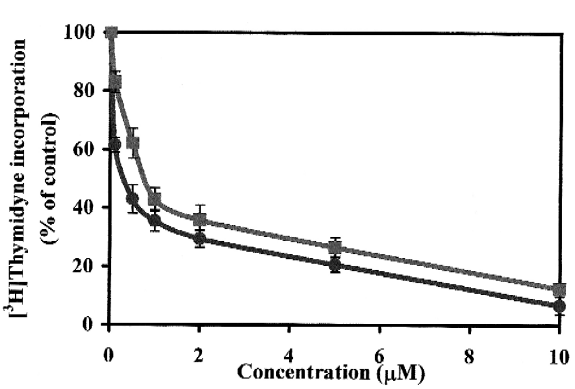

Fig. 4. DNA synthesis in confluent human skin fibroblasts cultured for 24 h with different concentrations of daunorubicin (●) and doxorubicin (■).

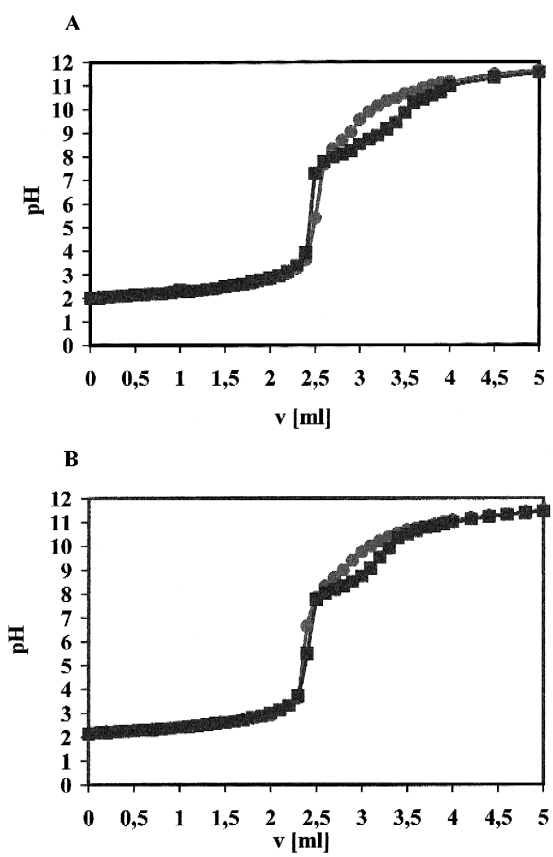

Fig. 6. Titrating curves for daunorubicin (●) and daunorubicin–Mn(II) complex (■) (panel A) and for doxorubicin (●) and doxorubicin–Mn(II) complex (■) (panel B) with 0.1 mol/l sodium hydroxide in the presence of 0.1 mol/l hydrochloric acid. Results represent the mean values from three independent experiments.

Table 2
Values of protonation constant for daunorubicin (log K) at some titrating fraction (a) and respective pH of the solution

a	pH	$[H^+]$	$[OH^-]$	log K_1
$C_L = 8.53 \times 10^{-4}$ M, $0 < a < 1$				
0.33	8.10	7.94×10^{-9}	1.26×10^{-6}	7.80
0.40	8.20	6.31×10^{-9}	1.58×10^{-6}	8.03
0.44	8.30	5.01×10^{-9}	1.99×10^{-6}	8.20
0.56	8.51	3.09×10^{-9}	3.24×10^{-6}	8.62
0.60	8.57	2.69×10^{-9}	3.72×10^{-6}	8.75
0.67	8.68	2.09×10^{-9}	4.79×10^{-6}	9.00
			log K_1 (mean ± S.D.)	8.40 ± 0.46
$C_L = 8.46 \times 10^{-4}$ M, $1 < a < 2$				
1.33	9.89	1.28×10^{-10}	7.76×10^{-5}	10.62
1.40	9.97	1.07×10^{-10}	9.33×10^{-5}	10.91
1.44	10.01	9.77×10^{-11}	1.02×10^{-4}	11.28
1.56	10.14	7.24×10^{-11}	1.38×10^{-4}	12.75
1.60	10.20	6.30×10^{-11}	1.58×10^{-4}	13.89
1.67	10.25	5.62×10^{-11}	1.78×10^{-4}	17.61
			log K_2 (mean ± S.D.)	12.84 ± 2.64

Since prolidase is a metallopeptidase (thus requiring manganese for catalytic activity) and anthracyclines are known as chelators of divalent cations, we considered that the chelating ability of anthracyclines may be an underlying mechanism for anthracycline-induced inhibition of prolidase activity. Supporting evidence came from the observation that another imidodipeptidase–prolinase (which do not require any metal for catalytic activity) is not inhibited by either anthracyclines (Fig. 5).

Complexes of daunorubicin and doxorubicin with Mn(II) were made and the constant stability and composition of a such complexes were determined. As can be seen from Fig. 6A and B, the final titrating points of hydrochloric acid were found at 2.5 and 2.4 ml of sodium hydroxide for daunorubicin and doxorubicin, respectively. It represents the initial titrating volume for the drugs and zero value of the drugs titrating fraction ($a = 0$). The final titrating points of daunorubicin were found at 2.95 ml ($a = 1$) and 3.4 ml ($a = 2$) of sodium hydroxide. The final titrating points of doxorubicin were found at 2.85 and 3.75 ml, respectively. In order to determine the constant values for the complex formation and its composition, the value of the protonation constant of the ligand was determined. Using the daunorubicin and doxorubicin titrating curve, the values of the protonation constants were calculated as: log $K_1 = 8.40$ and log $K_2 = 12.84$ for daunorubicin (Table 2), log $K_1 = 8.60$ and log $K_2 = 10.22$ for doxorubicin (Table 3). These values were confirmed by Schwarzenbach graphic curve (Inczedy, 1979).

Table 3
Values of protonation constant for doxorubicin (log K) at some titrating fraction (a) and respective pH of the solution

a	pH	$[H^+]$	$[OH^-]$	log K_1
$C_L = 8.55 \times 10^{-4}$ M, $0 < a < 1$				
0.33	8.14	7.24×10^{-9}	1.38×10^{-6}	7.84
0.44	8.32	4.78×10^{-9}	2.09×10^{-6}	8.22
0.56	8.48	3.31×10^{-9}	3.02×10^{-6}	8.59
0.67	8.51	2.13×10^{-9}	4.68×10^{-6}	8.98
0.78	8.82	1.51×10^{-9}	6.61×10^{-6}	9.39
			log K_1 (mean ± S.D.)	8.60 ± 0.61
$C_L = 8.44 \times 10^{-4}$ M, $1 < a < 2$				
1.17	9.74	1.82×10^{-10}	5.50×10^{-5}	9.23
1.28	9.99	1.02×10^{-10}	9.77×10^{-5}	9.81
1.39	10.21	6.16×10^{-11}	1.62×10^{-4}	10.04
1.44	10.30	5.01×10^{-11}	1.99×10^{-4}	10.62
1.56	10.45	3.55×10^{-11}	2.82×10^{-4}	11.38
			log K_2 (mean ± S.D.)	10.22 ± 0.82

Table 4
Average number of ligands (n) corresponding to the concentration of free ligand (L) for some values of titrating fractions (a) considering the reactions of protonation (α) that occur during daunorubicin (DNR) titration

$C_{DNR} = 9.44 \times 10^{-4}$ M, $C_{Mn(II)} = 3.21 \times 10^{-4}$ M					
a	$[H^+]$	$\alpha_{L(H)}$	$[L]$	$-\log [L]$	n
0.33	$10^{-7.91}$	2.57×10^5	3.07×10^{-9}	8.51	0.48
0.44	$10^{-7.97}$	1.95×10^5	3.78×10^{-9}	8.42	0.64
0.67	$10^{-8.07}$	1.23×10^5	5.11×10^{-9}	8.29	0.98
0.89	$10^{-8.24}$	5.62×10^4	9.33×10^{-9}	8.03	1.31
1.12	$10^{-8.52}$	1.55×10^4	2.69×10^{-9}	7.57	1.64
1.33	$10^{-8.73}$	5.89×10^3	5.42×10^{-9}	7.27	1.95
1.56	$10^{-8.88}$	2.95×10^3	7.16×10^{-9}	7.14	2.28
1.78	$10^{-9.15}$	8.52×10^2	1.30×10^{-9}	6.89	2.60

Based on the values of the total concentration of daunorubicin (DNR) or doxorubicin (DOX) [C_{DNR} or C_{DOX}] and free ligand [L], total concentration of Mn(II) [$C_{Mn(II)}$] and the average ligand number [n], considering the protonation reactions [$\alpha_{L(H)}$] (Table 4 for daunorubicin and Table 5 for doxorubicin) the composition of the daunorubicin–Mn(II) complex was determined as 3:1 (Fig. 7A) and that of the composition of the doxorubicin–Mn(II) complex was 2:1 (Fig. 7B). The constant stability value for the investigated complexes were calculated for daunoru-

Table 5
Average number of ligands (n) corresponding to concentration of free ligand (L) for some values of titrating fractions (a) considering the reactions of protonation (α) that occur during doxorubicin (DOX) titration

$C_{DOX} = 9.46 \times 10^{-4}$ M, $C_{Mn(II)} = 3.22 \times 10^{-4}$ M					
a	$[H^+]$	$\alpha_{L(H)}$	$[L]$	$-\log [L]$	n
0.33	$10^{-7.90}$	1053	7.530×10^{-7}	6.12	0.47
0.44	$10^{-8.01}$	635.8	1.167×10^{-6}	5.93	0.63
0.67	$10^{-8.17}$	304.7	2.076×10^{-6}	5.68	0.97
0.89	$10^{-8.31}$	161.4	3.299×10^{-6}	5.48	1.28
1.06	$10^{-8.49}$	71.47	6.390×10^{-6}	5.19	1.52
1.17	$10^{-8.73}$	24.65	1.698×10^{-5}	4.77	1.64
1.28	$10^{-9.06}$	6.359	6.679×10^{-5}	4.18	1.62

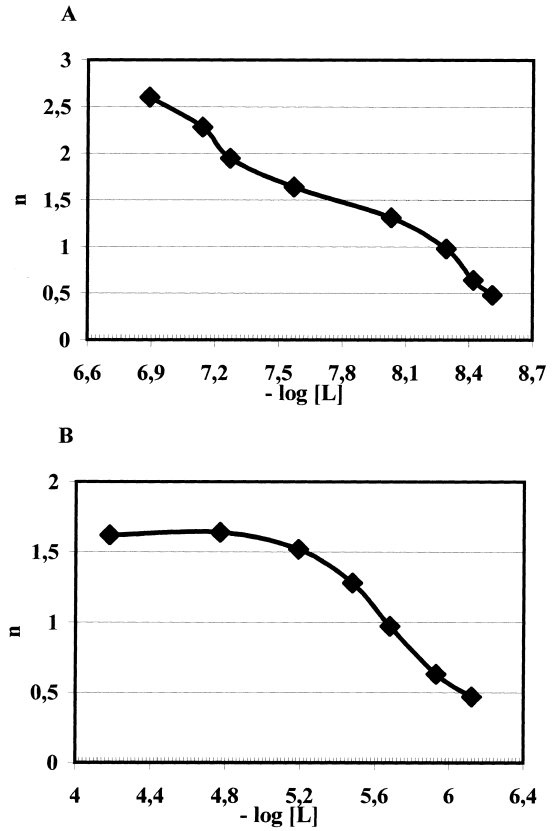

Fig. 7. The diagram determining the composition and the constants of the daunorubicin–Mn(II) complex (A) and of the doxorubicin–Mn(II) complex stability (B). [*L*]—Concentration of free ligand (equilibrium concentration), *n*—average number of ligands.

bicin as $\beta_{av} = (1.74 \pm 0.01) \times 10^{23}$, and for doxorubicin $\beta_{av} = (1.99 \pm 0.025) \times 10^{11}$.

4. Discussion

One of the recognized side effects of some anthracyclines (daunorubicin or doxorubicin) administration is poor wound healing (Bland et al., 1984). Biochemical observations have further indicated that poor wound healing in anthracycline-treated animals is a result of the impairment of collagen synthesis (Devereux et al., 1979; Sasaki et al., 1987). However, the mechanism for the phenomenon is not fully understood.

The data presented here show that daunorubicin and doxorubicin induce a strong inhibition of prolidase activity in cultured human skin fibroblasts. Simultaneously, the decrease in the enzyme activity was correlated to the decrease in biosynthesis of protein susceptible to the action of bacterial collagenase. The estimated IC_{50} for the inhibition of prolidase activity in skin fibroblast cultures were about 0.3 and 10 μM for daunorubicin and doxorubicin, respectively, while the IC_{50} for the inhibition of collagen biosynthesis were found to be 1 and 15 μM for daunorubicin and doxorubicin, respectively. The concentration of the drugs used in our experiments and the period of cell exposure to the drugs are clinically relevant (Chan et al., 1978; Ozols et al., 1980). It is known that 2 h after intravenous infusion of anthracyclines (average therapeutical doses 60 mg/m^2 body surface), the drug plasma concentration is 15–50 ng/ml and the drug tissue concentration is about 5–20 μg/g, suggesting rapid transport of the drugs into cells. The half-life of anthracyclines is reported to be 18–26 h. The intracellular therapeutical concentration of anthracyclines is, therefore, about 10–30 μM—which is within the range of concentrations tested in our studies.

Cell viability measured by tetrazoline test after 24-h incubation with above concentrations of daunorubicin or doxorubicin revealed no significant differences from the control fibroblasts cultured without anthracyclines, suggesting the specificity of daunorubicin- and doxorubicin-induced effects. Therefore, it seems that the inhibition of collagen biosynthesis by both anthracyclines is mostly due to the inhibition of prolidase activity. Decrease in enzyme activity due to the treatment of confluent cells with daunorubicin or doxorubicin was not accompanied by any difference in the amount of enzyme protein recovered from these cells as shown by Western immunoblot analysis. It suggests that the inhibitory effects of anthracyclines on prolidase activity are probably a post-translational event. We have found that the phenomenon may be due to the ability of daunorubicin or doxorubicin to form stable complex with Mn(II), a metal required for prolidase activity. The constant value for complex formation were around 1.74×10^{23} and around 1.99×10^{11} for daunorubicin and doxorubicin, respectively. This may explain the mechanism of prolidase activity decrease in fibroblasts treated with the anthracyclines. However, the constant stability values of the complexes of daunorubicin with manganese (II) and doxorubicin with manganese (II) are lower than those with iron (II) (Beraldo et al., 1985). It means that anthracycline–iron (II) complexes are more stable than anthracycline–manganese (II) complexes. These results support the data of Irving and Williams (1953), which classified divalent metal ions according to the stability of the complex formation as follows: Mn < Fe < Co < Ni < Cu.

The mechanism of daunorubicin—as well as doxorubicin-induced inhibition of prolidase activity may be also due to the anthracycline-induced free radical formation (as a result of complex formation) and subsequent damage to the enzyme protein. It is well documented that quinone reduction and the subsequent generation of free radicals and reactive oxygen species are implicated in the toxicity of doxorubicin action (Bland et al., 1984; Young et al., 1981).

The primary biological function of prolidase involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen synthesis (Jackson et al., 1975; Chamson et al., 1989; Yaron and Naider, 1993). It is evident that the absence of

prolidase will severely impede the efficient recycling of collagen proline. The clinical symptoms related to collagen deficit, which led Goodman et al. (1968) to liken the conditions to lathyrism, can be partially attributed to this effect. On the other hand, enhanced liver prolidase activity was found during the fibrotic process (Myara et al., 1987). It suggests that prolidase, which provides proline for collagen biosynthesis, may regulate the turnover of collagen and may be a rate-limiting factor in the regulation of collagen production. Recently, a link has been found between collagen production and prolidase activity in cultured human skin fibroblasts treated with anti-inflammatory drugs (Miltyk et al., 1996) during experimental aging of these cells (Pałka et al., 1996), fibroblasts chemotaxis (Pałka et al., 1997) and cell surface integrin receptor ligation (Pałka and Phang, 1997). Thus, as a result of daunorubicin- and doxorubicin-induced inhibition of prolidase activity, collagen biosynthesis is decreased. Moreover, the anthracyclines inhibited DNA synthesis at concentrations that were shown to inhibit prolidase activity and collagen synthesis. However, decrease in prolidase activity cannot be explained as a result of DNA suppression since expression of prolidase in fibroblasts treated with the anthracyclines (as shown by Western immunoblot analysis) was the same as in control cells. Therefore, it seems that the decrease in prolidase activity may not be related to the inhibition of DNA synthesis. On the other hand, the anti-mitotic effect of anthracyclines in vivo may result in the decrease of tissue fibroblasts' number (Bland et al., 1984)—contributing to the decrease in overall collagen production by these cells. Our results do not provide an experimental basis for the measurement of such a possibility, since in order to provide optimal conditions for assays of collagen biosynthesis (Makela et al., 1990) and prolidase activity (Myara et al., 1985) we used confluent (not proliferating) cells, where anthracyclines are less effective than in proliferating cells. Nevertheless, it seems likely that during the process of in vivo wound healing, the growth of proliferating cells may be significantly suppressed by anthracyclines.

We suggest that both phenomena (decrease in cell number and decrease in prolidase activity) are responsible for the suppression of collagen synthesis in cells exposed to anthracyclines. It may explain the potential mechanism of poor wound healing among patients administered with anthracyclines. The data presented here give rise to the possibility that daunorubicin- and doxorubicin-induced decrease in collagen biosynthesis may be mostly due to the inhibition of prolidase activity through the chelation of manganese metal required for prolidase activity.

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