The secondary alcohol and aglycone metabolites of doxorubicin alter metabolism of human erythrocytes

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Abstract

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Anthracyclines, a class of antitumor drugs widely used for the treatment of solid and hematological malignancies, cause a cumulative dose-dependent cardiac toxicity whose biochemical basis is unclear. Recent studies of the role of the metabolites of anthracyclines, i.e., the alcohol metabolite doxorubicinol and aglycone metabolites, have suggested new hypotheses about the mechanisms of anthracycline cardiotoxicity. In the present study, human red blood cells were used as a cell model. Exposure (1 h at 37°C) of intact human red blood cells to doxorubicinol (40 μM) and to aglycone derivatives of doxorubicin (40 μM) induced, compared with untreated red cells: i) a ~2-fold stimulation of the pentose phosphate pathway (PPP) and ii) a marked inhibition of the red cell antioxidant enzymes, glutathione peroxidase (~20%) and superoxide dismutase (~60%). In contrast to doxorubicin-derived metabolites, doxorubicin itself induced a slighter PPP stimulation (~35%) and this metabolic event was not associated with any alteration in glutathione reductase, glutathione peroxidase, catalase or superoxide dismutase activity. Furthermore, the interaction of hemoglobin with doxorubicin and its metabolites induced a significant increase (~22%) in oxygen affinity compared with hemoglobin incubated without drugs. On the basis of the results obtained in the present study, a new hypothesis, involving doxorubicinol and aglycone metabolites, has been proposed to clarify the mechanisms responsible for the doxorubicin-induced red blood cell toxicity.

Introduction

Doxorubicin (DOX), an anthracycline drug widely used for the treatment of solid and hematological malignancies (1), causes cumulative dose-dependent cardiac toxicity characterized by irreversible dilated cardiomyopathy and congestive heart failure (2).

Although several biochemical mechanisms have been proposed to account for the cardiotoxicity of this drug, the exact mechanism is not clear. Biochemical and physiological data favor the hypothesis that DOX causes the formation of free radicals that stimulate lipid peroxidation and alter cellular integrity (3-5). Alternative mechanisms

of DOX-induced toxicity have implicated DOX metabolites other than semiquinone free radicals. Recently, it has been demonstrated that in human cardiac cytosol, DOX is primarily converted to a secondary alcohol metabolite, i.e., doxorubicinol (DOXol) and/or to a broad panel of aglycones (see Figure 1) by an NADP(H)-dependent mechanism (6). *In vivo* and *in vitro* studies have demonstrated that DOXol may be involved in several mechanisms of toxicity in heart tissue, which include inhibition of the Ca^{2+}/Mg^{2+} ATPase of sarcoplasmic reticulum, the F0-F1 proton pump of mitochondria and the Na^+-K^+ ATPase and Na^+-Ca^{2+} exchanger of sarcolemma (7,8). Toxic effects on a number of mitochondrial functions (9,10) have been reported for the aglycone derivatives of DOX.

Moreover, *in vitro* and *in vivo* studies have shown that several parameters indicative of red blood cell function and integrity might be negatively affected by anthracycline drugs. In fact, changes in membrane ionic permeability (11), alteration of sodium transporters (12), inhibition of inositol lipid metabolism (13), increase in lipid peroxidation (14,15), and alterations of membrane structure (16) have been reported. However, although several studies on the effects of anthracyclines on red blood cells have been performed, none of them were focused on the effects of the anthracycline metabolites other than semiquinone free radicals. Earlier reports have identified secondary alcohol and aglycone metabolites of DOX in human plasma as the major metabolites following intravenous administration of this drug (17,18). Thus, it seemed appropriate to investigate the interaction of DOX metabolites with intact red blood cells and the consequent effects on their energy metabolism. A complete understanding of the functional alterations induced in human red blood cells not only by DOX but also by its main metabolites might lead to the development of new therapeutic uses of this drug.

Material and Methods

Chemicals

[2-^{13}C]-Glucose was purchased from Cambridge Isotopes Laboratories (Andover, MA, USA). DOX hydrochloride and DOXol were obtained by Pharmacia & Upjohn (Erlangen, Germany) and were dissolved in distilled water. The derived aglycones were prepared by heating the parent compounds at 100ºC for 30 min in the presence of 2 N acetic acid and extracting the cleavage products with chloroform (19) and were dissolved in dimethyl sulfoxide (DMSO). Purity was checked by [^{1}H]-NMR (data not shown). All other reagents were of the highest grade available and were obtained from Sigma (St. Louis, MO, USA).
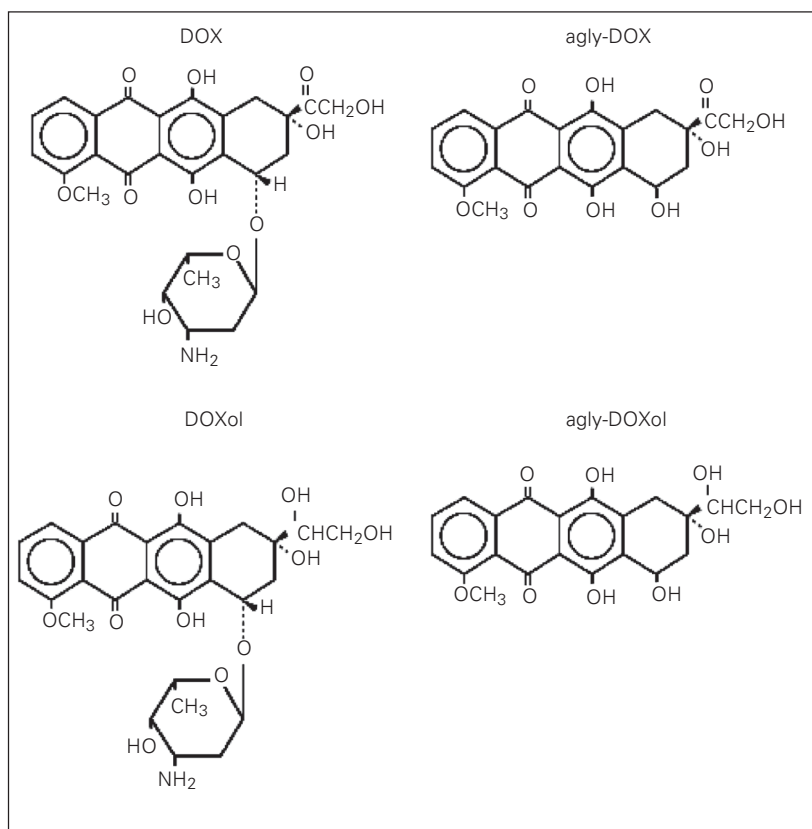

Figure 1. Chemical structures of doxorubicin (DOX), doxorubicinol (DOXol), aglycone-DOX (agly-DOX) and aglycone-DOXol (agly-DOXol).

Preparation of red blood cells

Heparinized fresh human blood was obtained from consenting informed healthy donors and processed immediately. Plasma was separated by centrifugation at 2500 g for 5 min. Erythrocytes were washed four times with HEPES buffer (25 mM Hepes, 1 mM NaH_2PO_4, 110 mM NaCl, 5 mM KCl, and 2 mM $MgCl_2$), pH 7.4, and 290 ± 5 mOsm/kg, measured with an Osmostat OM-6020 apparatus (Daiichikagakuco Ltd., Kyoto, Japan). The buffy coat together with part of the upper erythrocyte layer was removed and discarded after each washing step. After the washing procedure, the packed cells were gently resuspended with the HEPES buffer to obtain a 50% (v/v) hematocrit. Subsequently, they were divided into 5 aliquots: four were incubated with the drugs, i.e., DOX, DOXol, aglycone-DOX (agly-DOX) and aglycone-DOXol (agly-DOXol), and one was left untreated (control). The concentrations of the drugs were 40 μM. After 1 h at 37ºC in the dark, cell suspensions were washed twice with HEPES washing buffer (2500 g for 3 min at 4ºC) and used for the metabolic determinations. At the end of incubation, hemolysis was determined by measuring spectrophotometrically (Cary 3E, Varian, Palo Alto, CA, USA) the hemoglobin (Hb) concentration in the supernatant solutions obtained by centrifugation at 2500 g for 5 min at 4ºC. Methemoglobin (MetHb) levels were determined spectrophotometrically in lysed cells according to the method of Zijlstra et al. (20).

Determination of the pentose phosphate pathway

The pentose phosphate pathway (PPP) was determined by [13C]-NMR spectroscopy performed on lysates of cells incubated with [2-13C]-glucose. The following procedure was used to perform [13C]-NMR measurements: both drug-treated and untreated intact red blood cells were suspended in HEPES buffer (50%, v/v) and the cell suspensions were incubated with 15 mM [2-13C]-glucose for 6 h at 37ºC in the dark, as reported by Misiti et al. (21). At the end of the incubation time, samples were checked for pH and MetHb concentration and then immediately mixed with an equal volume of cold 12% $HClO_4$ solution (w/w). Denatured material was discarded after centrifugation (2000 g, 10 min, 4ºC) and the acid supernatant was transferred to the NMR tube for [13C]-NMR determinations. Experiments were performed using autoclaved plastic ware, glassware and buffers. Each step of sample preparation was performed in a sterile chamber to avoid sample contamination.

[13C]-NMR measurements

[13C]-NMR experiments were performed with a Gemini 300 Varian spectrometer, operating at 74.62 MHz and 25ºC, using a 5-mm diameter tube. A 45º pulse, an acquisition time of 0.8-s and 1-s recovery between pulses were used. Spectra were broadband decoupled from protons and a line broadening of 1 Hz was applied before Fourier transformation. A sealed capillary containing a 50% ^2H_2O/methanol (v/v) solution was inserted into the NMR tube. Methanol resonance (49.9 ppm) was used as chemical shift reference. The PPP activity was assessed by measuring the ratio between the signals corresponding to lactate molecules labeled at C-3 (methyl group) and at C-2 (methyne group) generated from the metabolism of [2-13C]-glucose (22).

Glutathione peroxidase, superoxide dismutase, glutathione reductase and catalase activities

Antioxidant enzymes were measured at 37ºC in hemolysates obtained from intact red blood cells after treatment with the drugs and from untreated intact cells. Glutathione

peroxidase (GPx) was determined in a coupled assay in which the rate of tert-butyl hydroperoxide-dependent NADP(H) oxidation at 340 nm was monitored by the method of Beutler (23). Superoxide dismutase (SOD) was assayed by monitoring the autoxidation of 6-hydroxydopamine at 490 nm by the method of Heikkila (24). Glutathione reductase (GR) was assayed spectrophotometrically by measuring the rate of NADP(H) oxidation at 340 nm (25). Catalase (CAT) activity was assayed using hydrogen peroxide as a substrate (23).

Determination of oxygen equilibrium curves for hemoglobin

Human Hb (HbA) was purified by lysing a packed preparation of washed human erythrocytes, followed by the addition of 2 volumes of cold hypotonic buffer. The stroma was removed by centrifugation at 12,000 *g* for 30 min and HbA purity was checked by isoelectrofocusing in 8 M urea. Purity was always higher than 97%. Organic and inorganic phosphates (i.e., stripped Hb) were removed by passing the hemolysate first through a Sephadex G-25 column, equili-

brated with 10 mM Tris buffer, pH 8.0, containing 0.1 M NaCl, and then through a column of mixed-bed ion-exchange resin (BioRad AG 501X8, Hercules, CA, USA).

Oxygen binding isotherms were determined by the tonometric method (26) at a protein concentration of 5 mg/ml. The study of the functional properties was performed in 0.1 M HEPES buffer plus 100 mM NaCl and 10 mM 2,3-diphosphoglyceric acid at pH 7.4 and 37ºC. The drugs were used at 10 µM concentration. The formation of MetHb after each measurement was less than 5%.

Titration of the sulfhydryl groups in Hb samples was performed according to the method of Boyer (27).
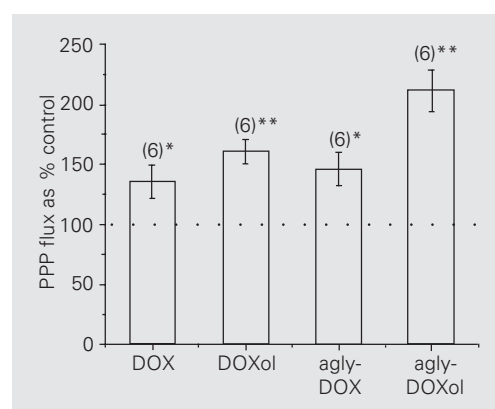
Statistical analysis

The significance of differences between the control and experimental groups was determined by the two-tailed Student *t*-test.

Results

Effect of the drugs on the metabolic pathways of red blood cells

When human erythrocytes were incubated for 1 h in the dark at 37ºC in HEPES buffer in the presence or absence of 40 µM DOX, 40 µM DOXol, 40 µM agly-DOX and 40 µM agly-DOXol, a higher PPP glucose flux was observed in the drug-treated red blood cells compared to control cells. In particular, as shown in Figure 2, DOX caused a 35% PPP flux increase, whereas DOXol, agly-DOX and agly-DOXol caused a 60, 45 and 110% increase in PPP flux, respectively, compared to control. No changes of PPP flux were observed (data not shown) when red blood cells were incubated with DMSO for 1 h at 37ºC in the dark, rouling out the possibility that DMSO itself might be responsible for the observed metabolic modifications.

Figure 2. DOX, DOXol, agly-DOX and agly-DOXol stimulate the pentose phosphate pathway (PPP) in human red blood cells. Human red blood cells were incubated for 1 h at 37ºC in the presence and absence of 40 µM DOX, 40 µM DOXol, 40 µM agly-DOX and 40 µM agly-DOXol. After drug treatment, intact red blood cells were washed and incubated with [2-^{13}C]-glucose at 37ºC for 6 h. The PPP activity was assessed by measuring the ratio between the signals corresponding to lactate molecules labeled at C-3 (methyl group) and at C-2 (methyne group) generated from the metabolism of [2-^{13}C]-glucose. Data are reported as percent of controls (no drugs, control cells = 100 ± 5%, N = 12) and as means ± SEM of the number of observations in separate experiments indicated in parentheses. The absolute value of PPP flux in the absence of any drug was 2.70 ± 0.15 nmol min^{-1} ml^{-1} RBC (N = 12). The statistical significance of the effect of DOX, DOXol, agly-DOX and agly-DOXol treatment was determined by the paired Student *t*-test (*P < 0.05; **P < 0.01). For abbreviations, see legend to Figure 1.

Effect of the drugs on red blood cell enzymes

To test the possibility that the alteration of erythrocyte metabolism following exposure to DOX, DOXol, agly-DOX and agly-DOXol might be linked to variation in GPx, SOD, GR or CAT activity, we performed an analysis of the activity of these enzymes in hemolysates. Figure 3 reports the effects of DOX, DOXol, agly-DOX and agly-DOXol on intracellular GPx activity. As can be seen, all DOX metabolites tested decreased GPx activity. In the presence of DOXol, agly-DOX and agly-DOXol, the inhibition of GPx activity was 19, 17 and 23%, respectively, of that observed in control cells. In contrast, in intact red blood cells exposed to DOX, GPx activity was unaltered. Although we employed a conventional GPx assay system that used GR as a coupling enzyme, DOX, DOXol, agly-DOX and agly-DOXol did not affect the assay system (see Figure 3). Figure 4 shows the effects of DOX, DOXol, agly-DOX and agly-DOXol on intracellular SOD. SOD activity showed significant differences between controls and drug-treated red blood cells. In erythrocytes exposed to DOXol, agly-DOXol and agly-DOX, the inhibition of SOD activity was 60% of control. Contrary to DOX-derived metabolites, DOX itself had no significant effects on SOD activity. Figure 4 reports the effects of DOX, DOXol, agly-DOXol and agly-DOX on intracellular CAT. As can be seen, CAT activity was not significantly affected after 1 h of incubation with DOX, DOXol or its corresponding aglycone metabolites. These results are consistent with the suggestion that DOX and its metabolites may have different targets within the red blood cell.

Oxygen equilibrium measurements

Table 1 reports the change in P_{50} for Hb induced by DOX, DOXol, agly-DOX and agly-DOXol at pH 7.4 and 37ºC. It is evident that the presence of the drugs caused a 22% increase in oxygen affinity compared to control.

This suggests that DOX and its main metabolites may interact with Hb modifying its binding to oxygen, and consequently causing reduced tissue oxygenation. To test the possibility that alteration of Hb oxygen binding properties following exposure to DOX, DOXol, agly-DOX and agly-DOXol might be linked to the oxidation of thiol groups, we titrated the ß-93 cysteines of HbA treated with the drugs using ρ-hydroxymercuribenzoate (PMB) (data not shown). The results obtained demonstrated that, in contrast to untreated HbA, in samples incubated with DOX, DOXol
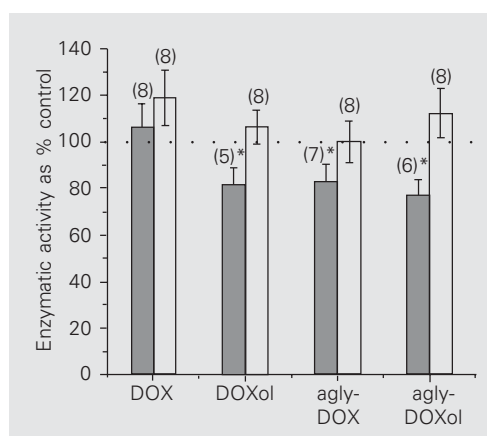

Figure 3. Effect of DOX, DOXol, agly-DOX and agly-DOXol on glutathione peroxidase (closed columns) and glutathione reductase (open columns) activity of human red blood cells. Human red blood cells were incubated for 1 h at 37ºC in the presence and absence of 40 µM DOX, 40 µM DOXol, 40 µM agly-DOX and 40 µM agly-DOXol, before hemolysis and enzymatic activity determinations as described in Material and Methods. Data are reported as percent of control activity (no drugs, control cells = 100 ± 7%, N = 6) and as means ± SEM of the number of observations in separate experiments indicated in parentheses. The statistical significance of the effect of DOX, DOXol, agly-DOX and agly-DOXol treatment was determined by the paired Student *t*-test (*P < 0.05). For abbreviations, see legend to Figure 1.
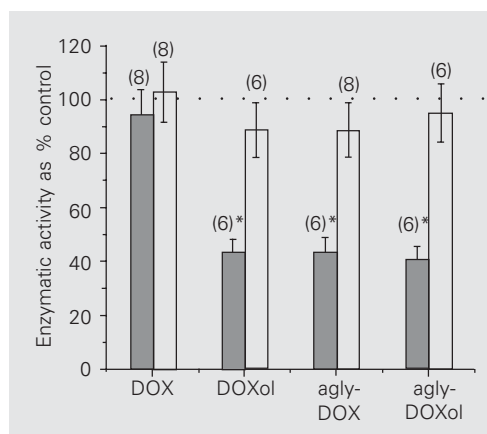

Figure 4. Effect of DOX, DOXol, agly-DOX and agly-DOXol on superoxide dismutase (closed columns) and catalase (open columns) activity of human red blood cells. Human red blood cells were incubated for 1 h at 37ºC in the presence and absence of 40 µM DOX, 40 µM DOXol, 40 µM agly-DOX and 40 µM agly-DOXol, before hemolysis and enzymatic activity determinations as described in Material and Methods. Data are reported as percent of control activity (no drugs, control cells = 100 ± 10%, N = 8) and as means ± SEM of the number of observations in separate experiments indicated in parentheses. The statistical significance of the effect of DOX, DOXol, agly-DOX and agly-DOXol treatment was determined by the paired Student *t*-test (*P < 0.01). For abbreviations, see legend to Figure 1.

and aglycones the -SH groups were not titratable, demonstrating a specific modification of this amino acid group by the drugs.

Effects of drugs on Pmethemoglobin levels and hemolysis degree

MetHb levels determined in intact red cells after treatment with anthracycline drugs and their metabolites were 6.3 ± 4.3, and the values for control cells were 5.6 ± 6.0%, N = 6, P = 0.3. MetHb levels did not change significantly even during [2-^{13}C]-glucose incubation (6 h at 37ºC). Percent hemolysis was always less than ~2%.

Discussion

The present study clearly demonstrates that not only DOX but also its main metabolites, i.e., the secondary alcohol DOXol and aglycone derivatives of DOX, dramatically affect the red blood cell energy metabolism, stimulating the PPP. These results are in agreement with previous studies that reported a dose-dependent activation of PPP in intact human erythrocytes after exposure to adriamycin and daunomycin exposure (28). Henderson et al. (28) proposed that DOX could

Table 1. Oxygen affinity of hemoglobin A in the absence and in the presence of doxorubicin (DOX), doxorubicinol (DOXol), aglycone-DOX (agly-DOX) and aglycone-DOXol (agly-DOXol).

Samples	P_{50}
HbA (control)	23.0
HbA + 10 µM DOX	18.3*
HbA + 10 µM DOXol	18.2*
HbA + 10 µM agly-DOX	18.0*
HbA + 10 µM agly-DOXol	17.9*

Hemoglobin A (HbA) was incubated in 0.1 M HEPES buffer, 0.1 M NaCl in the presence of 10 mM 2,3-diphosphoglyceric acid at pH 7.4 and 37ºC. The average SEM was ± 8% for P_{50} values (N = 6). Data (P_{50}) are reported in mmHg. The statistical significance of the effect of DOX, DOXol, agly-DOX and agly-DOXol treatment was determined by the paired Student *t*-test (*P < 0.05).

undergo cyclic oxidation-reduction within the red blood cell acting as a mediator of electron transfer from NADP(H) via semiquinone to oxyhemoglobin. Thus, the direct oxidation of NADP(H) by DOX could account for the larger portion of the PPP stimulation that we observed in intact red blood cells following DOX exposure. Therefore, the cited investigators proposed that the reactive oxygen species generated during the oxidation-reduction cycle of anthracyclines might oxidize intracellular glutathione and provide further PPP stimulation. Bates and Winterbourn (29) proposed that $O_2^{-\bullet}$ and adriamycin semiquinone may be generated directly by reaction of adriamycin with oxyhemoglobin. However, although many *in vitro* studies devoted to the understanding of the biochemistry have emphasized the toxic effects of anthracyclines within the red blood cells, the molecular basis of these effects is not fully understood. Alternative mechanisms of DOX-induced toxicity have implicated DOX metabolites other than semiquinone free radicals. This is the case for DOXol, a hydroxy metabolite formed upon a two-electron reduction of the C-13 carbonyl group in the side chain of DOX (7,8,30) and for the aglycone metabolites (9,10). Thus, in the present study, we used red blood cells as a cell model in order to acquire more information on the biological activity of DOXol, agly-DOX and agly-DOXol. It should also be noted that, although the concentrations of the drugs used in this study would not be achievable in the blood stream in normal human usage, in many earlier *in vitro* studies concerning the effects of anthracyclines or other drugs on red blood cell, the drugs were used at similar or higher concentrations (13,16,28,29,31). Here, we show that both DOXol and the aglycone derivatives of DOX increased the PPP flux in human red blood cells. It is important to remark that DOXol as well as its corresponding aglycone metabolite were at least 1.8 times more potent than the parent compound in the stimulation of

the PPP glucose flux. Recent studies carried out to identify the mechanisms responsible for the anthracycline-induced cardiotoxicity have demonstrated that DOXol might indeed be more potent than DOX in causing metabolic dysfunction (7,8).

Due to the high levels of Hb within the red blood cells, it is possible that, under our experimental conditions, the interaction of some fractions of DOX and its metabolites with Hb may represent another important metabolic event occurring within the cell after drug exposure. Thus, we determined the O_2 affinity of Hb treated with DOX and its metabolites in order to assess whether the interaction of Hb with the drugs may modify its oxygen binding properties. Under physiological conditions, we demonstrated an increase in the O_2 affinity of drug-treated Hb compared to free-drug Hb. This finding, plus the impossibility to titrate the -SH groups in drug-treated Hb, is probably due to the chemical modification exerted by anthracycline drugs on the cysteine residues of HbA. In this regard, it is known that the oxygen affinity of HbA is higher than normal when cysteines ß-93 are chemically modified (32).

This result raises the new hypothesis of interplay between anthracyclines and Hb in addition to the recent suggestion regarding the existence of two binding sites for DOX on Hb (33,34). However, because the O_2 affinity of Hb is a crucial factor in determining O_2 delivery in the blood, in cancer patients treated with DOX a higher O_2 affinity of blood might favor pulmonary O_2 loading but impair O_2 release to tissues. Although further study is required to clarify this point, we suggest that this finding might be relevant to the understanding of the mechanisms of toxicity induced by anthracyclines.

Previous studies have demonstrated that the toxic effects of DOX are believed to be mediated by free radicals (3-5). Thus, in an attempt to better clarify the mechanisms underlying the PPP stimulation and the increased O_2 affinity of Hb observed in red blood cell following exposure to DOX and DOX metabolites, we assessed the activities of different red cell antioxidative enzymes, including GPx, SOD, CAT and GR. Our data showed that DOX treatment had no significant effects on the red cell GPx, SOD, CAT and GR enzymes, in agreement with *in vitro* reports that red cell enzymes of the reduction systems are not inactivated by DOX (35). In contrast to DOX, DOXol and the aglycone derivatives of DOX only inhibited the red cell GPx and SOD enzymes. In this respect, we suggest that a large accumulation of oxygen reactive species within the red blood cells after treatment with DOXol and aglycone derivatives of DOX might represent an inevitable consequence of impairment of the red cell GPx and SOD enzymes induced by DOX metabolites. Thus, the oxidation of sulfhydryl groups, induced by the oxygen reactive species accumulated within the red blood cell, may account for the larger portion of the PPP stimulation and for the increased O_2 affinity of Hb that we observed in intact red blood cells following DOXol and aglycone metabolites exposure.

Furthermore, the present data allow us to suggest that differences in PPP activity which exist among cell types may lead to variability in the sensitivity of cells to toxicity induced by DOX metabolites. This is consistent with earlier findings showing that multidrug resistant cells have an elevated PPP activity (36,37).

On the basis of the present findings, we hypothesize that in cancer patients treated with anthracyclines, as plasma levels of DOXol and aglycone metabolites increase during prolonged exposure to the drug, an impairment of the activity of the red blood cell antioxidant enzymes may progressively alter the redox status of the cell, inducing important alterations in the red blood cell functions. Furthermore, the results of this study support the hypothesis that DOX metabolites other than semiquinone free radicals might play an important role in the mechanisms responsible for DOX-induced cell toxicity.

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