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COMMENTARY

ANTIOXIDANT AND PROOXIDANT FUNCTIONS OF DT-DIAPHORASE IN QUINONE METABOLISM

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Bioactivation of quinones by quinone reductases has been studied extensively in recent years within the context of biochemical toxicology and pharmacology. Of crucial importance in this connection is the evaluation of the chemistry of the individual quinones and of the molecular mechanisms by which they are activated. The physiological and toxicological properties of quinones—whether naturally occurring, or chemotherapeutic agents, or derived from the oxidative metabolism of exogenous or endogenous aromatic compounds—can be understood as a consequence of their inherent chemical reactivity. Two closely related properties of these compounds are essential for the understanding of these effects: first, their ability to undergo reversible oxido-reduction reactions with formation of a semiquinone intermediate and, second, their electrophilic character, which enables them to undergo nucleophilic attack. These chemical features are relevant to rationalize the biological consequences of quinone activation by enzymatic reduction and nucleophilic addition, the latter being formally a two-electron transfer process.

This commentary is concerned with the enzymatic activation of quinones by DT-diaphorase [NAD(P):H-(quinone acceptor) oxidoreductase; EC 1.6.99.2] and the implications of this reaction for cellular detoxification and toxicity, i.e. the antioxidant and prooxidant functions of DT-diaphorase. A brief review of the main kinetic properties of DT-diaphorase is pertinent to this discussion; a more detailed survey has been published elsewhere [1].

In 1958, Ernster and Navazio [2] reported the occurrence of a highly active diaphorase in the soluble fraction of rat liver microsomes. They named the enzyme DT-diaphorase, and in subsequent years Ernster and his colleagues [3, 4] provided data regarding enzyme kinetics, purification, assay conditions, activators and inhibitors, and comparison with other NAD(P)H-oxidizing flavoproteins. DT-diaphorase is a unique flavoenzyme in several aspects: first, it displays a nonspecific reactivity towards NADH and NADPH and shows a broad electron acceptor specificity, catalyzing the reduction of quinones, quinone epoxides, quinoneimines, certain aromatic nitro compounds, aromatic C-nitroso compounds, azo dyes, and hexavalent chromium [see Refs. 1, 5 and 6]. Second, DT-diaphorase is strongly inhibited by dicumarol and other oral anticoagulants [which are considered competitive inhibitors of the enzyme with respect to NAD(P)H]. Third, and perhaps the most striking feature of DT-diaphorase relevant to this commentary, is its so-called ability to catalyze "obligatory" two-electron transfers. The reduction potential of the DT-diaphorase flavins has not been studied in detail, although this parameter is regarded as a critical factor in the reduction of quinones by electron-transferring flavoproteins. Despite the lack of information on the redox properties of this flavoprotein, it is generally accepted that hydroquinones are formed during DT-diaphorase catalysis. The release of a hydroquinone from the active site of this enzyme was hypothesized to involve two one-electron transfers: the expected semiquinone intermediate may not be removed easily from the active site and may be reduced to its hydroquinone form by a subsequent one-electron transfer [7].

DT-diaphorase has been associated with different aspects of research encompassing its function in the detoxification of quinones and the prevention of mutagenesis and carcinogenesis, its role in vitamin K-dependent carboxylation, and its biosynthesis, induction and coordinate transcriptional regulation with other Phase II enzymes. These and other aspects were the subject of a conference in Stockholm in 1986 [see Ref. 8] and subsequent review articles [1, 5, 6, 9, 10]. More recently, an extensive discussion was presented on the potential role of DT-diaphorase in enzyme-directed bioreductive drug activation with emphasis on cancer chemotherapy [11, 12], a concept recognized and developed by Sartorelli and his colleagues [13] over 20 years ago.

The view of DT-diaphorase as a flavoenzyme actively involved in the detoxification of quinones, hence, as an antioxidant enzyme, is based mainly on two of the properties mentioned earlier: its apparent catalysis of two-electron transfers and the observation that dicumarol, an inhibitor of DT-diaphorase, enhanced toxicity in several experimental

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models. The former property, although lacking chemical foundation, is considered crucial to its role in cytoprotection against toxic chemicals [14] as opposed to the one-electron activation of quinones yielding semiquinone radicals. The latter aspect deserves further comment: a protective function of DT-diaphorase was suggested by studies showing a dicumarol-dependent potentiation of quinone toxicity *in vivo*, in cell cultures, in isolated cells, and in perfused organs. This approach is rather controversial, because dicumarol elicits more effects at a cellular level than those that can be simply ascribed to a specific inhibition of DT-diaphorase, such as uncoupling of oxidative phosphorylation and inhibition of enzymes such as glucuronyl transferases [15], xanthine oxidase, xanthine dehydrogenase [16], glutathione transferases and glutathione peroxidase II [17]. In addition, the concentration of dicumarol required to inhibit DT-diaphorase varies with the quinone electron acceptor [18]. Thus, high doses of dicumarol may be required to inhibit the DT-diaphorase-mediated reduction of less efficient electron acceptors, and this might affect biochemical processes in cells other than DT-diaphorase-catalyzed reactions [18]. Site-directed mutagenesis of rat liver DT-diaphorase [19] and the use of photoaffinity probes [20] identified a Tyr 128 residue as the dicumarol-binding site, different from a glycine-rich sequence that served as an NAD(P)H-binding site [19]. Flavones, effective in the nanomolar range and binding to sites different from those of dicumarol, appear to be a new type of inhibitor of DT-diaphorase [21].

On the other end, the involvement of DT-diaphorase in metabolic pathways leading to cytotoxicity also has been documented: DT-diaphorase not only catalyzes the activation of quinone-based anticancer compounds to hydroquinones, which rearrange into bioalkylating agents [11, 12], but it also activates nitro compounds to carcinogenic nitrosamines [22, 23] and facilitates the cytosolic reduction of dinitropyrines (thus enhancing mutagenicity studied in the Ames test) and that of partially reduced metabolites to penultimate mutagenic species [24]. DT-diaphorase appears to constitute a major part of the total dinitropyrine nitroreductase activity (induced by Aroclor 1254 pretreatment) [25].

The purpose of this commentary is to revise the established dogma that purports DT-diaphorase as an antioxidant enzyme protecting against quinone toxicity and to survey the actual functions of DT-diaphorase in itself and in connection with superoxide dismutase. It is expected that a critical analysis of the reactions catalyzed by DT-diaphorase will increase the understanding of the actual mechanisms of action of this enzyme with emphasis on its role in quinone activation. For the sake of convenience, three types of hydroquinones formed during DT-diaphorase activity can be distinguished (Fig. 1): (**I**) redox-stable hydroquinones; (**II**) redox-labile hydroquinones that subsequently autoxidize with formation of reactive oxygen species. This process can be either inhibited or stimulated by superoxide dismutase, and (**III**) hydroquinones that readily rearrange to potent electrophiles participating in bioalkylation reactions. These three instances share a common activation by DT-diaphorase; however, the inherent chemical reactivity of the hydroquinone formed is independent of the type of catalysis and is strictly an expression of its functional group chemistry, i.e. its substitution pattern. Pathway **I** (Fig. 1), along with a concerted activity of DT-diaphorase with superoxide dismutase (pathway **IIa**), indicates an *antioxidant* or protective function against quinone toxicity. Conversely, stimulation of hydroquinone autoxidation by dismutase (pathway **IIb**) and rearrangement of hydroquinones to alkylating agents (pathway **III**) substantiate the participation of DT-diaphorase in biochemical processes leading to an expression of quinone toxicity supported by either oxidative stress (a *prooxidant* activity) or bioalkylation.

I DT-DIAPHORASE-CATALYZED FORMATION OF REDOX-STABLE HYDROQUINONES

Ample evidence has accumulated that establishes a protective role for DT-diaphorase against the toxicity of quinones. This evidence originates from cellular experimental models utilizing simple quinones of the *p*-benzo- or naphthoquinone series either unsubstituted or bearing alkyl substituents. The biochemical basis for the protective role of DT-diaphorase in quinone cytotoxicity—with reference to this type of quinones—involves the following aspects.

First, DT-diaphorase catalysis circumvents semiquinone formation by one-electron quinone reductases and, hence, the oxyradical production during autoxidation of the latter [14]. This is substantiated by many reports showing that the prevalence of one-electron transfer activation of quinones over DT-diaphorase catalysis is associated with a high steady-state concentration of oxyradicals leading to cytotoxicity. These experimental models utilized unsubstituted or alkyl-substituted *p*-benzo- or 1,4-naphthoquinones and quinoneimines in isolated hepatocytes or endothelial cells [26–34] and, in some cases, the prevalence of one-electron reduction pathways (associated with cytotoxicity) was supported by ESR studies identifying semiquinone and oxygen radicals. The experimental support for a role of DT-diaphorase against the toxicity of these quinones stems largely from models entailing inhibition of the enzyme by dicumarol. A comment on the various enzymes (other than DT-diaphorase) inhibited by dicumarol was given above. The two-electron reduced form of menadione generated during DT-diaphorase catalysis is usually considered a redox stable hydroquinone [14]. However, it was reported recently that the reduction of menadione by purified DT-diaphorase resulted in $HO^{\cdot}$ formation and that dicumarol inhibited $HO^{\cdot}$ production and DNA single-strand breaks in MCF-7 cells supplemented with this quinone [35]. The antioxidant function of DT-diaphorase is usually addressed in connection with prevention of quinone cytotoxicity, and failure of the enzyme to prevent other types of oxidative stress does not necessarily antagonize its potential antioxidant properties. For example, DT-diaphorase activity increased along

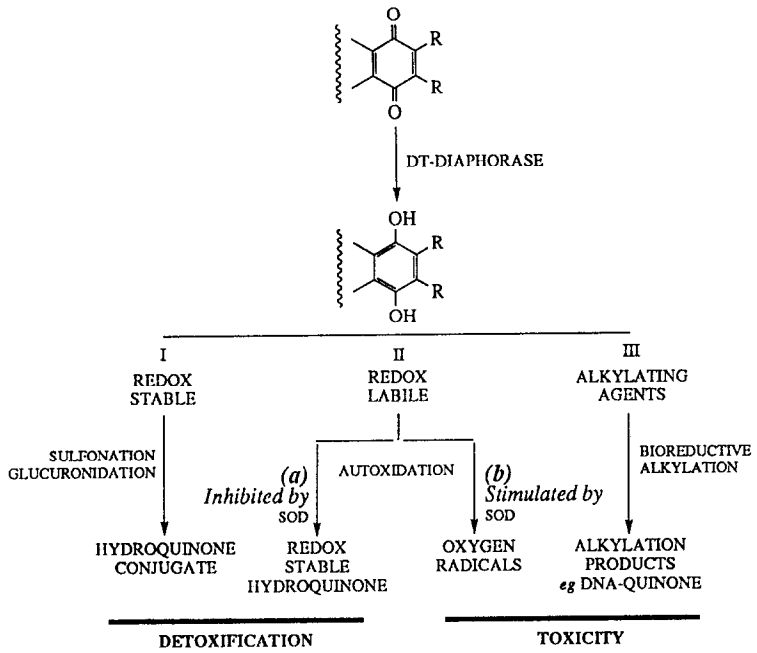

Fig. 1. Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism. The reactivity of the hydroquinone formed during DT-diaphorase catalysis, (**I**) redox stable, (**II**) redox labile, or (**III**) alkylating agents, is a function of the substitution pattern (R) of the quinone.

with the activities of other antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) during the late gestational rat fetal lung; however, this increase in DT-diaphorase activity failed to protect against hyperoxia in adult rats [36]. This might be obvious when considering the type of oxidants that are present in hyperoxic conditions.

Second, aromatization of the quinonoid ring via DT-diaphorase catalysis decreases the electrophilic character of the quinone, thereby restricting its participation in arylation reactions. Hence, DT-diaphorase indirectly prevents cytotoxicity originating from arylation reactions. For example, the different ability to undergo 1,4-Michael addition with cellular GSH and/or alkylate protein thiols (rather than their ability to undergo redox cycling) appears responsible for the cytotoxic effects of *p*-benzoquinones with alkyl or halogen substituents as well as for the dichloro-substituted naphthoquinone, dichlone [37–40]. GSH reacts readily with quinones possessing the appropriate substitution pattern and inhibits efficiently the covalent binding of [^{3}H]-menadione to calf thymus DNA [28]. This effect is understood in terms of a competition of diverse nucleophiles for the quinone and reveals the facility of the reaction of the thiol with menadione resulting in thioether derivative formation. Studies on the contribution of arylation reactions and oxidative stress to quinone-mediated cytotoxicity suggested that arylation of cellular nucleophiles might be a more effective mechanism than redox cycling [41]. This, of course, depends on cell-specific defense mechanisms against oxidative stress: hepatocytes seem to be endowed with defenses difficult to overwhelm, whereas the inability of myocytes to endure oxidative stress was summoned to explain the cardiotoxicity of Adriamycin® [42]. Some effects of dicumarol could also be cell specific: dicumarol did not elicit any effect on the menadione-induced DNA single-strand formation in cultured hepatocytes, whereas it potentiated this process in human fibroblasts [43].

Third, the predominant feature of the enzyme, i.e. to catalyze the formation of hydroquinones, furnishes the chemical requisites to facilitate conjugation with glucuronide or sulfide, thus facilitating metabolic inactivation and cell disposition as water-soluble products. Although this view is chemically correct, evidence for a concerted activity of DT-diaphorase and UDP glucuronyl transferases in quinone detoxification is still missing. The dicumarol-sensitive glucuronidation of benzo[*a*]-pyrene-2,6-quinone suggests the involvement of DT-diaphorase in this process [44]. However, interpretation of this effect is not straightforward, because it could originate from the inhibition of a specific UDP glucuronyl transferase along with that of DT-diaphorase [18]. The one-electron activation of quinones, as accomplished by NADPH–cytochrome P450 reductase catalysis, may also facilitate conjugation with glucuronide provided that the semiquinone species disproportionates readily to quinone and hydroquinone [45] and that disproportionation reactions prevail over autoxidation.

Strong evidence for an antioxidant function of DT-diaphorase has been furnished in connection with the deactivation of quinones derived from the oxidative metabolism of benzene in the two main

types of bone marrow stroma cells, macrophages and fibroblastoid cells [46]. These studies are a good example of the necessity of concerted enzymatic activities to observe protection against quinone cytotoxicity. The susceptibility of macrophages to benzene-derived metabolites may be accounted for in terms of their efficient activation via a peroxidase-catalyzed reaction and of a low activity of DT-diaphorase (which, as mentioned earlier, would prevent arylation reactions) [47]. Also, UDP glucuronyl transferase activity (which facilitates upon conjugation the metabolic inactivation and elimination of the hydroquinone) is higher than that of DT-diaphorase in bone marrow macrophages; thus, the latter enzyme may represent a rate-limiting step in the detoxification of benzene-derived quinones in these cells [48]. These differences in enzyme levels explain the selective toxicity of the benzene metabolite hydroquinone to macrophages (relative to fibroblastoid cells) [47].

Fourth, in line with an antioxidant role for DT-diaphorase is its induction along with other detoxification (Phase II) enzymes [49]. This aspect of DT-diaphorase research has been studied intensively following the early observation that induction of DT-diaphorase by small doses of polycyclic hydrocarbons and azo dyes conferred subsequent protection against the cytotoxic effects of quinones [50]. Induction of DT-diaphorase results from enhanced transcription and is triggered by a wide variety of xenobiotics: a common denominator of all inducers thus far is that they are electrophiles that can be classified as Michael acceptors, whose potency as inducers correlates with their efficiency in Michael reactions [51]. A 41-bp enhancer element appears to mediate inducer activity of these compounds [49]. These inducers up-regulate DT-diaphorase at the transcriptional level through the xenobiotic responsive element (XRE; mediated by the Ah receptor and activated by planar aromatic compounds) or through the antioxidant responsive element (ARE; mediated by members of the Jun/Fos protooncogene families and activated by phenolic antioxidants such as *tert*-butylhydroquinone) [52, 53]. Activation of the ARE may involve an oxidatively regulated protein, similar to the oxy R protein that regulates antioxidant defense in bacteria and yeast [54]. A model for the mechanism of regulation of human DT-diaphorase gene expression in tumor cells hypothesizes the transfer of a "redox signal" from unknown redox proteins, which in turn modify the Jun and Fos proteins for greater affinity towards the AP1 site within the human ARE, resulting in the transcriptional activation of the DT-diaphorase gene expression [55].

II DT-DIAPHORASE-CATALYZED FORMATION OF REDOX-LABILE HYDROQUINONES

A simple example of the influence of the substitution pattern on the chemical reactivity of hydroquinones is provided by menadione (2-methyl-1,4-naphthoquinone) and its glutathionyl thioether (2-methyl-3-glutathionyl-1,4-naphthoquinone). DT-diaphorase catalyzes the reduction of the former to a redox-stable hydroquinone [14, 56] and that of the latter to a redox-labile hydroquinone, which autoxidizes at substantial rates [56]. This cannot be explained by changes in the reduction potential of the quinone elicited by a glutathionyl substituent: the $E(Q/Q^{\cdot -})$ values of menadione and its thioether derivative, i.e. -203 and -192 mV, respectively, differ only slightly [57].

Depending on the effect of superoxide dismutase on the free radical decay pathways of the redox-labile hydroquinones formed during DT-diaphorase catalysis, two types of compounds could be distinguished: redox-labile hydroquinones whose autoxidation is inhibited by superoxide dismutase and those whose autoxidation is stimulated by superoxide dismutase.

DT-diaphorase and Superoxide Dismutase: A Concerted Antioxidant Activity

DT-diaphorase-catalyzed reduction of naphthoquinones bearing glutathionyl, methoxyl, and hydroxyl substituents (the latter in the quinonoid ring) yields redox-labile hydroquinones [56, 58]. Also, 2,3-epoxy-*p*-benzo- and 1,4-naphthoquinones autoxidize readily (probably via a 2-OH-substituted intermediate) following their reduction by DT-diaphorase [59, 60].

Propagation of hydroquinone oxidation proceeds via $O_2^{\cdot -}$ according to the following reactions:

$$Q^{\cdot -} + O_2 \underset{b}{\overset{f}{\rightleftharpoons}} Q + O_2^{\cdot -} \qquad (1)$$

$$QH^- + H^+ + O_2^{\cdot -} \rightarrow Q^{\cdot -} + H_2O_2 \qquad (2)$$

Reaction 2 and, thus, propagation of hydroquinone oxidation, is inhibited by superoxide dismutase, which catalyzes the fast disproportionation of $O_2^{\cdot -}$ to H_2O_2 (reaction 3). Formation of the latter

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (3)$$

species is minimal under these circumstances, for the steady-state concentration of $O_2^{\cdot -}$ is negligibly small: it is formed during semiquinone autoxidation and consumed during hydroquinone oxidation (Fig. 2). Depending on the physico-chemical properties of the semiquinone, the former reaction proceeds at fast rates; the equilibrium constant, K_1, for most naphthoquinones referred to in this section is above unity [i.e. the reduction potential $E(Q/Q^{\cdot -})$ values are more negative than the $E(O_2/O_2^{\cdot -})$ value]. Reaction 2 is expected to proceed fairly rapidly through a mechanism analogous to the oxidation of ascorbate and phenols by $O_2^{\cdot -}$ [61]. Although a good correlation has been observed between the one-electron reduction potential of various quinones and their ability to be reduced by the one-electron transfer flavoprotein, NADPH–cytochrome P450 reductase [62, 63], there is no correlation between the reduction potential of quinones and their rate of reduction by DT-diaphorase. Neither could correlations be established based on the partition coefficient of the quinones or the occurrence of electron-donating or withdrawing substituents that modulate the transfer of reducing equivalents onto the quinonoid ring [56].

The scheme in Fig. 2 emphasizes the role of

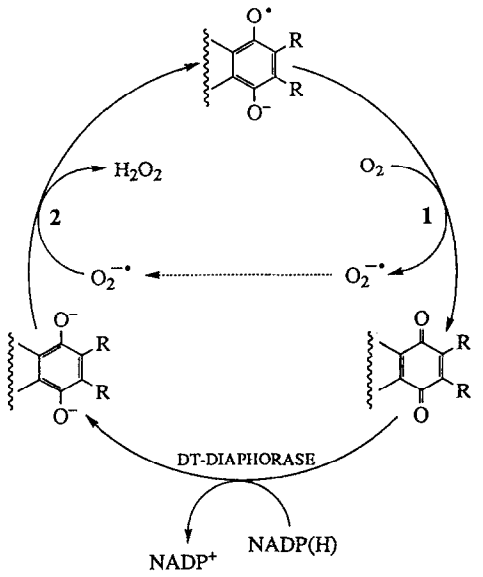

Fig. 2. Superoxide radical-mediated propagation of hydroquinone autoxidation. Numbers 1 and 2 in the figure correspond to equations 1 and 2 in the text.

$O_2^{\cdot -}$ as a propagating species in hydroquinone autoxidation in a manner analogous to that described for autoxidation of 6-hydroxydopamine [64], dialuric acid, divicine and isouramil [65], and some hydroquinones generated during DT-diaphorase catalysis [58].

Thus, despite the formation of redox-labile hydroquinones during DT-diaphorase catalysis, the concerted activity of this enzyme with superoxide dismutase results in inhibition of H_2O_2 formation and enhancement of the steady-state concentration of the hydroquinone species. An example of the antioxidant character of the DT-diaphorase/superoxide dismutase concerted activity was provided by the suppression of oxygen radical formation arising from the redox transitions of the dopamine-derived *o*-quinone [66]. Adrenochrome, an *o*-quinone derived from the autoxidation of adrenaline and which exhibits neurotoxic and cardiotoxic properties, is also reduced efficiently by rat brain and liver DT-diaphorase [67]. Interestingly, a recent report showed induction of DT-diaphorase and Cu,Zn-superoxide dismutase, but not of Mn-superoxide dismutase, when human blood mononuclear cells were exposed to visible light [68].

Protection against quinone cytotoxicity (regardless of whether the formation of redox-labile or redox-stable hydroquinones is favored) does not appear to be solely a function of DT-diaphorase activity. The concerted activities of DT-diaphorase and superoxide dismutase and DT-diaphorase and UDP glucuronyl transferase are some examples. In addition, the higher resistance to the deleterious effects of 2,3-dimethoxy-1,4-naphthoquinone in the eukaryotic cell line 3T3-GGT appears to be dependent on a γ-glutamyl transpeptidase-mediated increase of the utilization of extracellular GSH [69].

Quinone thioethers, such as the glutathionyl conjugates of 1,4-naphthoquinones, are particularly interesting because they form readily upon sulfur nucleophilic attack on the quinones and because they are substrates for quinone reductases. A distinction should be made between the redox-labile semiquinone and hydroquinone thioethers: the autoxidation of the former is enhanced by superoxide dismutase, whereas that of the latter is inhibited by the enzyme [58]. Also, at variance with alkylating quinones which do not redox cycle, GSH does not confer protection against this type of quinone-mediated oxidative stress [70].

Stimulation of Hydroquinone Autoxidation by Superoxide Dismutase

DT-diaphorase catalyzes the formation of redox labile hydroquinones with a particular redox chemistry. At variance with what was described in the previous section, the oxidation of these hydroquinones is enhanced by superoxide dismutase. These features are an expression of the physico-chemical properties of the quinone and are understood as follows [71].

First, the reduction potential of the $Q/Q^{\cdot -}$ couple for these compounds is less negative than that of the $O_2/O_2^{\cdot -}$ couple; hence, the equilibrium constant K_1 (reaction 1 above) is below unity. k_1b prevails over k_1f, and $O_2^{\cdot -}$ is expected to reduce the quinone to the semiquinone at fairly high rates, thereby enhancing the steady-state concentration of the latter. This view is supported by a careful study using 2,5-bis-(1-aziridinyl)-benzoquinone derivatives with different reduction potentials [the $E(Q/Q^{\cdot -})$ values ranged between +3 and −160 mV] [72]. These derivatives inhibited the formation of $O_2^{\cdot -}$ and H_2O_2 during xanthine oxidase catalysis and, hence, the xanthine oxidase-mediated DNA strand break. These effects were explained partly by the efficient removal of $O_2^{\cdot -}$ by some quinones as in reaction 1*b*. This *in vitro* study is important when considering the relative contributions of free radical mechanisms and alkylation reactions to the cytotoxicity exerted by chemotherapeutic agents. The reactions are complex and require consideration of multiple competing radical decay processes, such as semiquinone and $O_2^{\cdot -}$ disproportionation [72]. Trapping of $O_2^{\cdot -}$ by this type of antitumour quinones (as in reaction 1*b*) does not exclude the formation of H_2O_2, which would ensue according to reaction 4 below. Reactions 1*b* and 4 suggest a small steady-state concentration of $O_2^{\cdot -}$ in this redox cycling model, at variance with that of H_2O_2 [72].

$$Q^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow Q + H_2O_2 \qquad (4)$$

Second, the steady-state concentration of the semiquinone may be further enhanced by the prevalence of the comproportionation over the disproportionation reaction ($k_5b > k_5f$). For example, the decay of the semiquinone form of diaziquone via disproportionation proceeds at rates that are slow for quinone radicals, that is, 2–3 orders

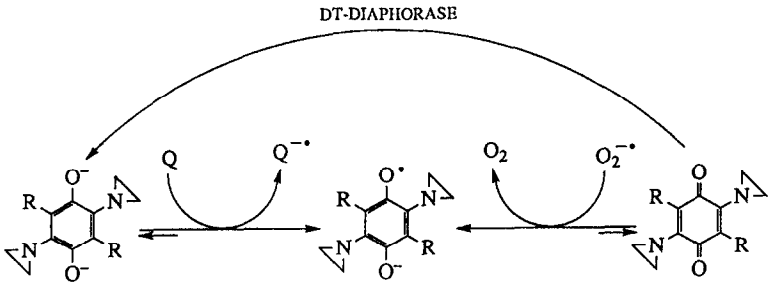

Fig. 3. Role of semiquinone species in DT-diaphorase-catalyzed reactions.

of magnitude slower than those reported for mitomycin and Adriamycin [73].

$$Q^{\cdot -} + Q^{\cdot -} \underset{b}{\overset{f}{\rightleftharpoons}} Q + Q^{2-} \qquad (5)$$

The two features described above contribute to an enhanced steady-state concentration of the semiquinone species, which is expected to play a crucial role in the redox transitions of this type of quinones. This view is summarized in the scheme in Fig. 3. In compliance with these properties, some of the semiquinone species in this group can be detected by ESR in aerobic conditions.

A third property could potentially contribute to an enhanced steady-state concentration of the semiquinone species: when suitable functional groups are present, they can hydrogen bond strongly with neighboring carbonyl groups of the quinone moiety; intramolecular hydrogen bonding tends to stabilize the semiquinone by decreasing hydrogen bonding with solvent molecules, as in the case of hydroxyl-substituted, aromatic-ring naphthosemiquinones [74] and aziridinyl-substituted benzoquinones [75]. Some hydroxyl-substituted, aromatic-ring naphthosemiquinones are stable in anaerobiosis over a limited pH range, but the ESR signal rapidly disappears in the presence of O_2 [74].

These concepts, in principle, may be summoned to account for the chemical reactivity of diverse redox-labile hydroquinone forms of the anticancer compound generated by DT-diaphorase catalysis: diaziquone (AZQ), bioreductive alkylating naphthoquinones bearing substituents at C_2 such as—CH_2Cl,—CH_2OCH_3, and—CH_2OH, and naphthol derivatives with both quinone and diol functionalities, such as naphthazarin and juglone. The features described above contribute to different extents to the redox transitions of these compounds during DT-diaphorase catalysis.

Diaziridinylbenzoquinones. The antitumour activity of the first compound, diaziquone, is attributed to its quinone moiety and aziridinyl substituents; the former participates in redox cycling processes and, hence, oxygen radical production, whereas the latter is essential for the alkylating properties of this compound. Diaziquone is reduced to the hydroquinone form by either purified DT-diaphorase or DT-diaphorase activity in the S9 fraction of MCF-7 human breast carcinoma cells [75–79]. $HO^{\cdot}$ formation during the course of AZQ activation by the S9 fraction of MCF-7 cells was suppressed by dicumarol, thus suggesting a DT-diaphorase-supported redox cycling of the quinone [76]. Autoxidation of the hydroquinone species entails the formation of diverse radicals, such as $O_2^{\cdot -}$, $HO^{\cdot}$, and that of the semiquinone form of diaziquone ($AZQ^{\cdot -}$) [78, 79]. The observation of a semiquinone signal is explained by the concepts outlined in Fig. 3.

Taken together, DT-diaphorase appears to be involved in the activation of AZQ to radical species and responsible for plasmid DNA strand breakage. It is also likely that oxygen radicals may underline, in part, the cytotoxicity of diaziquone towards Chinese hamster ovary cells [80] and hepatocytes [81]. Conversely, DT-diaphorase activity confers protection to isolated hepatocytes during the metabolism of trenimon (an aziridinylbenzoquinone derivative) [82], whereas the enzyme potentiates toxicity by this compound in L5178Y murine lymphoblastic cells [83]. These controversial observations suggest that DT-diaphorase is not unique in contributing to the expression of, or protection against, quinone toxicity and that cell specific factors are likely to play a significant role in these processes.

Aziridinylbenzoquinones bearing halogen (—Cl,—F, or—Br) at C_3 and C_6 autoxidize readily following their enzymic activation or glutathione nucleophilic substitution. This process is associated with DNA strand breaks, which are inhibited by superoxide dismutase.* DNA strand break formation may proceed in a manner entailing a semiquinone-dependent reduction of metal ligands bound at the DNA surface and leading to site-specific, $HO^{\cdot}$ production. Of note, the role of DT-diaphorase in enzyme-directed bioreductive drug development should be considered carefully when quinones containing halogen substituents are evaluated. These compounds, along with the alkylating agent 2-chloromethyl-naphthoquinone, inhibit DT-diaphorase in a dose-dependent manner and with a kinetic behavior similar to that described for dichlorophenolindophenol [84].

Superoxide dismutase enhances the autoxidation of the hydroquinone form of AZQ and suppresses

* Goin J, Butler J and Cadenas E, Manuscript submitted for publication.

the ESR signal of $AZQ^{\cdot -}$ [79] and, therefore, this enzyme facilitates the oxidative decay of the semiquinone. This effect is explained in terms of a displacement of the equilibrium of reaction 1 towards the right-hand side upon rapid removal of $O_2^{\cdot -}$ (i.e. $k_1 f \gg k_1 b$) [85]. GSH, on the other hand, facilitates the reductive decay of the semiquinone: the thiol also suppresses the ESR signal of $AZQ^{\cdot -}$ and promotes hydroquinone formation [71, 79]. This is accounted for by the electron transfer depicted in reaction 6. This reaction is thermodynamically unfavorable for the case of aziridinylbenzosemiquinones (as well as other semiquinones) despite the low reduction potential of these species [e.g. $E(AZQ^{\cdot -}/AZQ^{2-}) = -39$ mV and $E(GS^{\cdot}, H^+/GSH) = +850$ mV]. The thermodynamic restriction is overcome kinetically when the equilibrium of reaction 6 is displaced towards the right upon the rapid removal of thiyl radicals upon conjugation with thiolate (see below) [see Ref. 86].

$$Q^{\cdot -} + RS^- + H^+ \rightarrow QH_2 + RS^{\cdot} \qquad (6)$$

Therefore, catalytic amounts of superoxide dismutase and large amounts of GSH are able to determine the fate of the semiquinone form of AZQ in terms of an oxidative and a reductive pathway, respectively. Accordingly, both superoxide dismutase and GSH abolish the AZQ semiquinone ESR signal [71, 79].

2-Alkylnaphthoquinones. A salient feature of the redox transitions encompassed by the one- and two-electron catalysis of the bioalkylating agent 2-methylmethoxy-1,4-naphthoquinone is the different contribution of disproportionation and autoxidation reactions, respectively. In the former case, semiquinone disproportionation prevails, whereas during DT-diaphorase catalysis autoxidation is the main free radical decay pathway. These differences are abrogated by superoxide dismutase, which enhances autoxidation during NADPH–cytochrome P450 catalysis to a maximal value. DT-diaphorase-mediated activation of 2-methylmethoxy-1,4-naphthoquinone leads to calf thymus DNA strand break formation, a process partially sensitive to superoxide dismutase and/or catalase [87].

Hydroxy-substituted, Aromatic Ring Naphthoquinones. Naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) is the basic unit of tetracyclic antitumour antibiotics such as daunorubicin, Adriamycin, and carminomycin. Naphthazarin and its thioether derivative are efficiently reduced by one-electron reductase and DT-diaphorase, and in both instances superoxide dismutase enhances the formation of oxyradicals [88, 89]. As a corollary, these compounds have proved to be highly cytotoxic quinones in isolated hepatocytes [90].

The role of DT-diaphorase in catalyzing the formation of redox-stable (Fig. 1, pathway **I**) and redox-labile (Fig. 1, pathway **IIb**) hydroquinones is illustrated by the following examples. Pretreatment of replicating cells with dicumarol enhanced the cytotoxicity of 1,4-naphthoquinone (redox stable) but did not elicit changes in the cytotoxicity of 5-hydroxy- and 5,8-dihydroxy-naphthoquinones [91]. The doxorubicin resistance developed by human leukemia cell lines (HL-60), involving slightly increased superoxide dismutase and DT-diaphorase activities, did not prevent the cytotoxic effects of 5-hydroxy- and 5,8-dihydroxy-1,4-naphthoquinones [92]. Doxorubicin, on the other hand, was not a substrate for purified rat Walker 256 tumor DT-diaphorase, but for an NADPH–cytochrome P450 reductase present in Sp107 rat mammary carcinoma [93].

Anthraquinone-based Antitumor Agents. Activation of anthraquinone-based antitumor agents, such as mitoxantrone, daunorubicin, and ametantrone, by DT-diaphorase was associated with the formation of $O_2^{\cdot -}$, H_2O_2 and $HO^{\cdot}$ as indicated by ESR spin-trapping studies [94]. The cytotoxicity and genotoxicity of several hydroxy-substituted anthraquinones to V79 cells were decreased (rather than enhanced) by dicumarol, thus suggesting a role for DT-diaphorase in the activation of these compounds within a process that entailed the formation of oxyradicals [95].

Redox-labile Hydroquinones and Thiol Reactivity

Thiol reactivity is a central feature of quinone cytotoxicity, and two key reactions can be invoked to account for these effects. First, alkylation reactions resulting in quinone-thioether formation and supported by the electrophilic character of quinones and their high reactivity towards sulfur nucleophiles, i.e. nonprotein and protein thiols. The chemical considerations for the sulfur addition and substitution chemistry of quinones have been surveyed in an excellent review article [96]. Second, thiol oxidation by oxyradicals generated during quinone metabolism. Thiol arylation and oxidation account for the following aspects of quinone cytotoxicity.

Many quinone-thioethers derived from the interaction of quinones with GSH possess biological activity. A clear example is the ability of the menadione-glutathionyl conjugate to redox cycle following its activation by one-electron [97, 98] and two-electron [56] transfer flavoproteins. Glutathionyl adducts of some quinoneimines bind to calf thymus DNA [99], and certain halogen-containing quinone thioethers are potent and selective nephrotoxicants [100–103].

Oxidation (rather than alkylation) of thiols is observed during the exposure of hepatocytes to various quinones: the fall of reduced glutathione (GSH) and the concomitant rise in oxidized glutathione (GSSG) precede the decrease in protein sulfhydryls [26, 41]. The mechanisms underlying cytoskeletal damage differ depending on whether the quinone acts primarily by oxidative stress or alkylation [26]: oxygen radicals ensuing from quinone-supported redox cycling mediate an oxidation-dependent actin cross-linking (sensitive to dithiothreitol) [104]. Likewise, a similar mechanism was brought forward to account for the marked changes observed in the intracellular pyridine nucleotides [105], which seem to be a requirement for Ca^{2+} release [106, 107].

Alkylation and thiol oxidation are not necessarily independent processes: extensive GSH oxidation by oxygen radicals accompanies the thiol nucleophilic addition to 2-methyl-1,4-naphthoquinone alkylating

agents [108], and semiquinone thioethers result from these interactions [109, 110].

The redox properties of the hydroquinones generated during DT-diaphorase-catalyzed reactions (as in pathways **I** and **IIa, b** in Fig. 1) are critical to the understanding of thiol reactivity and of the cytotoxic implications for these processes. Although these reactions are complex and their features not fully understood, their analysis requires consideration of the interdependent redox transitions involving quinones (reaction 7), oxygen (reaction 8), and the thiol/disulfide system (reaction 9):

$$QH_2 \leftrightharpoons Q^{\cdot -} \leftrightharpoons Q \quad (7)$$

$$O_2 \leftrightharpoons O_2^{\cdot -} \rightarrow H_2O_2 \rightarrow HO^{\cdot} \quad (8)$$

$$RS^- \rightarrow RS^{\cdot} \rightarrow RSSR \quad (9)$$

$O_2^{\cdot -}$ (or $HO_2^{\cdot}$), $HO^{\cdot}$, and the semiquinones formed during the hydroquinone redox transitions can facilitate with different efficiencies the $RS^- \rightarrow RS^{\cdot}$ transition. The thermodynamic and kinetic aspects involved in thiol oxidation by semiquinones were referred to above in reaction 6. Conjugation of thiyl radicals with thiolate (reaction 10) is a critical factor in these reactions [see Refs. 96 and 111] resulting in disulfide anion radical formation, which subsequently autoxidizes with formation of $O_2^{\cdot -}$ (reaction 11). These relationships, which apply to some extent to models entailing the autoxidation of

$$RS^{\cdot} \rightarrow RS^- \rightarrow RSSR^{\cdot -} \quad (10)$$

$$RSSR^{\cdot -} + O_2 \rightarrow RSSR + O_2^{\cdot -} \quad (11)$$

dihydropyrimidines and alloxan [65] and the reactivity of 1-naphthoxyl radical with GSH [112], have been critically analyzed by Wardman [86, 111] and further discussed in connection with $O_2^{\cdot -}$ reactivity by Winterbourn [113] and on thermodynamic grounds by Koppenol [114]. When quinones are concerned, $RS^{\cdot}$ and $RSSR^{\cdot -}$ can decay by pathways other than those shown in reactions 10 and 11: methyl-substituted *p*-benzohydroquinones are readily oxidized ($k_{12} \sim 10^7$ $M^{-1}sec^{-1}$) by $GS^{\cdot}$, and benzoquinone and methyl-substituted derivatives are reduced to semiquinones by $GSSG^{\cdot -}$ at high rates ($k_{13} \sim 10^8$–10^9 $M^{-1}sec^{-1}$) [115].

$$RS^{\cdot} + QH_2 \rightarrow RSH + Q^{\cdot -} + H^+ \quad (12)$$

$$RSSR^{\cdot -} + Q \rightarrow RSSR + Q^{\cdot -} \quad (13)$$

Within the context of this commentary, it is important to characterize the modulation of thiol reactivity by superoxide dismutase within pathways involving DT-diaphorase activity. The effect of superoxide dismutase on thiol oxidation permits one to address quinone reactivity, and its further implications for cytotoxicity, in terms of their bioreductive activation systems and the inherent redox transitions. The $RS^- \rightarrow RS^{\cdot}$ transition is mainly coupled to semiquinone (reaction 6) and $O_2^{\cdot -}$ reduction; although neither reaction is expected to occur at appreciable rates, there are numerous reports documenting their occurrence in biological settings.

During the thiol-mediated *reductive decay* of the semiquinone (reaction 6), the focus of superoxide dismutase activity is centered on a reaction down the free radical chain, i.e. displacing the equilibrium of reaction 11 via reaction 3. Thus, the radical character is transferred according to the sequence in reaction 14 and, finally, via disproportionation to a non-radical product, H_2O_2. Two oxidizing radicals ($Q^{\cdot -}/Q^{2-}$ and $GS^{\cdot}/GS^-$, with positive $E^{o\prime}$ values) and two reducing radicals ($GSSG/GSSG^{\cdot -}$ and $O_2/O_2^{\cdot -}$ with negative $E^{o\prime}$ values) are generated during this sequence. The overall effect is expressed as an enhancement of GSSG and H_2O_2 accumulation by superoxide dismutase.

$$Q^{\cdot -} \rightarrow GS^{\cdot} \rightarrow GSSG^{\cdot -} \rightarrow O_2^{\cdot -} \quad (14)$$

Thiyl radical formation during thiol oxidation by $O_2^{\cdot -}$ is also suppressed by superoxide dismutase. However, in these instances the effect entails prevention of thiyl radical formation, and this is expressed as an inhibition of GSSG accumulation by the enzyme.

These features are inherent in the metabolism of the 2-methylmethoxy-naphthoquinone bioalkylating agent by one- and two-electron transfer flavoproteins [87]. GSSG accumulation was stimulated and inhibited by superoxide dismutase during the activation of the quinone by NADPH–cytochrome P450 reductase and DT-diaphorase, respectively. These diverse effects do not necessarily reflect the mode of activation of the quinone, i.e. one- versus two-electron transfers. The steady-state concentrations of semiquinone and $O_2^{\cdot -}$ appear to be crucial factors contributing to the prevalence of either pathway. Under situations in which $[Q^{\cdot -}]_{ss} > [O_2^{\cdot -}]_{ss}$, superoxide dismutase favors the radical transfer in the sequence in reaction 14. Conversely, when $[Q^{\cdot -}]_{ss} < [O_2^{\cdot -}]_{ss}$, superoxide dismutase prevents thiyl radical formation and GSSG accumulation. These relationships are summarized in Fig. 4. In addition to the decay pathways for thiyl radicals described here, it should be considered that these radicals are potent oxidants capable of H abstraction and initiation of lipid peroxidation [116].

In summary, the molecular mechanisms described in this section illustrate a complex network of free radical reactions involving multiple competing radical decay processes and redox reactions that are not necessarily irreversible. The antioxidant or prooxidant features inherent in the concerted and individual activities of DT-diaphorase and superoxide dismutase and the further implications for thiol oxidation require (a) consideration of the redox activity of the primary hydroquinone product (an expression of its functional group chemistry), (b) recognition of different radical species and their relative steady-state concentrations, (c) identification of prevalent free radical decay pathways, and (d) characterization of the multiple points in the free radical chain where superoxide dismutase can exert its activity.

III DT-DIAPHORASE-MEDIATED FORMATION OF BIOALKYLATING AGENTS

Enzyme-mediated aromatization of quinones leads to loss of their electrophilic character and, hence,

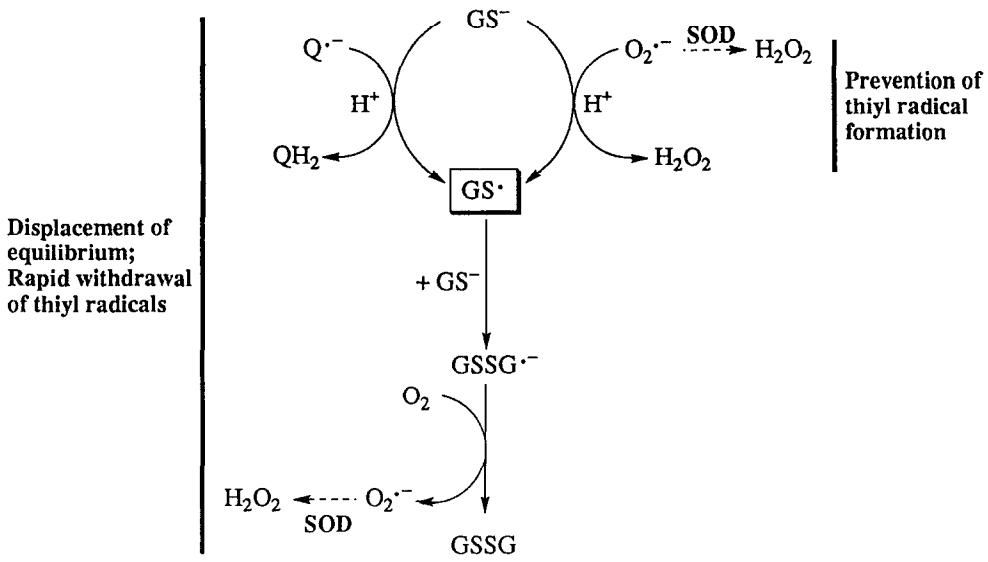

Fig. 4. Effect of superoxide dismutase on the redox transition of thiyl, semiquinone, and superoxide radicals.

restricts their participation in arylation reactions. This is usually considered as part of the protective functions of DT-diaphorase against quinone cytotoxicity (Fig. 1, pathway **I**). This assumption is correct and usually applies to hydroquinone species with simple substitution patterns. Conversely, the two-electron enzymic reduction fulfills the chemical requirements for activation of quinones to potent electrophilic compounds upon facilitation of oxidative elimination of a leaving group (with formation of a quinone methide) or aziridinyl ring opening, and subsequent electrophilic attack on cellular nucleophiles [11–13] (Fig. 1, pathway **III**).

The implications of quinone methide formation during DT-diaphorase-catalyzed activation of mitomycin C and, consequently, its bioalkylating activity are well documented by numerous studies using cell lines rich or deficient in DT-diaphorase activity and evaluating the sensitivity of cytotoxicity to dicumarol. Despite this, no correlation seems to exist between DT-diaphorase levels and sensitivity to mitomycin C, whereas a correlation was found for the case of EO9, an indoloquinone bearing aziridinyl substituents in mouse and human colon carcinomas [117–119]. On the other hand, studies carried out with purified DT-diaphorase failed to show reduction of this chemotherapeutic agent. This controversy was resolved recently by Ross and his colleagues: an excellent discussion and a brief historical perspective on the subject were covered in a recent review [12]. Briefly, mitomycin C was reduced effectively by DT-diaphorase at pH 5.8, and the pH-dependent metabolism of mitomycin C was explained by the ambivalent behavior of the quinone methide intermediate: at near physiological pH, the methide functioned as an electrophile alkylating and inhibiting the enzyme, whereas at low pH values, the quinone methide was converted into a nucleophile upon rapid deprotonation, leading to 2,7-diaminomitosene formation. In the latter instances, no inhibition of DT-diaphorase occurred [120, 121].

There seems to be a general consensus that DT-diaphorase activity has only a limited role in the cellular activation of mitomycin C under hypoxic conditions, whereas the enzyme appeared to activate the drug efficiently in aerobiosis [122, 123]. Despite the selective activation of mitomycin C by DT-diaphorase in aerobiosis, the hydroquinone form apparently does not autoxidize, thus excluding the participation of oxyradicals in DNA damage [121]. Indeed, free radical contribution to mitomycin cytotoxicity is a controversial issue: mitomycin metabolism does not involve oxyradical-mediated cytotoxicity (e.g. DNA strand breaks) in EMT6 cells [124, 125], whereas oxygen radicals underlie mitomycin cytotoxicity in other cell lines and in lung and heart [126, 127]. On the other hand, multiple drug level resistance in HT29R13 cells is partly accounted for by increased enzyme activities involved in drug or oxidant detoxification, such as glutathione transferase-π and -α glutathione reductase, and glutathione peroxidase [128]. Augmentation of the glutathione peroxidase pathway was suggested to render certain tumors resistant to the anticancer quinones upon maintaining intracellular peroxide homeostasis during a putative oxidative stress induced by quinone-containing anticancer agents [129]. Also, the cytotoxicity originating from continuous mitomycin C treatment was enhanced modestly by GSH depletion (DT-diaphorase activity was not affected by treatment of cells with buthionine sulfoximine to deplete GSH) [130]. Taken together, these observations suggest that cell resistance to mitomycin C may not be the sole function of DT-diaphorase activity and that oxidant formation may be among the requisite conditions to observe antitumour activity.

In addition to DT-diaphorase, other enzymes are actively involved in mitomycin C activation. In hypoxic conditions, mitomycin C (and the indoloquinone EO9) are reduced presumably by one-electron transfer reductases to an extent similar or greater than that observed with DT-diaphorase in hypoxia [119]. Xanthine dehydrogenase from EMT6 mouse mammary tumors catalyzes the conversion of mitomycin C to 2,7-diaminomitosene in aerobic and hypoxic conditions. Oxyradical formation during xanthine dehydrogenase-catalyzed activation of mitomycin is less efficient than that observed during the xanthine oxidase-catalyzed process [131–133]. Interestingly, dicumarol inhibited the xanthine dehydrogenase-mediated conversion of xanthine to uric acid but potentiated the metabolism of mitomycin C by the enzyme [16]. From these observations, it is clear that dicumarol does not modulate mitomycin C metabolism solely through the inhibition of DT-diaphorase.

As mentioned earlier, aziridinyl N protonation and ring opening are the key chemical steps in the activation of aziridinylbenzoquinones to electrophilic species. These processes require quinonoid ring aromatization via one- or two-electron flavin reductases with subsequent formation of alkylating agents producing DNA cross-links. The two-electron reduction of AZQ was shown to be accomplished by DT-diaphorase purified from rat liver or human HT-29 cells [121] and by the rat liver enzyme expressed in *Escherichia coli* [79]. A trend between the rate of reduction by DT-diaphorase of a diaziridinylbenzoquinone analog and its ability to induce toxicity in HT-29 cells, rich in DT-diaphorase, supports a role for this enzyme in the bioreductive activation of these compounds [134]. Studies on DT-diaphorase-mediated activation of the bifunctional alkylating agents 2,5-diaziridinylbenzoquinone (DZQ) and 3,6-bis-substituted analogs revealed alterations in DNA cross-linking and sequence selectivity following the activation of these compounds by DT-diaphorase [135, 136]. Although evidence has accumulated on aziridinylbenzoquinone-supported redox cycling and the accompanying DNA strand breakage [77, 78], the contribution of oxidative stress to aziridinylbenzoquinone anticancer activity has not been explored in detail.

In general, the role of free radicals in antitumour quinone reactivity and of their implications for cancer cell and systemic cytotoxicity has been randomly established without emphasis on a particular chemotherapeutic quinone. Some of the compounds listed in this section, although generally termed bioalkylating agents, can exert their cytotoxic effects by a free radical mechanism: the hydroquinone forms of 3-bromo-2-methyl-1,4-naphthoquinone [137], AZQ [76–81], and 2-methylmethoxy-1,4-naphthoquinone [87] autoxidize readily following their activation by DT-diaphorase, contrary to the established dogma that two-electron transfers yield redox-stable hydroquinones. Moreover, DNA strand breakage during DT-diaphorase catalysis of the latter is 5-fold higher than that observed during the one-electron activation of the quinone [87]. DT-diaphorase activity in MCF-7 S9 fraction catalyzed the activation of AZQ, menadione, and certain anthraquinones resulting in $HO^{\cdot}$ production; conversely, $HO^{\cdot}$ formation observed during activation of mitomycin C was not mediated by DT-diaphorase [138]. Metabolism of EO9 by rat Walker and human HT29 tumor cell DT-diaphorases [118, 139] resulted in single-strand breaks in plasmid DNA [140]; given the chemical mechanisms leading to single-strand breakage, it could be assumed that free radicals play a significant role in the metabolism of EO9 by DT-diaphorase. As described above, these processes operate by a two-electron redox cycle supported by DT-diaphorase and involve multiple competing free radical decay pathways. It is clear that both mechanisms entailing formation of alkylating species targeted towards DNA and oxygen radicals should be considered when addressing the cytotoxic effects of these chemotherapeutic compounds.

CONCLUSIONS

The DT-diaphorase-catalyzed hydroquinone formation is not a superficially simple process: advances in our understanding of the cellular functions of DT-diaphorase and assignment of this activity to antioxidant or prooxidant pathways require careful elucidation of structure–activity relationships and characterization of hydroquinone chemical reactivity. Hydroquinone reactivity, an expression of its functional group chemistry, can serve to rationalize the cytotoxic effects of different quinones and aid drug design targeted toward enzymes or cells. Subtle modifications in the functional groups of quinones lead to situations where activation via DT-diaphorase is not only associated with either prevention or potentiation of quinone cytotoxicity, but it also results in different types of cytotoxic mechanisms.

On a more general note, it is necessary to recognize that the protective or prooxidant functions of DT-diaphorase cannot be viewed in a simplistic manner such as one- versus two-electron enzymatic activation. Oxidative stress is a characteristic feature of quinone cytotoxicity, and understanding of the overall biological activity of DT-diaphorase requires analysis of its activity within metabolic pathways involving multiple enzymes. Establishing partners for DT-diaphorase, such as superoxide dismutase within the context of hydroquinone autoxidation [58, 66] and UDP glucuronyl transferase within hydroquinone deactivation and elimination [44, 47], seems essential to the understanding of the biological role of this enzyme. The relative cellular ratios of these concerted activities, as well as those providing substrates for DT-diaphorase, can lead to the recognition of rate-limiting steps that are critical for the expression of cytotoxicity.

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