Doxorubicin inhibits oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by a lactoperoxidase/H₂O₂ system by reacting with ABTS-derived radical

Krzysztof J. Reszka *, Bradley E. Britigan

Research Service, VA Medical Center, Cincinnati, OH 45230, USA
Department of Internal Medicine, University of Cincinnati, 231 Albert Sabin Way, ML 0557, Cincinnati, OH 45267-0557, USA

Abstract

The effect of doxorubicin on oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by lactoperoxidase and hydrogen peroxide has been investigated. It was found that: (1) oxidation of ABTS to its radical cation (ABTS$^{\cdot+}$) is inhibited by doxorubicin as evidenced by its induction of a lag period, duration of which depends on doxorubicin concentration; (2) the inhibition is due to doxorubicin hydroquinone reducing the ABTS$^{\cdot+}$ radical (stoichiometry 1: 1.8); (3) concomitant with the ABTS$^{\cdot+}$ reduction is oxidation of doxorubicin; only when the doxorubicin concentration decreases to a near zero level, net oxidation of ABTS could be detected; (4) oxidation of doxorubicin leads to its degradation to 3-methoxysalicylic acid and 3-methoxyphthalic acid; (5) the efficacy of doxorubicin to quench ABTS$^{\cdot+}$ is similar to the efficacy of p-hydroquinone, glutathione and Trolox C. These observations support the assertion that under certain conditions doxorubicin can function as an antioxidant. They also suggest that interaction of doxorubicin with oxidants may lead to its oxidative degradation.
Published by Elsevier Inc.

Keywords: ABTS; Anthracyclines; Doxorubicin; EPR; Glutathione; Hydroquinone; Inactivation; Lactoperoxidase; Oxidation; Trolox C

The biological action of the anticancer anthracyclines doxorubicin (DOX, Adriamycin) and daunorubicin (DNR)[1] is frequently linked to their ability to induce oxidative stress through generation of free radicals and ROS. This property results from the presence in the drugs' structures of a quinone moiety (Fig. 1, ring C), which may undergo metabolic reduction and, via aerobic redox cycling, generate superoxide and, subsequently, other more reactive forms of oxygen [1–3]. It is believed that ROS may play a role in the anthracycline-induced cardiotoxicity [4].

Several recent studies demonstrated that anthracyclines also possess reducing capabilities since they react with oxidants. This is not surprising, as the drugs contain in their chromophores an electron-donating hydroquinone moiety (Fig. 1, ring B). For example, DOX and DNR have been shown to undergo oxidation by microperoxidase/H₂O₂ [5], the oxo-ferryl form of myoglobin [6,7] and hemoglobin [8]. In the presence of nitrite, acetaminophen or salicylic acid lactoperoxidase (LPO) and myeloperoxidase (MPO) also oxidize the drugs [9–11]. It has also been demonstrated that DOX inhibits cardiac lipid conjugated dienes and lipid hydroperoxide formation in blood plasma from the coronary sinus and femoral artery of cancer patients [12]. DOX also inhibits lipid peroxidation in model systems containing myoglobin and H₂O₂ [6] and protects cardiomyocytes against iron-mediated toxicity [13]. These observations suggest that anthracyclines can function as antioxidants.

The above reports prompted us to explore further the putative antioxidant properties of anthracyclines. In the

---

* Corresponding author. Fax: +1 513 558 0852.
E-mail address: reszkakj@ucmail.uc.edu (K.J. Reszka).

[1] *Abbreviations used:* DNR, daunorubicin; DOX, doxorubicin; LPO, lactoperoxidase; MPO, myeloperoxidase; ABTS, 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonate).

R: -H   Daunorubicin (DNR)
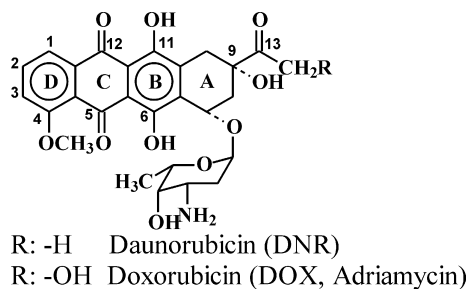R: -OH   Doxorubicin (DOX, Adriamycin)

Fig. 1. Structures of DOX and DNR.

present study, we investigated the effect of DOX on oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by LPO and hydrogen peroxide (H_2O_2). ABTS is an excellent substrate for peroxidases [14–16] and is frequently used to study antioxidant properties of natural compounds [17–19] and electron transfer reactions involving free radicals [20]. Oxidation of ABTS affords a persistent radical cation, ABTS$^{·+}$, which intensely absorbs light in the visible region of the spectrum (λ_{max} 415, 730 nm). Antioxidant properties of a tested compound are usually assayed by measuring its inhibitory action on the rate of the appearance of ABTS$^{·+}$, and/or induction and duration of lag period preceding the appearance of the radical. Alternatively, the testing compound can be added to the preformed ABTS$^{·+}$ radical. This causes reduction of the radical, which is manifested as a decrease of the absorbance at specific wavelength. The magnitude of this decrease is correlated with the amount of the antioxidant used. Here, we used both these approaches to investigate the antioxidant properties of DOX and compared them with those of p-hydroquinone, glutathione and Trolox C.

Materials and methods

Chemicals

ABTS (diammonium salt) was from Aldrich (Milwaukee, WI). LPO, H_2O_2 (30%), p-hydroquinone (p-QH$_2$), Trolox C, glutathione (GSH, reduced form) and 3-methoxysalicylic acid (3MeSA) were purchased from Sigma (St. Louis, MO). α-D(+)-Glucose (Gl) was from ACROS Organics (New Jersey, USA) and glucose oxidase (GO) (Type X, 100 U/mg) from MP Biochemicals, Inc. (Solon, OH). 3-Methoxyphthalic acid (3MePA) was a gift from Mr. Wagner (University of Iowa). DOX (hydrochloride form), solution for injection (2 mg/mL) was from Ben Venue Laboratories, Inc. (Bedford, OH). The concentrations of stock solutions of the reactants were determined spectrophotometrically using $\varepsilon_{240} = 39.4 \, M^{-1} \, cm^{-1}$ for H_2O_2 [21], $\varepsilon_{412} = 1.12 \times 10^5 \, M^{-1} \, cm^{-1}$ for LPO [22] and $\varepsilon_{480} = 1.15 \times 10^4 \, M^{-1} \, cm^{-1}$ for DOX [23]. The concentration of ABTS was determined using $\varepsilon_{340} = 3.6 \times 10^4 \, M^{-1} \, cm^{-1}$ and that of ABTS$^{·+}$ using $\varepsilon_{415} = 3.6 \times 10^4 \, M^{-1} \, cm^{-1}$ [24], or $\varepsilon_{730} = 1.5 \times 10^4 \, M^{-1} \, cm^{-1}$ [17].

Spectrophotometric measurements

Absorption spectra were measured using an Agilent diode array spectrophotometer model 8453 (Agilent Technologies, Inc., Chesterfield, MO). Measurements were performed in phosphate buffer pH 7.0

(50 mM) at room temperature. Typically the reaction was initiated by addition of a small aliquot of H_2O_2 stock solution (2.5 μL) as the last component to a sample consisting of ABTS, DOX and LPO in buffer. When glucose/glucose oxidase (Gl/GO) was used to generate H_2O_2, GO (1 μL) was added last to a final concentration of 0.05 U/mL. Using ABTS and LPO, the rate of H_2O_2 generation by Gl/GO was determined to by 9 μM H_2O_2/min. The formation or the decay of the ABTS$^{·+}$ radical was measured at 415 and 730 nm during 10 or 20 min reaction. Concomitant changes in the concentration of DOX were determined measuring absorbance at 480 nm during the same time period. Data were collected in 5 s intervals during continuous stirring of the sample in a spectrophotometric cuvette (1 cm light path). All experiments were repeated at least twice.

Preparation and reactions of ABTS$^{·+}$ radical

The ABTS$^{·+}$ radical was prepared by reacting ABTS (100 μM) with H_2O_2 (14 μM) in the presence of LPO (26 nM). This generated ~25 μM ABTS$^{·+}$. The progress of the ABTS$^{·+}$ formation was monitored at 415 and 730 nm collecting data every 5 s. When the A_{415} stabilized, an aliquot of DOX (Trolox C, p-QH$_2$ or GSH) stock solution was added and changes in A_{415} were continuously recorded. The amount of ABTS$^{·+}$ lost was determined from ΔA_{415} and converted to μM using $\varepsilon_{415} = 3.6 \times 10^4 \, M^{-1} \, cm^{-1}$. Calculations based on ΔA_{730} and using ε_{730} (ABTS$^{·+}$) $= 1.5 \times 10^4 \, M^{-1} \, cm^{-1}$ gave similar results. When DOX was used, concomitant changes in absorbance at 480 nm were also measured for the same period of time to assess effects of the reaction on the drug. The rate of ABTS$^{·+}$ reduction was calculated based on ΔA_{415} during the initial fast phase of the reaction (first 5 s after the addition of a reducing agent DOX, p-QH$_2$, Trolox C or GSH).

EPR measurements

EPR spectra were recorded using a Bruker EMX EPR spectrometer (Bruker BioSpin Co., Billerica, MA), operating in X band and equipped with a high sensitivity resonator ER 4119HS. Samples (total volume 250 μL) were prepared in pH 7.0 buffer and the reaction was initiated by addition of H_2O_2 as the last component. In cases, in which H_2O_2 was generated by Gl/GO, GO was added last. The sample was transferred to a flat aqueous EPR cell and recording was started 1 min after initiation of the reaction. Spectra of ABTS$^{·+}$ were recorded using microwave power 5.17 mW, modulation amplitude 0.2 G, receiver gain 2×10^5, conversion time 40.96 ms, time constant 81.92 ms and scan rate 80 G/41.94 s. The effect of DOX was assessed by recording EPR spectra of ABTS$^{·+}$ in 1 min intervals from samples containing various amounts of the drug. The EPR spectrum of a DOX-derived radical was recorded using microwave power 40 mW, modulation amplitude 2 G. Other parameters remained unchanged.

Mass spectrometry

Samples for MS analysis were prepared by oxidation of DOX by LPO/H_2O_2 in the presence of ABTS in phosphate buffer, pH 7.0. When oxidation of DOX was nearly completed (A_{480} reached minimum), samples were acidified to pH ~ 1 with 12 M HCl and extracted with ethyl acetate. The organic phase was evaporated under a stream of nitrogen and the remaining dry materials were dissolved in methanol or in water. The samples were then diluted at a ratio of 1–5 in 50/50 (vol/vol) MeOH/H_2O, or 50/50 (vol/vol) MeOH/CHCl$_3$ for those that were not soluble in MeOH. The sample solutions were then directly infused into the mass spectrometer using a syringe pump at a flow rate of 5 mL/min. The instrument used was Micromass (Waters) Q-TOF 2 mass spectrometer, which was tuned and calibrated to acquire spectra in negative ion mode. The acquired data were processed using MassLynx 4.0 software. Identification of DOX degradation products was accomplished by comparing with mass spectra and fragmentation pattern of authentic 3MeSA and 3MePA samples.