Oxidation of Anthracyclines by Peroxidase Metabolites of Salicylic Acid

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ABSTRACT

Oxidation of anthracyclines leads to their degradation and inactivation. This process is carried out by peroxidases in the presence of a catalytic cofactor, a good peroxidase substrate. Here, we investigated the effect of salicylic acid, a commonly used anti-inflammatory and analgesic agent, on the peroxidative metabolism of anthracyclines. We report that at pharmacologically relevant concentrations, salicylic acid stimulates oxidation of daunorubicin and doxorubicin by myeloperoxidase and lactoperoxidase systems and that efficacy of the process increases markedly on changing the pH from 7 to 5. This pH dependence is positively correlated with the ease with which salicylic acid itself undergoes metabolic oxidation and involves the neutral form of the acid (pKf = 2.98). When salicylic acid reacted with a peroxidase and H2O2 at acid pH (anthracyclines omitted), a new metabolite with absorption maximum at 412 nm was formed. This metabolite reacted with anthracyclines causing their oxidation. It was tentatively assigned to biphenyl quinone, formed by oxidation of biphenol produced by dimerization of salicylic acid-derived phenoxyl radicals. The formation of this product was inhibited in a concentration-dependent manner by the anthracyclines, suggesting their scavenging of the salicylate phenoxyl radicals. Altogether, this study demonstrates that oxidation of anthracyclines is mediated by peroxidase metabolites of salicylic acid, such as phenoxyl radicals and the biphenol quinone. Given that cancer patients undergoing anthracycline chemotherapy may be administered salicylic acid-based drugs to control pain and fever, our results suggest that liberated salicylic acid could interfere with anticancer and/or cardiotoxic actions of the anthracyclines.

Acetylsalicylic acid (aspirin, ASA) (Fig. 1) is a commonly used analgesic and anti-inflammatory drug. ASA acts by inhibiting cyclooxygenase (COX) and lipooxygenase enzymes and suppresses release of prostaglandins. In vivo, ASA is rapidly metabolized to salicylic acid (SA, 2-hydroxybenzoic acid) (Fig. 1), which, although unable to inhibit cyclooxygenase activity, still exerts anti-inflammatory properties. Recent studies suggest that SA and related nonsteroidal anti-inflammatory drugs (NSAIDs) possess preventive and therapeutic anticancer properties (Sotiriou et al., 1999; Andrews et al., 2002; Gwyn and Sinicrope, 2002; Thun et al., 2002). These functions are partly due to their inhibition of COX-2, an inducible form of COX that is overexpressed in cancer cells (Hussain et al., 2003). Studies have shown that SA possesses both anti- and prooxidant properties, which are unrelated to its inhibition of COX. The antioxidant functions of SA are due to its ability to intercept reactive oxygen and nitrogen products. It has been shown that in cell-free systems SA scavenges, ‘OH radicals generated chemically (Fenton system) and radiolytically, to produce hydroxylated products 2,5- and 2,3-dihydroxybenzoic acids (Maskos et al., 1990). These reactions seemed to be so specific that dihydroxybenzoic acids have been used as an index of generation of ‘OH in vitro and in vivo (Floyd et al., 1986; Sagone and Husney, 1987; Davis et

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ABBREVIATIONS: ASA, acetylsalicylic acid (aspirin); COX, cyclooxygenase; SA, salicylic acid; NSAID, nonsteroidal anti-inflammatory drug; MPO, myeloperoxidase; DXR, doxorubicin (Adriamycin); DNR, daunorubicin; LPO, lactoperoxidase; HOOC-SA-OH, –OOC-SA-OH, HOOC-SA-O; neutral and anionic forms of salicylic acid and the respective phenoxyl radical; SA-BPH, biphenol form of SA; SA-BPQ, biphenol quinone form of SA; Q-OOH2, Q-OOH, and Q-O, the quinone-hydroquinone moiety of anthracyclines, and the corresponding semiquinone and di-quinone forms; EPR, electron paramagnetic resonance.
SA reacts with peroxynitrite (Kaur et al., 1997), quenches singlet oxygen (Kalyanaraman et al., 1993), and inhibits superoxide/NO-dependent low density lipoprotein oxidation (Hermann et al., 1999a).

Similar to other phenolic compounds, SA is a substrate for peroxidases. HRP/H$_2$O$_2$ and methemoglobin/H$_2$O$_2$ oxidize SA to the corresponding phenoxyl radical, as demonstrated using EPR (Shiga and Imaizumi, 1973, 1975). Incubation of SA with metmyoglobin/H$_2$O$_2$ affords 2,3- and 2,5-dihydroxybenzoic acids (Galaris et al., 1988). SA stimulates low density lipoprotein oxidation by MPO/H$_2$O$_2$, through the intermediacy of SA-derived phenoxyl radicals (Hermann et al., 1999b). Ascorbate peroxidase and lactoperoxidase metabolize SA at acidic pH (Kvaratskhelia et al., 1997; Muraoka and Miura, 2005). Stimulated granulocytes induce decarboxylation of SA; however, no major role for MPO in this process was envisaged (Sagone and Husney, 1987).

It has also been reported that NSAIDs modulate the cytotoxic action of anticancer agents (Inchiosa and Smith, 1990; Duffy et al., 1998). This aspect of the biochemistry of NSAIDs is of particular interest given that during chemotherapy, cancer patients may also be administered NSAIDs. Earlier, we have reported that acetaminophen, a phenolic compound and the active ingredient of the popular analgesic drug Tylenol, stimulates oxidation of the anticancer anthracyclines doxorubicin (DNR) and daunorubicin (DNR) by peroxidases (Reszka et al., 2004). Because the reaction leads to degradation of anthracyclines and loss of their anticancer and cytotoxic activities, better understanding of this process and mechanisms involved may be important for clinical oncology. It was of interest to find out whether other phenolic compounds also stimulate oxidative degradation of DNR(DXR).

We were particularly interested in SA since it may be used by cancer patients undergoing anthracycline chemotherapy. We report that at pharmacologically relevant concentrations (<2 mM; Stead and Moffat, 1983), SA efficiently stimulates oxidative degradation of DNR(DXR) by LPO(MPO)/H$_2$O$_2$ systems especially at acidic pH. We also show that the peroxidative metabolism of SA gives rise to a redox-active product, presumably of a biphenol type, which also mediates oxidation of anthracyclines.

Materials and Methods

Chemicals. DNR (hydrochloride form) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health (Bethesda, MD). Pharmaceutical preparation of DXR (hydrochloride form) solution for injection (2 mg/ml) (Ben Venue Laboratories, Inc., Bedford, OH) was purchased from The University of Iowa Hospitals and Clinics Pharmacy (Iowa City, IA). LPO from bovine milk (EC 1.11.1.7), H$_2$O$_2$ (30%), SA (sodium salt), and ASA were obtained from Sigma-Aldrich (St. Louis, MO). MPO (1 mg/ml stock solution), isolated from human neutrophils, was a generous gift from Dr. Jerrold Weiss (University of Iowa). All chemicals were of the highest purity available. The stock solution of DNR (10 mM) was prepared in deionized water and stored at 4°C. The concentrations of the reactants were determined spectrophotometrically using appropriate extinction coefficients, $e$$_{412}$ = 39.4 M$^{-1}$ cm$^{-1}$ for DNR(O$_2$; Nelson and Kiesow, 1972), $e$$_{412}$ = 1.12 × 10$^5$ M$^{-1}$ cm$^{-1}$ for LPO (Jenzer et al., 1986), and $e$$_{150}$ = 1.15 × 10$^4$ M$^{-1}$ cm$^{-1}$ for DNR(DXR) (Chaires et al., 1982). Because DNR and DXR tend to form dimers in aqueous solutions, in the present study, they were used in low micromolar concentrations (<10 μM) to assure that they were present predominantly as monomers.

Spectrophotometric Measurements. Oxidation of anthracyclines was studied by measuring their absorption spectra at designated time points. The spectra were measured using an Agilent diode array spectrophotometer model 8453 (Agilent Technologies, Palo Alto, CA). Samples were prepared in phosphate buffers (50 mM) for pH 6.0 to 8.0 and acetic buffers (50 mM) for pH < 6. All measurements were performed at room temperature. Typically the reaction was initiated by addition of a small aliquot of H$_2$O$_2$ (5 or 10 μl) as the last component to a sample consisting of DNR(DXR), SA, and LPO (or MPO) in buffer solution. Time course measurements were carried out following changes in absorbance at 480 nm ($\lambda$$_{max}$ for DNR and DXR). Data were collected in 2-, 5-, or 10-s intervals during continuous stirring of the sample in a spectrophotometric cuvette (1-cm light path). All experiments were repeated at least twice.

The initial rate of DNR(DXR) oxidation by MPO/H$_2$O$_2$/SA ($V$$_0$) was determined from the initial linear portion of the $A$$_{412}$ versus time traces using the method of linear regression. When DNR(DXR) was oxidized by LPO/H$_2$O$_2$ in the presence of SA, curves of a Z-shape were recorded. They were characterized by the maximum rate ($V$$_{max}$) determined by linear fitting to the portion of the curve with the largest slope.

Oxidation of SA by LPO/H$_2$O$_2$ and MPO/H$_2$O$_2$ was determined by recording absorption spectra of its metabolite showing maximum absorption at 412 nm and by measuring time course of its formation in buffers of various pHs and at various anthracycline concentrations. Concentrations of the neutral form of SA, HOOC-SA-OH, were determined using the known total concentration of the salicylate, the $pK_a$ of the SA carboxylic group of 2.98 (Lide, 2004), and the actual pH of the sample solution.

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 were prepared in pH 7.1 or 5.1 buffers (total volume, 250 μl), and the reaction was initiated by addition of H$_2$O$_2$ as the last component. To facilitate detection of radicals, 400 μM DNR was used in these experiments. Similar experiments were carried out with DXR. The sample was transferred to a flat aqueous EPR cell, and recording was started 1 min after initiation of the reaction (H$_2$O$_2$ addition). The spectra were recorded using microwave power (40 mW), modulation amplitude (2 G), receiver gain (2 × 10$^6$), conversion time (40.96 ms), time constant (81.92 ms), and scan rate (80 G/41.92 s). Spectra shown (see Fig. 10) are average of seven scans and represent results of typical experiments.

Fig. 1. Structures of ASA, SA, and anthracyclines. HOOC-SA-OH designates the neutral form of SA.
Effect of Salicylic Acid on Oxidation of Anthracyclines by MPO/\(\text{H}_2\text{O}_2\). The effect of SA on the peroxidative metabolism of DXR(DNR) was initially studied using the conditions previously established for acetaminophen (Reszka et al., 2004). However, when DXR in pH 7.0 buffer was exposed to MPO/\(\text{H}_2\text{O}_2\) in the presence of micromolar concentrations of SA, no changes in the absorption spectrum of DXR were observed, suggesting that under these conditions, DXR was not oxidized. In contrast, oxidation of DXR became evident when the reaction was carried out at acidic pH. Figure 2 shows spectra recorded at selected time points following the addition of \(\text{H}_2\text{O}_2\) to DXR/MPO/SA at pH 5.46. The decrease in intensity of the drug's characteristic absorption band at 480 nm indicates that the drug undergoes oxidation. When \(A_{480}\) reached a near zero level (indicating that almost all DXR was consumed), a new absorption band with maximum at 412 nm began to emerge. As will be shown later, this new band originates not from DXR, but from SA, and the corresponding metabolite is assigned the symbol X.

Dependence of the reaction on pH was studied next by recording the time course of \(A_{480}\) changes in buffers of various pHs at constant initial concentration of SA of 0.5 mM. It may be seen that the rate of the reaction increases as the pH decreases (Fig. 3, inset A). Because the SA carboxylic group (\(pK_a = 2.98\)) (Lide, 2004) is the only group that can be affected by changes in pH in the studied pH range of approximately 7 to 5, the observed stimulatory effects are attributed to the higher concentration of the neutral (nonionized) form of salicylic acid, HO-SA-COOH, at acidic pH. Indeed, the initial rate of DXR oxidation \((V_i)\) depends linearly on [HO-SA-COOH], with the latter being calculated for a given pH (Fig. 3, main panel). Similar results were obtained for DNR (data not shown). This dependence of DXR(DNR) oxidation on pH is completely opposite to that observed in the presence of acetaminophen, in which the maximum stimulation was observed at near neutral pH, and no effect was observed at pH ~5 (Reszka et al., 2004). We emphasize that ionization of the DXR(DNR) hydroquinone group does not change in this pH range (\(pK_a\) of first ionization of the drugs' hydroquinone moiety is ~9.5, Razzano et al., 1990); accordingly, its redox potential should remain invariant at these pHs. These results further support the idea that the observed dependence on pH should be linked to ionization status of the cofactor and not the anthracyclines.

Simultaneously with measurements of drug oxidation, we measured the formation of the species X versus pH. Inset B in Fig. 3 shows the time course of absorption changes at 412 nm at various pHs. It is apparent that the appearance of the species X is well correlated with the complete oxidation of the anthracycline. Figure 4 shows that the initial rate of DXR oxidation measured at various total [SA] but at one pH (5.25) changes linearly with [HO-SA-COOH], additionally supporting the idea that the neutral form of SA is involved in the reaction.

Importantly, SA at a very low concentration stimulated degradation of substantially higher amounts of DXR. For example, [HO-SA-COOH] of 0.99 \(\mu\)M (100 \(\mu\)M [SA] total at pH 5.0) was sufficient to decrease DXR concentration from 18.8 \(\mu\)M by 16.1 \(\mu\)M or by ~85.8% \((n = 2; [\text{LPO}]) = 24 \, \text{nm}, [\text{H}_2\text{O}_2] = 55 \, \text{mM}\). This suggests that the SA metabolite had to redox cycle several times to accomplish this level of degradation. When ASA was used instead of SA, oxidation of DXR by MPO/\(\text{H}_2\text{O}_2\)/ASA at various pHs. Plot of the initial rate of DXR oxidation \((V_i)\) (\(\text{d}A_{480}/\text{dt}\)) versus [HOOC-SA-OH]. Mean ± S.D. from two independent experiments. Inset A, time course of \(A_{480}\) changes at pH 5.0, 5.5, 5.4, 5.76, 5.82, and 7.02 (traces a–f, respectively). Inset B, concomitantly measured \(A_{115}\) versus time changes attributed to species X. [DXR] = 9.3 \(\mu\)M, [SA] = 0.5 mM, [H\(_2\)O\(_2\)] = 52 \(\mu\)M, [MPO] = 0.5 \(\mu\)g/ml.

2 This refers only to low, pharmacological concentrations of SA because oxidation of anthracyclines was readily accomplished at pH 7.0 when using high, cytotoxic concentrations of SA (10 mM).
tyrosine, or various phenolic compounds, e.g., phenol (hydroxybenzene), biphenol quinone (SA-BPQ). It is known that oxidation of sponding biphenols (SA-BPH), which then are oxidized to the SA-derived phenoxyl radicals (HOOC-SA-OH) and recombination of the primary metabolites of HOOC-SA-OH, formed at acidic pH. This species is presumably formed by SA may also involve its secondary metabolite that is readily oxidized, which is observed neither at neutral nor acidic pHs.

Effect of Salicylic Acid on Oxidation of Anthracyclines by LPO/H2O2. We also studied the capacity of LPO to support the reaction because this peroxidase is highly effective in oxidation of phenolic compounds (Monzani et al., 1997). Figure 5, inset A, shows that the rate of DNR oxidation by LPO/H2O2/SA increases as pH decreases; however, in contrast to the system with MPO, the A480 versus time traces assume Z-shape, indicating that the process is autocatalytic. The time course of DNR oxidation recorded at constant pH of 5.0 but at various [SA] also assumes Z-shape (Fig. 5, inset B). Figure 5, main panel, shows that the maximal rate of the reaction (Vmax) increases linearly with [HO-SA-COO] for DXR (data not shown). Based on these observations, we infer that the stimulatory action by SA may also involve its secondary metabolite that is readily formed at acidic pH. This species is presumably formed by recombination of the primary metabolites of HOOC-SA-OH, the SA-derived phenoxyl radicals (HOOC-SA-O·), to corresponding biphenols (SA-BPH), which then are oxidized to biphenol quinone (SA-BPQ). It is known that oxidation of various phenolic compounds, e.g., phenol (hydroxybenzene), tyrosine, or p-cresol, leads to formation of the corresponding dimers, which are also substrates for peroxidases (Bayse et al., 1972; Sawahata and Neal, 1982; Marquez and Dunford, 1995; Monzani et al., 1997).

Measurements of the position of the enzyme’s Soret band during turnover were conducted next. Addition of H2O2 (5 μM) to DXR, SA, and LPO (0.46 μM) in pH 5.0 buffer caused the peak at 412 nm (ferric LPO) to shift to 430 nm (LPO compound II).3 In this form, the enzyme lived for ~45 s, after which it returned to native LPO. The corresponding spectral lines intersect at 421 nm, consistent with conversion of LPO-II to native LPO (Jenzer et al., 1986). During the LPO-II lifetime, the A480 decreased by ΔA480 = 0.041, which corresponds to the loss of 3.6 μM DXR (Fig. 6A). The presence of LPO in the form of compound II during the reaction suggests that reduction of LPO-II by HOOC-SA-OH is the rate-limiting step. When the same experiment was repeated at pH 7.0, the decrease at 480 nm was very small (Fig. 6B) (∆[DXR] = 0.59 μM in 75 s), and the peak at 435 nm was observed for at least 20 min. This result confirms that reaction of ionized SA (HO-SA-COO-) with LPO-II at pH ~ 7 is very slow. When SA was omitted, the amount of DXR degraded at pH 7.0 was nearly the same as when SA was present, further confirming that SA is inactive at this pH (data not shown).

Oxidation of Salicylic Acid by MPO and LPO Systems. We observed that oxidation of SA/DXR(DNR) at acidic pH by either LPO/H2O2 or MPO/H2O2 generated species X, but only when the anthracycline was depleted. This suggested to us that X could be derived from SA and not from DXR or DNR. Therefore, we next studied the formation of this metabolite in the absence of the anthracyclines.

Reaction of SA with MPO/H2O2 in pH 5.1 buffer generated spectrum with λmax at 412 nm (Fig. 7), which is attributed to the species X. The absorbance at 412 nm, after reaching maximum starts to decrease, suggesting that X is unstable (Fig. 7, inset A). The formation of this metabolite is pH-dependent as the rate of its formation increases sharply upon changing pH from 6.4 to 5.0 (Fig. 7, inset A). No peak at 412 nm was formed at pH ~ 7.0 and above, during prolonged oxidation of DXR(DNR) was observed neither at neutral nor acidic pH.

Fig. 5. Oxidation of DNR by LPO/H2O2/SA. Plot of the maximal rate of DNR oxidation (Vmax) (dA480/dt)max versus [HOOC-SA-OH] for the reaction at pH 5.0. Inset, typical A480 versus time traces recorded at [SA] of 25, 50, 100, 150, and 200 μM (total concentrations) (traces a–e, respectively) at pH 5.0. [DNR] = 10 μM, [H2O2] = 25 μM, [LPO] = 22 nM. Inset B, time course of A480 changes at pH 7.0, 5.97, 5.77, 5.53, 5.45, and 5.25 (traces a–f, respectively). [DNR] = 10 μM, [H2O2] = 25 μM, [LPO] = 22 nM, [SA] = 0.25 mM (total).

Fig. 6. Absorption spectra and position of the LPO Soret band observed during oxidation of DXR by LPO/H2O2/SA in pH 5.0 buffer (A) and pH 7.0 buffer (B). [DXR] = 11.4 μM, [LPO] = 0.46 μM, [SA] = 25 μM (total), [H2O2] = 5 μM. Spectra 1 to 14 in A were recorded 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 75 s after H2O2 addition. Spectra 1 to 8 in B were recorded 0, 5, 10, 15, 20, 35, 60, and 75 s after H2O2 addition. No changes in A480 were observed at longer times of the reaction.
observation. Thus, efficient metabolism of SA by MPO occurs only at acidic pH, with maximum efficiency at pH 5.0, the lowest pH used in our experiments. The initial rate of the formation of X changes linearly with [HOOC-SA-OH] (Fig. 7, inset B). Similar observations were made when LPO was used instead of MPO. We tentatively assign the product X to SA-BPQ and analog of 4,4'-biphenol quinone generated during enzymatic oxidation of phenol (hydroxybenzene) (Sawahata and Neal, 1982). We note that the $V_i$ versus pH relationship determined here for SA is opposite to that found for other phenolics, for which decrease in pH was associated with decrease in $V_i$ (Marquez and Dunford, 1995; Monzani et al., 1997).

**Effect of Anthracyclines on the Formation of Species X.** Based on the observations that MPO/LPO/H$_2$O$_2$/SA oxidizes DNR(DXR) that MPO/LPO/H$_2$O$_2$ oxidizes SA and also that the SA metabolite (species X) appears only when DNR(DXR) is depleted, we asked how formation of X depends on anthracyclines. Therefore, we studied formation of this product as a function of [DNR]. It was expected that if X, and/or its precursors, reacts with DNR, the appearance of the 412-nm band should depend on [DNR]. Figure SA shows $A_{412}$ versus time traces observed at [DNR] of 0, 3.9, 7.2, and 14.8 $\mu$M. The figure shows that there is a distinctive [DNR]-dependent lag period, preceding the formation of X. Concomitantly measured changes in absorbance at 480 nm indicate that in contrast to the formation of X, oxidation of DNR starts immediately after the H$_2$O$_2$ addition (Fig. 8B). These results suggest that a precursor of X, or the X itself, may react with DNR. The latter possibility was studied next.

**Oxidation of Anthracyclines by the Peroxidase Metabolite of SA, Compound X.** Subsequent experiments were designed to find out whether the species X itself can react directly with DNR (DXR). The metabolite was prepared by oxidation of SA in the absence of anthracyclines, and when the absorption at 412 nm reached a maximum level, a small aliquot of DNR solution was injected. Immediately after the DNR addition, the absorbance at 412 nm decreased (Fig. 9A), suggesting that the metabolite X was reduced. Subsequent addition of H$_2$O$_2$ almost fully recovered X. The cycle, reduction of X by DNR and its reoxidation by H$_2$O$_2$, was repeated several times, without any significant loss of the compound X. This observation strongly suggests that X has redox properties similar to that of a quinone/hydroquinone couple. Simultaneously measured changes in absorbance at 480 nm indicate that the added DNR was completely oxidized by X.
that DNR was completely oxidized (Fig. 9B). Similar responses of X were observed when DNR was replaced by DXR, reduced glutathione, ascorbate, NADH, or azide (data not shown), confirming that the species is a powerful oxidant.

**EPR Study.** EPR measurements were carried out to find whether oxidation of the anthracyclines by peroxidases in the presence of SA generates free radicals. When DNR(DXR) was incubated with MPO/H$_2$O$_2$ in the presence of SA in pH 5.1 buffer, the EPR signal shown in Fig. 10, trace A, was observed. The signal line width of 0.195 mT and g of 2.00479 are close to those reported previously for DNR(DXR) radicals in other peroxidizing systems (Reszka et al., 2004, 2005a). No signal was detected when SA was omitted (Fig. 10, trace B) or when the reaction was carried out at pH 7.0 (data not shown).

The dependence of the signal on pH corroborates our results of spectrophotometric measurements. These results are consistent with the mechanism whereby the anthracycline hydroquinone moiety undergoes oxidation to the corresponding semiquinone by an SA-derived metabolite(s). We did not observe any EPR signals from control samples consisting of SA/peroxidase/H$_2$O$_2$ in acidic buffer. Although oxidation of SA by HRP/H$_2$O$_2$ or methemoglobin/H$_2$O$_2$ generates phenoxyl radicals (Shiga and Imaizumi, 1973, 1975; Marquez and Dunford, 1995; Monzani et al., 1997; Hermann et al., 1999b); accordingly, oxidation of SA should yield the corresponding phenoxyl radical, HOOC-SA-O’, as described by eq. 1, using MPO as a typical peroxidase. The resulting radicals may dimerize, forming a corresponding biphenol (SA-BPH) (eq. 2), or react with other substrates. We propose that in the presence of DXR or DNR, the SA-derived phenoxyl radicals react with the quinone-hydroquinone group (Q-QH$_2$) of the anthracyclines, causing its oxidation to a semiquinone radical (Q-QH) (eq. 3). During this reaction, the phenoxyl radical is reduced back to HOOC-SA-OH.

![Figure 10](image)

**Discussion**

The structure of anthracyclines contains a hydroquinone moiety (Fig. 1, ring B in DXR and DNR); therefore, they are susceptible to oxidation. However, LPO/H$_2$O$_2$ and MPO/H$_2$O$_2$ alone do not oxidize the anthracyclines. This is despite the fact that p-hydroquinone itself is a very good substrate for these enzymes. The purpose of our study was to determine whether SA can stimulate oxidation of DXR and DNR by these enzymatic systems. The rationale for this was that: SA is the major metabolite of the commonly used analgesic and anti-inflammatory drug aspirin, ASA. It may be used by cancer patients undergoing chemotherapy, so both SA and the anthracyclines may colocalize in tissues. SA is a phenolic compound, and the peroxidative metabolism of phenols affords reactive metabolites, phenoxyl radicals, which can react with other substrates causing their oxidation. Our earlier study showed that in the presence of acetaminophen, also a phenolic compound, both isolated MPO and MPO-rich HL-60 cells readily oxidize anthracyclines (Reszka et al., 2004). Because oxidation of anthracyclines leads to their inactivation (Cartoni et al., 2004; Reszka et al., 2005b), this reaction may be of clinical importance.

Our study shows that SA stimulates oxidation of DNR(DXR) by peroxidases but does this in a pH-dependent fashion. The stimulatory effect increases as the pH decreases from 7 to 5, which parallels the dependence on pH of the peroxidative metabolism of SA itself. These observations suggest that oxidation of the anthracyclines is mediated by an SA metabolite and that the protonated (neutral) form of SA (HOOC-SA-OH) is the preferred substrate for peroxidases. Oxidation of phenols yields the respective phenoxyl radicals (Shiga and Imaizumi, 1973, 1975; Marquez and Dunford, 1995; Monzani et al., 1997; Hermann et al., 1999b); accordingly, oxidation of SA should yield the corresponding phenoxyl radical, HOOC-SA-O’, as described by eq. 1, using MPO as a typical peroxidase. The resulting radicals may dimerize, forming a corresponding biphenol (SA-BPH) (eq. 2), or react with other substrates. We propose that in the presence of DXR or DNR, the SA-derived phenoxyl radicals react with the quinone-hydroquinone group (Q-QH$_2$) of the anthracyclines, causing its oxidation to a semiquinone radical (Q-QH) (eq. 3). During this reaction, the phenoxyl radical is reduced back to HOOC-SA-OH.
that the SA-dependent oxidation of anthracyclines occurs at acid pHs is relevant to the situation in vivo since the extracellular pH of solid tumors, against which anthracyclines are frequently used, is acidic. pH as low as 6.1 occurs with some types of tumors (Gillies et al., 2002).

Oxidation of SA in acid solutions gives rise to a product X, which shows maximum absorption at 412 nm. Based on the known chemistry of phenoxyl radicals and using the simplest phenol (hydroxybenzene) as a reference, we tentatively identify this product as the respective biphenol quinone (SA-BPQ). This compound could be formed by enzymatic oxidation of a biphenol, which is the product of recombination of phenoxyl radicals. Although oxidation of phenol can produce 2,2'- and 4,4'-biphenol quinones, only the latter one shows the characteristic intense absorption at 398 nm (Sawahata and Neal, 1982). Therefore, by analogy to oxidation of phenol to 4,4'-biphenol quinone, the species X could be assigned to 5,5'-SA-BPQ, in which the >C = O functions are in para position. The structure of this compound and the proposed mechanism of its formation are shown in Fig. 11. The probable reason why this dimeric product is efficiently formed in acid solutions is the low pKₐ value of the −COOH group in SA phenoxyl radicals, −3 (Neta and Fessenden, 1974). Dimerization of these radicals is facile when their carboxyl group is protonated. In contrast, recombination of SA-derived phenoxyl radical anions (O-SA-COO⁻) may be hindered due to repulsion of their negative charges. Thus, as the pH increases from 5 to 7, the proportion of neutral radicals become so low that any dimers formed are below the detection limit (but see footnote 4).

We found that the SA derived biphenol quinone can be reduced by DNR (Fig. 9A). The resulting biphenol was reoxidized with H₂O₂ and reduced again by a second dose of the drug. The reduction of 5,5'-SA-BPQ was concomitant with DNR oxidation. Thus, in the peroxidase/H₂O₂/SA system, oxidation of a biphenol, which is the product of recombination of phenoxyl radicals and the SA-derived biphenol quinone (Fig. 11, steps 1 and 2, respectively). The former reaction seems to play a more important role since presence of anthracyclines inhibits formation of biphenols (Fig. 11, step 1).

We have previously shown that oxidation of anthracyclines can be inhibited by ascorbate or reduced glutathione (Reszka et al., 2004). Therefore, the efficacy of this reaction in vivo will certainly depend on the presence of endogenous antioxidants and may become evident under conditions of oxidative stress, when these antioxidants are depleted. We emphasize that redox cycling of anthracyclines might promote development of oxidative stress.

Our results show that oxidation of anthracyclines leads to their irreversible bleaching, suggesting a significant modification of their chromophores. In agreement with this, recent studies revealed that oxidation of anthracyclines leads to their degradation to low-molecular weight products, 3-methoxyphthalic acid and 3-methoxysalicylic acid (Bomgaars et al., 1997; Cartoni et al., 2004; Reszka et al., 2005b). This degradation could be mediated by the drug-derived semiquinone radicals (Q-QH⁻), which decay, presumably, by disproportion to the parent drug and the electron-deficient diquinone, Q-Q (2 Q-QH⁻ ⇌ Q-QH₂ + Q-Q). It seems likely that subsequent reactions of this diquinone species could give rise to the ultimate colorless stable products. We emphasize that semiquinone radicals generated by oxidation of anthracyclines (Q-QH⁻) differ from the better known radicals formed by metabolic reduction (QH-QH⁻). The latter can reduce O₂ to superoxide, which restores the drug to its original form (Kalyanaraman et al., 1980). In contrast, semiquinones formed by oxidation undergo structural modifications.

It has been reported that products of anthracycline oxidation are virtually nontoxic to human leukemia HL-60 cells, human prostate cancer PC3 cells, and rat heart cardiomyocytes (H9c2) (Reszka et al., 2005b). These results agree with lower toxicity of 3-methoxyphthalic acid in H9c2 cells reported by another group (Cartoni et al., 2004) and with the lower toxicity of photochemically degraded DXR in P388 murine leukemia cell line (Bomgaars et al., 1997). Altogether, these observations rise the possibility that oxidation of anthracyclines in vivo may suppress their therapeutic activity. Because cancer patients undergoing anthracycline chemotherapy may be administered salicylates to control pain and inflammation, possible complications and decreased anticancer activity of the drugs should be considered. One possible beneficial effect of the drugs’ degradation could be reduced cardiotoxicity as suggested by results of in vitro studies on toxicity of anthracycline degradation products in mouse cardiomyocytes (Cartoni et al., 2004; Reszka et al., 2005b). Together, these results suggest that it should be possible to modulate inactivation of the anthracyclines in vivo by pharmacological interventions, using stimulants, or inhibitors of peroxidative processes.

![Fig. 11. Proposed mechanism of the stimulatory action of SA in the oxidation of DNR(DXR) by peroxidases. Q-QH⁻ and Q-QH₂ designate the quinone-hydroquinone moiety of the anthracyclines (rings C and B) and the corresponding semiquinone radical, respectively. HOOC-SA-OH and HOOC-SA-O' are the protonated (neutral) forms of salicylic acid and the corresponding phenoxyl radical. 5,5'-SA-BPH and 5,5'-BPQ represent the SA-derived biphenol and the corresponding biphenol quinone.](image-url)
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References


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