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IS METABOLIC ACTIVATION OF TOPOISOMERASE II POISONS IMPORTANT IN THE MECHANISM OF CYTOTOXICITY?

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Abstract

The antitumor drugs doxorubicin and etoposide, a phodophyllotoxin derivative, are clinically active for the treatment of human malignancies. Because of their extreme effectiveness in the clinic, their modes of actions have been the subject of intense research for over several decades both in the laboratory and in the clinic. It has been found that both doxorubicin and etoposide (VP-16) act on topoisomerase II, induce DNA cleavage, and form double-strand breaks, causing tumor cell death. However, both of these drugs also undergo extensive metabolism in tumor cells and *in vivo* to various reactive intermediates that bind covalently to cellular DNA and proteins. Moreover, both drugs are metabolized to reactive free radicals that induce lipid peroxidation and DNA damage. However, the role of drug activation in the mechanism of cytotoxicity remains poorly defined. In this review, we critically evaluate the significance of metabolic activation of doxorubicin and etoposide in the mechanism of tumor cytotoxicity.

INTRODUCTION

The clinically active anticancer drugs doxorubicin (also known as adriamycin) and etoposide are used for the treatment of a wide variety of cancers [1]. Doxorubicin and etoposide (VP-16) act as topoisomerase II (Topo II) poisons and induce accumulations of enzyme-linked, double-strand breaks, which are highly cytotoxic to cells [2, 3]. Topoisomerases (I and II) are an important class of nuclear enzymes that are responsible for maintaining the topology of DNA; they are involved in DNA repair, transcription, replication and segregation of chromosomes [4–6]. Inhibition and/or interference with topoisomerase functions leads to cell death. *In vivo*, the mechanism(s) of tumor cell killing by doxorubicin and VP-16 are extremely complicated in spite of their known action as topo II poisons, and various other mechanisms have been proposed. Cytotoxicity of these drugs could also depend upon other factors, e.g., metabolic activation, as they undergo enzymatic activation to reactive species that either bind to or induce damage to cellular macromolecules. This review examines mechanisms of action based on metabolic activation (bioactivation) of these two anticancer drugs that may be important for their modes of action.

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DOXORUBICIN

Doxorubicin was first discovered in early 1970 and has become one of the most important and widely used anticancer drugs for the treatment of both solid and hematological malignancies [1, 7, 8]. It contains an anthraquinone chromophore (Figure 1) and an aminoglycoside. Because of the anthraquinone structure, which contains a quinone-hydroquinone moiety, it is readily reduced by a variety of enzyme systems [9–12], most notably, cytochrome P450-reductase, via one-electron reduction, forming a semiquinone radical (Figure 1). The semiquinone radical of doxorubicin is extremely unstable in the presence of molecular oxygen and undergoes a futile oxidation-reduction cycle forming superoxide radical and, ultimately a highly reactive oxidant, the hydroxyl radical (Figure 2) [9–16]. The formation and reaction of the reactive oxygen radicals with cellular macromolecules is the basis for many of the known toxicological and cytotoxicological effects of doxorubicin. Other enzymatic systems are also known to activate doxorubicin, e.g., xanthine oxidase, DT-diaphorase and nitric oxide synthase, to form reactive oxygen species. However, it is believed that the cytochrome P450 reductase system is the main enzymatic reductive pathway for the activation of doxorubicin [10]. There are two main consequences of this reductive activation of doxorubicin: a) generation of covalent binding species and b) formation of free radical species.

COVALENT BINDING

Enzymatic activation of the anthraquinone moiety of doxorubicin results in the formation of highly reactive alkylating species. Moore [17] proposed that the anticancer drugs mitomycin and anthracyclines can be bioreductively activated to reactive species capable of alkylating cellular macromolecules. We were the first to unequivocally show that doxorubicin and its related analog, daunomycin, alkylate DNA and cellular proteins *in vitro* and *in vivo* [18–22]. We proposed that following one-electron reduction of doxorubicin, various reactive intermediates are formed (Figure 1) that could alkylate cellular macromolecules [18, 19]. As both reduced glutathione and ethylxanthate inhibited this covalent binding, we proposed that a quinone methide, formed at C7 of the doxorubicin (Figure 1) molecule, was most likely the reactive species that alkylated DNA and proteins [19, 21]. The formation of quinone methide was proposed to result from a two-electron reduction of the quinone function of the doxorubicin. In addition to the one-electron reductases, DT-diaphorase, which reduces quinones via a two-electron reduction, also formed this reactive intermediate [21]. In addition, we proposed a C7 radical intermediate that is also generated from the one-electron reduction intermediate, the doxorubicin semiquinone radical [18, 19, 21]. Various investigators have now confirmed our earlier observations on the covalent binding of doxorubicin and daunomycin with DNA [23–27]. The structure of this adduct has also been elucidated [28].

Doxorubicin-DNA adducts are biologically active and have been shown to form at the gpc sites in DNA *in vitro* or in tumor cells [23, 29]. More interestingly, these doxorubicin-DNA adducts inhibit binding of the transcription factors and DNA polymerase, which may have significant implications for the mechanism of doxorubicin cytotoxicity for tumor cells [30]. Culeen and Phillips [23] also found that DNA adduct formation was significantly enhanced

in the presence of Fe^{2+} ions and suggested the intermediacy of Fe^{3+} -doxorubicin complexes in DNA-doxorubicin adduct formation. Skladanowski and Konopa [31, 32] have shown that doxorubicin and other related anthracyclines form interstrand DNA crosslinks at physiological concentrations and are found to have a close relationship with growth inhibition in a number of tumor cell lines. It is of interest to note that adducts were found to be active in doxorubicin-resistant cells [32], suggesting that the doxorubicin DNA-adducts, once formed, may not be a substrate for p-glycoprotein.

Koch and his collaborators have reported that formaldehyde was a key intermediate in the formation of certain doxorubicin-DNA adducts [33, 34]; these were found to be specific at the gpc sites of DNA and were similar to those observed by Luce et al. [35]. Koch et al. [33, 36] showed that formaldehyde-doxorubicin-DNA adducts are also formed in breast tumor cells. Formation of formaldehyde required induction of oxidative stress, i.e., reductive activation of doxorubicin in the presence of molecular oxygen and peroxidation of cellular membrane [34, 36]. The most important finding for this type of adduct is that they are formed at pharmacological concentrations of doxorubicin and are cytotoxic to tumor cells [34, 37]. Furthermore, preformed adducts (i.e., synthesized *in vitro*) are taken up and retained in the nuclei of tumor cells by both sensitive and resistant cells more efficiently than the parent drugs [36, 37]. The significance of covalent binding and cross-link formation of doxorubicin and related drugs in cytotoxicity has been reviewed [26].

FREE RADICAL FORMATION

It is well established that doxorubicin (and most anthracyclines) forms a semiquinone radical following its reductive activation both *in vitro* and in biological systems (Figure 2) [9–12, 16]. The semiquinone radical is reasonably stable under anaerobic conditions and can be detected by electron spin resonance [38, 39]. However, it reacts rapidly with molecular oxygen, generating superoxide anion, hydrogen peroxide, and ultimately hydroxyl radicals in the presence of transition metal ions [13, 15, 16, 38]. Superoxide and hydroxyl radicals are easily detected with EPR using DMPO as a spin-trapping agent [16, 38]. Free radicals, in particular reactive hydroxyl radical, have been shown to induce DNA damage. In addition, hydroxyl radical initiates significant peroxidation of membrane lipids, forming toxic metabolites that alkylate DNA and proteins [40–42]. However, the role of free radical intermediates in the mechanism of cytotoxicity of doxorubicin remains controversial [40, 43–45]. This controversy is due to several factors: First, doxorubicin is effective *in vivo* at nanomolar concentration (10^{-7} - 10^{-8} M), while the detection of free radicals requires significantly higher (micromolar) concentrations. Second, the formation of hydroxyl radicals requires the presence of free iron (or iron complexed with doxorubicin), and it is believed that there is very little free Fe^{3+} *in vivo* (as most of the iron is bound to proteins)

While these are valid arguments against the role of free radicals in doxorubicin cytotoxicity, they can be easily explained on the basis of recent significant supporting information. Detection of free radical species requires a higher concentration of doxorubicin due to the limited sensitivity of EPR detection. When reactive free radicals are generated in cells and tissues, most of them are rapidly destroyed due to reactions with high concentrations of reduced glutathione and other sulfhydryl compounds present in cells and tissues. There are a

number of publications that have shown the formation of doxorubicin-GSH conjugates in cells as well as increased formation of oxidized glutathione following doxorubicin treatment. Moreover, in most cellular systems there are significant amounts of superoxide dismutase, catalase and glutathione peroxidase, which eliminate superoxide, hydrogen peroxide and hydroperoxides, respectively, decreasing the possibility of detecting any free radicals. It is not surprising, then, that depletion of glutathione by BSO in most tumor cells, including doxorubicin-resistant tumor cells, significantly enhances hydroxyl radical formation and doxorubicin cytotoxicity, thus implicating free radicals in the mechanism of doxorubicin antitumor activities [46, 47].

Doxorubicin requires reductive activation to generate free radical species. It is possible that certain tumor cells and tissues may not have sufficient amounts of the cytochrome P450-reductase to activate doxorubicin to the semiquinone radical as we found, for example, in human breast tumor cells selected for doxorubicin resistance [48, 49]. Moreover, the activities of the detoxifying enzymes glutathione peroxidase and glutathione transferase are also significantly increased in these resistant cell lines and in nude mouse xenografts [49, 50]. Consequently, significantly fewer doxorubicin-dependent free radicals are detected [16, 49]. In another study, in tumor cells selected for low levels of resistance to doxorubicin, hydroxyl radical formation was found to be similar to that of the sensitive cells; however, a significant decrease in hydroxyl radical formation was observed in the highly resistant variants [51]. These observations, i.e., detectable levels of free radical formed in doxorubicin-sensitive cells but not in resistant cells, support the notion that free radicals do play a role in drug cytotoxicity. Recent advances in fluorescence detection have further increased sensitivity for the detection of H_2O_2 in tumor cells and tissues, and now one can easily detect H_2O_2 formed from sub-micromolar concentrations of doxorubicin [52]. However, interference resulting from high fluorescence quenching by DNA-bound doxorubicin remains a problem.

Further support for the free radical-mediated cytotoxicity of doxorubicin has come from the very interesting work of Oberley and coworkers, who were the first to show that overexpression of MnSOD, which catalyzes the conversion of superoxide anion radical ($O_2^{\cdot-}$) to hydrogen peroxide, inhibits the growth of tumor cells [53]. When combined with doxorubicin or ionizing radiation, MnSOD enhanced tumor cell killing by both doxorubicin and ionizing radiation [54, 55] and was further enhanced by BCNU, an inhibitor of glutathione reductase. These observations clearly indicate that H_2O_2 formed from superoxide anion radical is the key intermediate for tumor cell killing by doxorubicin and ionizing radiation [56]. Furthermore, Mimnaugh et al. [57] have reported that the doxorubicin-sensitive human breast MCF-7 cells were significantly more sensitive to H_2O_2 than the corresponding doxorubicin-resistant MCF-7/ADR^R cells, implicating a free radical mechanism in doxorubicin cytotoxicity.

It has been reported that intercalated doxorubicin does not undergo reductive activation and generate free radicals, casting more doubts on the role of free radicals in doxorubicin cytotoxicity as most of doxorubicin in tumor cells is bound to DNA. It should be pointed out that the chromophore of doxorubicin remains intact following covalent binding to DNA (as the semiquinone radical does not bind covalently to DNA); thus, DNA-doxorubicin mono-

adduct could be reductively activated in tumors and generate free radical species. In this scenario, any hydroxyl radicals generated would be significantly more damaging to cellular DNA, as they would be formed in close proximity to the DNA, leading to cell death. In this regard, Dikalov et al. [58] have reported that doxorubicin covalently bound to small nucleotides is reductively activated and generates hydroxyl radicals *in vitro*. While formation of doxorubicin covalently bound to small nucleotides needs to be confirmed in tumors and *in vivo*, it definitely opens the possibility that covalently bound doxorubicin forms free radicals *in vivo* and contributes to doxorubicin cytotoxicity.

The role of iron in doxorubicin cytotoxicity is complex; however some conclusions can be derived from various observations. While iron concentration *in vivo* is low, tumors tend to contain significantly higher concentrations of copper and iron metal ions [59, 60]. Research has shown that cell membranes are not permeable to Fe chelated with doxorubicin; thus, the complex cannot generate or reduce peroxide inside tumor cells (near DNA) to form free radical species and induce cell death. However, it is well known that Fe ions are constantly released from dying cells; these Fe ions could chelate free doxorubicin present in tumor cells and cause free radical-mediated toxicity. Furthermore, the semiquinone radical of doxorubicin has been shown to induce release of iron from ferritin under anaerobic conditions [61], suggesting that doxorubicin-Fe complexes can be formed in tumors and lead to the formation of free radical species.

Further support for doxorubicin free radical-mediated tumor cell kill comes from our recent studies (Sinha et al.; unpublished observations) where topo II catalytic and cleavage activities were inhibited by nitric oxide treatment. In spite of significant inhibition of topo II functions, the cytotoxicity of doxorubicin was not compromised in either human breast MCF-7 or human colon HT-29 cells. In contrast, under identical conditions of nitric oxide treatment, the cytotoxicity and DNA cleavage of VP-16 was significantly reduced in both cell lines. This would suggest an alternate mechanism for doxorubicin, not related to topo II, must be operative and most likely a free radical-dependent mechanism for tumor cell kill by doxorubicin.

ETOPOSIDE (VP-16,213)

VP-16 and its congener, VM-26 (Figure 3), semisynthetic derivatives of epipodophyllotoxin, are active against a wide variety of tumors, most significantly against lymphoma and testicular tumors [62]. While the molecular mechanism of the drug action is not clear, VP-16 induces topo II-mediated single- and double-stranded DNA breaks in tumor cells [4, 5, 63]. It is believed that this VP-16-induced DNA damage ultimately causes cell death. Unlike doxorubicin, VP-16 does not appear to intercalate into DNA. Like doxorubicin, however, VP-16 and VM-26 undergo facile metabolism by a number of enzymatic systems *in vitro* and *in vivo* to form reactive products. We were one of the first groups to show that VP-16 is *o*-demethylated by cytochrome P450, horseradish peroxidase and tyrosinase to *o*-dihydroxy VP-16 (Figure 3) and to highly reactive *o*-quinone-VP-16 [64–67]. A number of other laboratories have also confirmed these observations [68, 69]. Formation of these intermediates required one-electron oxidation of the 4-hydroxyl group of VP-16 to form the phenoxy radical of VP-16, which can be easily detected by EPR [14]. Biologically, it is

significant to note that the formation of these metabolites required the presence of the 4'-OH in VP-16, which is also required for the antitumor activity of VP-16, as the *o*-methylated VP-16 is inactive and does not interact with topo II to induce DNA cleavage [70].

Studies from our laboratory have shown that the DHVP and the VP-16-Q derivatives are less cytotoxic to MCF-7 cells and induce less topo II-mediated DNA cleavage than VP-16 [70]. However, when formed in cells or *in vivo*, the antitumor activities of these metabolites are not known at this time, and the difference observed in MCF-7 cells may simply be due to their high polarity compared to VP-16. Thus, it is possible that these derivatives are not taken up equally by cells or may not reach their critical cellular target(s). In cell-free systems, we have shown that DHVP rapidly chelates both copper and iron ions and, in the presence of H₂O₂, generates reactive hydroxyl radicals [71, 72]. Hydroxyl radicals formed from DHVP-metal complexes induced significant damage in DNA. Additionally, the DHVP derivative undergoes autoxidation *in vitro* to produce H₂O₂ and hydroxyl radical. The rate of hydroxyl radical formation was significantly enhanced in the presence of metal ions or at high pH [14]. In contrast to DHVP, the reactive *o*-quinone-VP-16 rapidly bound to cellular proteins and DNA [65, 66] and formed glutathione conjugates in cells and *in vivo* [73]. It has been shown that the *o*-quinone metabolite also alkylates topo II and inhibits its functions [74–76]. The role of VP-16-Q in drug toxicity is the subject of intense research [74, 76]. It has been suggested that secondary leukemia associated with VP-16 treatment is due to metabolism of VP-16 to the DHVP and VP-16-Q metabolites by cytochrome P-450 CYP3A4, as there was a strong correlation with patients with a polymorphism in the 5' promoter region of CYP3A gene (CYP3A4-W) and secondary leukemia [77, 78]. However, in another study of patients with treatment-related acute leukemia, Reling et al. [79] found no significant association with VP-16 metabolisms.

VP-16 induces oxidation of glutathione to GSSG both *in vitro* and *in vivo*. It has been shown that this oxidation results from the reaction of the phenoxy radical of VP-16 with glutathione, forming glutathionyl radical [73]. There are a number of important implications for these observations: (a) cells become vulnerable to oxidative stress due to increased depletion of GSH, which could result in lipid peroxidation and/or oxidation of cellular enzymes critical for cell survival, and (b) lipid peroxidation products (e.g., aldehydes) may alkylate DNA, causing inhibition of DNA synthesis and ultimately cell death. While VP-16 is an inhibitor of lipid peroxidation [80], the DHVP metabolite could induce lipid peroxidation in the presence of free copper or iron ions. This oxidative stress/glutathione depletion caused by the VP-16 radical or VP-16 metabolites may be the basis for the synergistic interactions observed with VP-16 and ionizing radiation or photosensitizers in tumor cells and in the clinic [81, 82].

Metabolism of VP-16 to its dihydroxy and *o*-quinone derivatives clearly has significant implications for VP-16-dependent toxicity and the induction of secondary leukemia in patients undergoing VP-16-based chemotherapy. However, the role of these metabolites in the cytotoxicity of VP-16 is not understood. While it is true that the metabolites are also capable of forming a topo II-DNA-drug complex, the decreased antitumor activities in tumor cells remains poorly resolved. The VP-16-Q, *in vitro*, is active against topo II, induces significant DNA cleavage, and binds irreversibly to topo II; it is thus anticipated to be as

active as the parent drug. Due to poor uptake and non-specific binding, the antitumor activities of these metabolites cannot be accurately determined in tumor cells. However, it is very reasonable to speculate that if these metabolites are formed near the target, e.g., DNA in nuclei, they may be more active than VP-16. Since it is possible to modulate the metabolic profile of CYP3A-mediated metabolism of VP-16 by using specific inhibitor or stimulators, the antitumor activity and/or toxicity of VP-16 can be studied and the roles of metabolic activation in VP-16 cytotoxicity can be further defined.

CONCLUSIONS

It is quite clear from various published data that the mechanisms of cytotoxicity of both doxorubicin and VP-16 in tumor cells and *in vivo* are not clear, but certain conclusions can be made. Doxorubicin is indeed metabolized by various enzymes in tumors and *in vivo*, resulting in the formation of the reactive semiquinone radical. Under hypoxic or reduced O₂ concentrations, the semiquinone radical is stable and then decomposes to form very reactive species that covalently bind to DNA and proteins. Irrespective of which reactive species are formed (a quinone methide or C₇ free radical or species derived from formaldehyde), covalent binding and cross-linking with cellular DNA will cause a myriad of unwanted biological stresses in tumor cells, ultimately causing cell death. This mechanism of tumor cell killing is independent of O₂, and as most tumor cells (except for hematologically derived tumors and lung tumors) are hypoxic in nature, the possibility of formation of the covalent binding species is significantly higher. Because doxorubicin accumulates in cellular nuclei and mitochondria due to its equilibrium with doxorubicin bound to DNA, the concentrations of free doxorubicin will be highest in nuclei and in mitochondria, and it will thus be more likely to undergo reductive activation in these cellular compartments. However, bioactivation of the drug would be rate limiting based on the presence or absence of the activating enzymes. Similar situations are also feasible under aerobic conditions; however, the formation of covalently binding species would be significantly limited due to competing reactions of the semiquinone radicals with O₂. At high O₂ concentrations, a pathway involving oxygen-derived free radical formation (O₂^{•-} and [•]OH) would dominate. However, formaldehyde- or alkenal-derived covalent binding of doxorubicin with DNA is also possible. While DNA-bound doxorubicin is not bioactivated *in vitro*, free radical species are detected in tumor cells. It should be emphasized that incubations for the detection of free radicals using EPR methods are carried out at micromolar concentrations for a very brief drug exposure time, while the cytotoxicity studies in tumor cells are carried out for days. It is not known at this time whether free radicals are continuously generated in tumor cells at low concentrations of doxorubicin, causing tumor cell death.

Thus, cellular bioactivation is an important mechanism for tumor cell killing by doxorubicin and certainly would depend upon cell type and O₂ concentrations. It is suggested that because tumor cell death is caused by doxorubicin at low concentrations, other mechanisms do not contribute significantly; thus, only one mechanism, the topo II-based mechanism, is important for doxorubicin. Furthermore, because oxygen-derived free radical formation requires the presence of O₂, and in highly hypoxic cells there is very little O₂, it is concluded that these free radicals are not formed. It should be made very clear here that under highly hypoxic conditions, there is little to no tumor cell killing by most drugs, including

doxorubicin, irrespective of mechanism. Furthermore, when cells are not dividing, the concentration of topo II is low as topo II is completely cell-cycle dependent, and at low topo II protein levels, doxorubicin will become inactive or tumors will show resistance to it.

The mechanism of tumor cell killing by VP-16 is certainly not as complicated as that of doxorubicin. Unless clear evidence is presented in the future in regard to activation of VP-16 to achieve cell death, the main mechanism remains one that is dependent upon its interactions with topo II and inducing DNA damage. While bioactivation of VP-16 to its dihydroxy and *o*-quinone derivatives is important, they are also topo II-active and quite possibly contribute to tumor cell killing. With recent observations, it appears that the bioactivation of VP-16 to the *o*-quinone derivative may contribute towards the known VP-16-induced secondary leukemia formation in patients undergoing VP-16-based chemotherapy.

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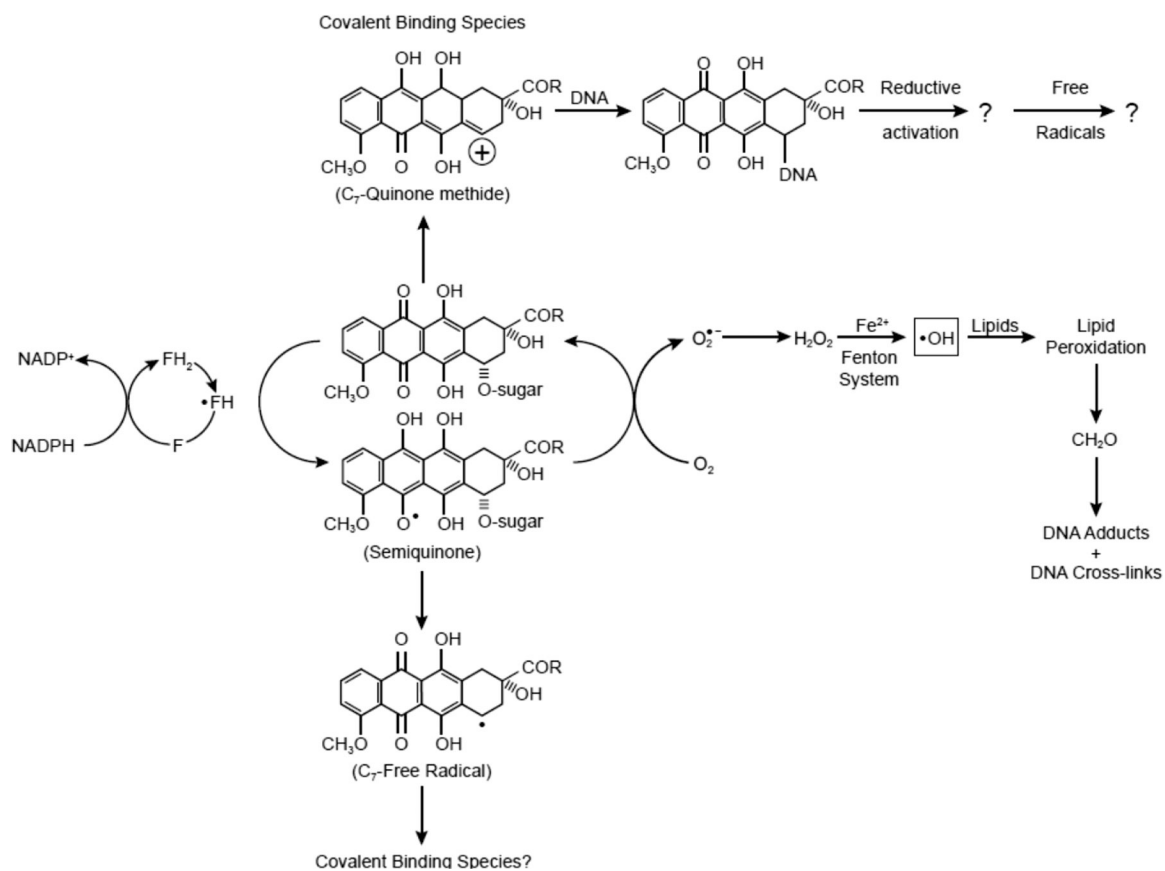


Figure 1: Proposed formation of alkylating species following enzymatic activation of doxorubicin. Also shown here is the intermediacy of formaldehyde in covalent binding of doxorubicin to DNA and possible redox-cycling of the mono-adduct and generation of hydroxyl radical at the DNA binding site.

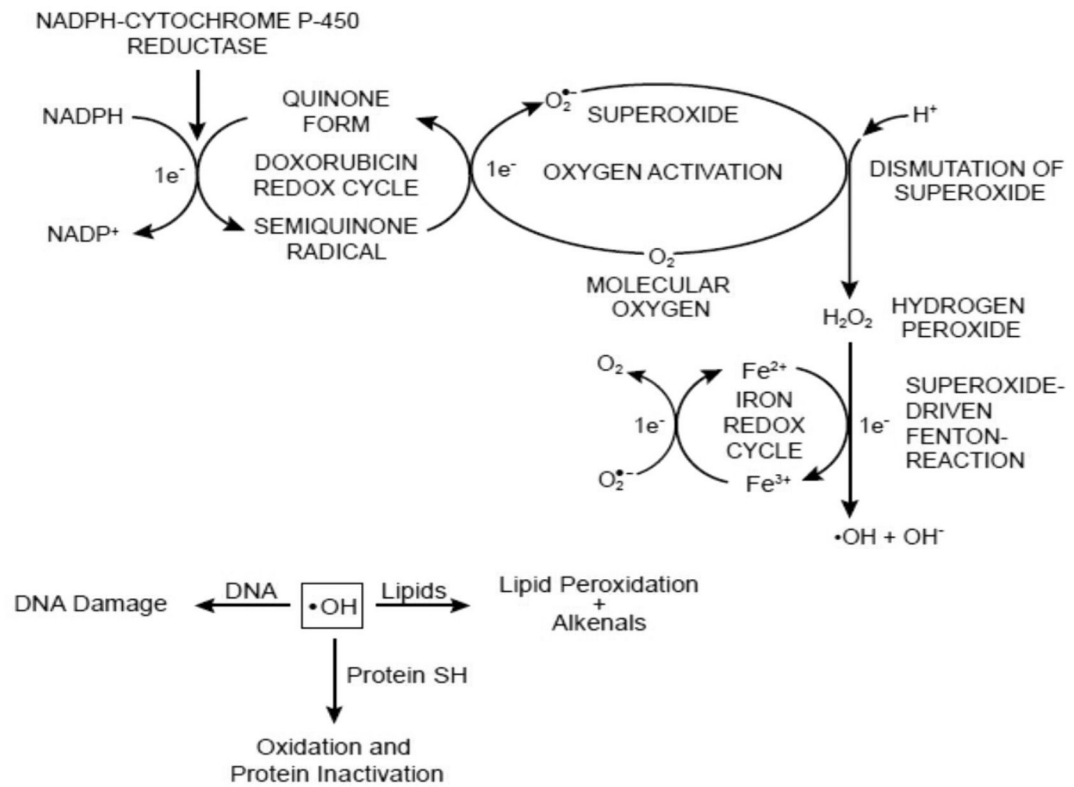
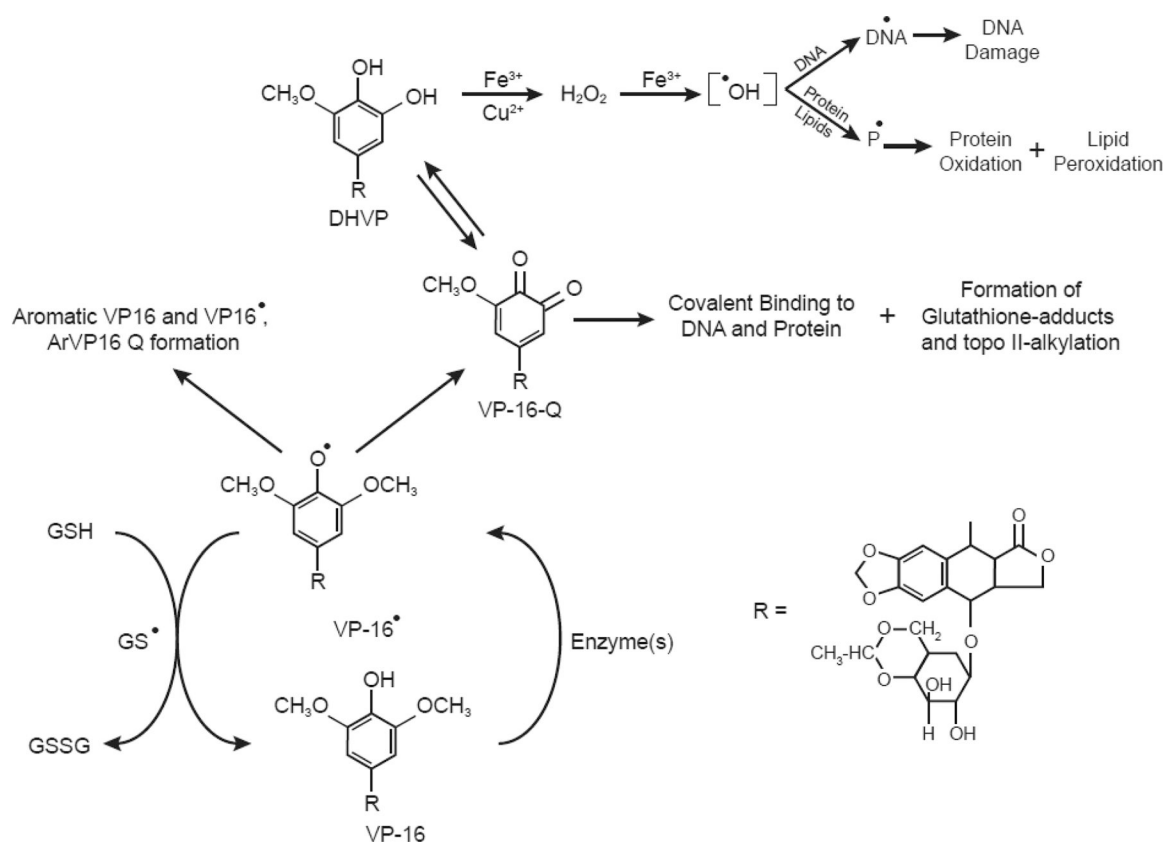


Figure 2: Formation of reactive oxygen species following reductive activation of doxorubicin and their implications in DNA damage, lipid peroxidation and protein oxidation.

**Figure 3:**

One-electron oxidation of VP-16 and formation of VP-16 phenoxy radical, *o*-quinone, and dihydroxy-VP-16. The reactive *o*-quinone derivative alkylates DNA and proteins, including topoisomerase II. The dihydroxy-VP-16 chelates metal ions and generates reactive oxygen species which induce DNA damage and protein oxidation [71, 72].