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COMMENTARY

DT-DIAPHORASE AND CANCER CHEMOTHERAPY

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Historical background

The first report documenting the discovery of DT-diaphorase, nowadays more properly referred to as NAD(P)H: (quinone acceptor) oxidoreductase (EC 1.6.99.2)†, was published in 1958 by Ernster and Navazio [1]. They described the activity of a diaphorase or electron transfer enzyme in the soluble fraction of rat liver homogenates, which catalysed the oxidation of NADH and NADPH (then referred to as *D*PNH and *T*PNH) with equal facility. Subsequently, Ernster and colleagues went on to partially purify and characterize this apparently unique enzyme [2–4]. In addition to its lack of specificity for either nicotinamide dinucleotide, a striking feature of DT-diaphorase was its extreme sensitivity to dicoumarol [2]. This property initiated the use of dicoumarol as a potent diagnostic inhibitor of DT-diaphorase activity and stimulated research to determine whether the enzyme played a role in the metabolism of vitamin K or its epoxide [5–7]. A further unique property among NAD(P)H quinone reductases is the ability of DT-diaphorase to catalyse obligatory two-electron reduction [8], and it is this feature which is crucial to its role in cytoprotection against toxic chemicals.

Numerous original research papers and reviews have been published on DT-diaphorase. These have focused on various basic aspects of the enzyme, including its structure, reaction mechanism, biosynthesis and regulation, and also on its classical role as a protective, cellular defense mechanism against the cytotoxic and mutagenic effects of a variety of structurally diverse, potentially lethal compounds, including quinones, quinone epoxides, quinoneimines, azo dyes and C-nitroso derivatives of arylamines. The purpose of this commentary is to highlight more recent biochemical and molecular advances which have increased our comprehension of the mechanism of action and regulation of DT-diaphorase and, in particular, its increasingly prominent role in current cancer chemotherapy.

Subcellular distribution and molecular biology

DT-diaphorase is a predominantly (> 90%) cytosolic enzyme [1–4]. Activity has also been detected in a number of subcellular organelles, including the endoplasmic reticulum [9], mitochondria [10] and Golgi apparatus [11]. The enzyme is widely distributed among organs, with particularly high levels being expressed in the liver, kidney and gastrointestinal tract [12–14].

The enzyme exists as a dimer of a molecular weight around 55,000 and contains two subunits of equal size and two molecules of FAD [8, 15]. At least two isofunctional forms of DT-diaphorase exist in mouse liver [16] and a minimum of three antigenically distinct variants can be detected in rat liver [17, 18]. In humans, genetic evidence indicates that the different forms of DT-diaphorase appear to be encoded by four gene loci (*DIA 1* to *DIA 4*) [19]. The product of one of these loci (diaphorase 4) has been characterized recently as a dioxin-inducible form of DT-diaphorase (termed NQO_1) [20]. It appears to account for the bulk of the DT-diaphorase activity measured in most tissues but does not occur in erythrocytes, which apparently have no measurable DT-diaphorase activity [21]. The gene for diaphorase 4 is located on chromosome 16 and is the orthologue of the rat NQO_1 gene [22]. Both the human and rat genes encode for a protein of 274 residues [22, 23]. Cloning, sequencing and expression of the human NQO_1 protein has shown that it is highly similar (84% homologous) to the rat NQO_1 protein [22] and that antisera against the rat enzyme cross-react with human NQO_1 [20].

More recently, a second constitutive human DT-diaphorase isozyme has been characterized (NQO_2) which is not induced by dioxin, and the gene encoding this product has been assigned to chromosome 6 [24]. The human NQO_2 cDNA codes for a protein of 231 amino acids, some 43 amino acids shorter than the NQO_1 protein [22, 24]. A comparison of the nucleotide sequences has demonstrated that the NQO_2 and NQO_1 cDNAs display 54% overall homology [24]. Interestingly, the NQO_2 gene locus is extremely polymorphic [24], and this finding may have important ramifications concerning inter-individual variation in both phar-

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† NAD(P)H:(quinone acceptor) oxidoreductase (EC 1.6.99.2) is the nomenclature recommended by the Enzyme Commission. DT-diaphorase is a trivial name for this oxidoreductase which has also been referred to previously as vitamin K reductase, phylloquinone reductase, menadione reductase, nicotinamide menadione oxidoreductase (NMOR), quinone reductase and quinone acceptor oxidoreductase. As suggested by Ernster (see Ref. 4), the more common term "DT-diaphorase" will be retained for the purpose of this commentary.

macodynamic and toxicological responses to certain xenobiotics.

Information regarding the substrate preferences of the different forms of DT-diaphorase is limited, but it is generally accepted that this class of enzymes displays marked overlapping substrate specificity [4, 15, 25]. Studies by Edwards and colleagues suggested that different forms of the enzyme may exhibit distinct affinities for the prototype substrates DCPIP and menadione [19], and similar conclusions were drawn by Segura–Aguilar and Lind using DCPIP, menadione, vitamin K_1 and benzo(*a*)pyrene-3,6-quinone [18]. Site-directed mutagenesis studies with rat NQO_1 have revealed regions which may be involved in NAD(P)H binding [26], and similar technology may prove useful in the elucidation of other domains which govern the binding of substrates for human DT-diaphorase.

Regulation of DT-diaphorase expression

The expression of many drug-metabolizing enzymes, including both phase I and phase II components, may be induced by a number of structurally dissimilar chemicals, including drugs, steroids, industrial chemicals and dietary constituents [27]. Depending on the resultant enzyme profile, enzyme induction may prove to be beneficial or deleterious to an organism [28]. In addition to being constitutively expressed in a variety of tissues, including stomach, kidney, lung, liver, colon and breast [4, 13, 14], DT-diaphorase may also be induced by polycyclic aromatic hydrocarbons [29], coumarins, fumarates, maleates, acrylates, cinnamates, dietary phenolic antioxidants and related compounds [30, 31]. Prochaska and Talalay [32] have conveniently categorized enzyme inducing agents into *bifunctional* and *monofunctional* inducers. Bifunctional inducers include compounds like polycyclic, planar aromatic hydrocarbons, azo dyes and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD*), which co-induce the activity of both phase I (most notably members of the cytochrome P450 1A family) and phase II enzymes [such as UDP-glucuronyl transferases, glutathione-*S*-transferases (GSTs) and DT-diaphorase]. Such compounds have been shown to mediate the induction of P450 1A enzymes through the Ah (*a*ryl *h*ydrocarbon) receptor [33, 34], and subsequent metabolism of these chemicals by this enzyme(s) may produce highly reactive, electrophilic metabolites [34]. By contrast, monofunctional inducers selectively increase the activities of phase II enzymes only [32]. Many monofunctional inducers have been shown to possess electrophilic centres or acquire them through metabolic activation [35, 36] and are classical Michael reaction acceptors [35].

These two classes of inducers are not mutually exclusive, as the metabolites of bifunctional inducers, such as β-naphthoflavone, may be regarded as monofunctional inducers *per se*. A series of structure–activity studies by Talalay, Prochaska and co-workers [30–32, 35, 36] have identified some of the molecular features required by inducers of DT-diaphorase. Interestingly, these workers also noted that the potency of monofunctional inducers of DT-diaphorase parallels their facility to act as substrates for the GSTs [37].

Pickett and colleagues have simultaneously reached conclusions similar to those of Prochaska and Talalay through the use of a complementary, molecular approach [38–40]. This group has identified two "responsive elements" or regulatory regions which are involved in the control of gene expression in the rat by planar aromatic compounds [38] and phenolic antioxidants [39, 40]. One of these, termed the "xenobiotic responsive element" (XRE), is a sequence within the 5′-flanking region of the rat GST Ya subunit gene which is also found in multiple copies in the 5′-flanking region of the gene encoding cytochrome P450 IAI (*CYPIAI*) [39]. The second region (termed the "antioxidant responsive element", ARE) is again present in the 5′-flanking region of the rat GST Ya subunit gene and is responsive to the metabolites of polycyclic aromatic hydrocarbons and phenolic antioxidants [39, 40]. The ARE is present in the 5′-flanking region of the DT-diaphorase (NQO_1) gene and appears to be involved in the constitutive expression of the GST Ya gene [40]. The core sequences of the two responsive elements XRE and ARE are distinct [40]. This is in accord with their disparate functions: ARE responds to reactive oxygen species (including hydrogen peroxide) produced by the redox cycling of metabolites, while the XRE is responsive only to planar aromatic compounds, although triggering of either regulatory region will result in elevations in both GST Ya and DT-diaphorase. The XRE functions as a transcriptional enhancer when Ah receptor ligands bind to the receptor in responsive cells. The ARE appears to be a regulatory element which activates genes encoding enzymes involved in the cellular defense mechanisms against oxidative stress and may function in a manner similar to that described for the regulators of superoxide- and hydrogen peroxide-inducible genes (*SoxR* and *OxyR*, respectively) recently characterized in bacteria [41].

Thus, DT-diaphorase can be induced in response to stimuli from polycyclic aromatic hydrocarbons, chemically reactive metabolites and reactive oxygen species, all of which have been implicated in carcinogenesis [34, 42, 43]. However, the system is also apparently regulated at other levels as steroids may potentiate (up to 80-fold) the coordinate induction of P450 1A1, GST and DT-diaphorase by polycyclic aromatic hydrocarbons *in vitro* [44]. High concentrations of dexamethasone may also induce directly an immunoreactive form of DT-diaphorase which has a low affinity for menadione, although the exact mechanism by which this occurs has yet to be defined [44].

DT-diaphorase and cancer

In view of the potential for DT-diaphorase to detoxify a wide range of chemically reactive metabolites and thereby protect the cell from the toxic and mutagenic effects of these species, together with its induction by a variety of pro-carcinogens, it

* Abbreviations: TCDD, tetrachlorodibenzo-*p*-dioxin; GSTs, glutathione-*S*-transferases; XRE, xenobiotic response element; ARE, antioxidant responsive element; AZQ, diaziquone; and MMC, mitomycin C.

is evident that a perturbation in the expression of this enzyme would be likely to occur during carcinogenesis. Indeed, both DT-diaphorase enzyme activity and the corresponding mRNA have been shown to be elevated markedly in rat hepatic preneoplastic nodules induced during multi-stage chemical carcinogenesis in the Solt–Farber model (6- to 7-fold higher than in surrounding normal tissue), along with mRNA for the GST Ya and Yb genes [45, 46]. The elevated mRNA has been shown to be the consequence of hypomethylation of the gene rather than the result of gene amplification [29, 38] and appears to be a reversible process since the levels of DT-diaphorase decrease in redifferentiated nodules [45]. In addition to DT-diaphorase and GST, other phase II enzymes are elevated during hepatocarcinogenesis, including UDP-glucuronyl transferase, while the activities of phase I enzymes are concomitantly decreased. These observations suggest that DT-diaphorase may provide an early cellular defense against tumorigenesis.

In addition to the hyperexpression of DT-diapharose in chemical carcinogen-induced hepatic preneoplastic nodules compared to surrounding normal tissue [29, 38, 45, 46], elevations in DT-diapharose have also been observed in rat brain during carcinogenesis [47], leading to suggestions that increased levels of DT-diapharose may be a common marker for neoplasia, at least in certain model systems [48, 49]. Moreover, there is now a considerable body of literature regarding the relative expression of DT-diaphorase in neoplastic versus normal tissue, including data not only from chemical carcinogenesis models [29, 38, 45, 46, 48, 50] but also from various cell lines [51–53], spontaneous tumours in rodents [54] and various human cancers [14, 55, 56]. These studies have collectively shown that DT-diaphorase is elevated not only in preneoplastic tissue but also in established tumours, although the majority of the information was obtained in rodent hepatoma models. However, the findings have been fortified recently in an important study by Cresteil and Jaiswal [57] which demonstrated a 20- to 50-fold increase in the levels of NQO_1 mRNA in human liver tumours and surrounding tissue as compared to the levels in samples from normal individuals.

Although these studies have consistently demonstrated an increase in DT-diaphorase in tumour with respect to normal tissues of the same organ, several papers have also been published to the contrary. For example, diethylstilbestrol-induced carcinogenesis in Syrian hamster kidney has been associated with marked decreases in the activities of both cytosolic and microsomal DT-diaphorases [58], although *in vitro* studies in a fibroblastic cell line from the same strain of species suggest that transformation of these cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine results in a marked elevation of DT-diapharose activity [49]. Similarly, although Schlager and Powis [14] clearly demonstrated that DT-diaphorase activity is increased in human tumours of the lung, liver, colon and breast, a more recent study has suggested that the enzyme may be elevated in some human colorectal tumours but decreased or unchanged in others [59]. The reasons for the apparent discrepancies between studies such as these are not fully understood. It may be that there are no hard and fast rules governing the expression of DT-diaphorase in tumour versus normal tissue. This may be accounted for by the marked genetic instability and biochemical heterogeneity of the cancer cell.

Possible species- and tissue-dependent regulation of expression of the enzyme may also be involved: for example, the study by Schlager and Powis [14] noted a marked decrease in the levels of human kidney tumour DT-diaphorase compared with the high levels observed in normal kidney. The diverse, multi-stage nature of neoplastic diseases in terms of their genetic, biochemical and cellular origin may also contribute to the variations in DT-diaphorase levels noted in different studies. In addition, it may well be that different classes of chemical carcinogens either decrease or increase DT-diaphorase depending on the tissue involved and the timing of the biopsy analysis. Furthermore, chemically induced cancers may affect DT-diaphorase expression in a different manner to neoplastic diseases initiated by other stimuli, such as viruses, radiation and spontaneous genetic changes in oncogenes and tumour suppressor genes.

The precise molecular basis for the alterations in DT-diaphorase expression seen in developing and established tumours remains poorly defined. Polymorphic loci on chromosome 6 (where NQO_2 is located) frequently show loss of heterozygosity during ovarian [60] and colorectal [61] cancer development. Loss of heterozygosity is associated with the unmasking of recessive mutations at tumour suppressor loci. The activity of NQO_2 may be important in carcinogen and anticancer drug metabolism, and it is therefore an intriguing possibility that somatic deletion at NQO_2 may play a role in tumour development and drug resistance. This hypothesis should be examined in more detail and the presence of several restriction fragment length polymorphisms at the NQO_2 locus [24] indicates that such a study should be technically feasible. Similarly, genetic alterations on chromosome 16, such as the loss of heterozygosity observed in breast cancer [62], may affect the expression of NQO_1 during tumour development.

Although not previously reported, the possibility also exists for amplication or translocation of DT-diaphorase in tandem with oncogenes and tumour susceptibility genes [63]. The effects of altered expression of positive and negative cancer genes on DT-diaphorase expression do not appear to have received much attention. It is interesting that the human NQO_1 promoter region contains putative binding sites for the AP-1 protein complex and the DNA-binding protein product of the *jun* oncogene [57]. The levels of these regulatory proteins can be altered during transformation, and future studies should be directed at defining their role in the regulation of the NQO_1 gene.

DT-diaphorase and the action of anticancer drugs

Although DT-diaphorase was originally considered to function as a protective enzyme which detoxifies a range of chemically reactive metabolites, recent

evidence has shown that this flavoenzyme, like many other drug-metabolizing systems, may paradoxically activate certain xenobiotics [64, 65]. In view of the general consensus that DT-diaphorase is over-expressed in tumour cells, particularly those of the liver, breast and colon [14, 31, 55–57] and the realization that DT-diaphorase may either activate or detoxify xenobiotics, depending on their chemistry [64, 65], it has become increasingly important to establish the role of DT-diaphorase in the sensitivity of tumours to anticancer drugs. Indeed, there has recently been a surge of interest in mechanistic toxicology studies designed to assess the role of DT-diaphorase in the bioactivation and/or detoxication of a number of established and experimental anticancer agents. These include the bioreductive alkylating aziridinyl benzoquinones such as diaziquone (AZQ), mitomycin C (MMC), and the indoloquinone EO9, together with the dinitrophenyl aziridine CB 1954 and the benzotriazine-di-*N*-oxide SR 4233. The structures of these compounds are shown alongside menadione in Fig. 1.

In addition to understanding the molecular mechanisms responsible for the sensitivity and resistance of tumour cells to these agents, two particular aspects of the role of DT-diaphorase in the metabolism of such compounds are of particular relevance to cancer chemotherapy. The first is the possibility that measurement of DT-diaphorase in tumour biopsies, known as "enzyme profiling", would provide a possible basis for selecting those patients most likely to benefit from a particular drug treatment. The second is the opportunity to design improved anticancer agents which would either be activated by DT-diaphorase in tumours or detoxified by the enzyme in normal tissues, a process we have termed "enzyme-directed bioreductive drug development" [66].

Diaziquone (AZQ)

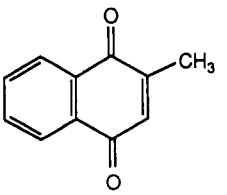
AZQ is one of a number of aziridinylbenzoquinone analogues synthesized at the National Cancer Institute, U.S.A., by Driscoll and co-workers [67–69]. Extensive screening of quinoid compounds for anti-tumour activity revealed that *p*-benzoquinone analogues containing aziridine moieties were among the most active against many tumour models [68]. AZQ was selected on the basis that it would penetrate the central nervous system, while retaining adequate water solubility to be administered parenterally [67, 68]. Subsequently, this bioreductive drug has entered clinical trials in the U.S.A., some of which have indicated activity in brain tumours and non-lymphocytic leukaemia [70, 71].

Aziridinylbenzoquinone anticancer drugs are believed to undergo activation to potent alkylating agents upon reduction of the quinone moiety [72]. Reduction of the quinone nucleus to either the one-electron reduction product, the semiquinone, or the two-electron reduction product, the hydroquinone, is thought to facilitate protonation of the aziridine ring and subsequent ring opening leading to the formation of the ultimate potent alkylating species [73]. Quinone bioreductive drugs may be enzymatically reduced by a direct two-electron reduction catalysed by DT-diaphorase [4, 15, 25] or via one-electron reduction by a variety of enzymes, including NADPH-cytochrome P450 reductase [72, 74]. Although either of these routes of bioactivation could, in theory, result in the formation of the ultimate toxic metabolite of AZQ, recent evidence has shown that DT-diaphorase may be a major reductase involved in the bioactivation of AZQ to genotoxic and cytotoxic metabolites in cancer cells [65, 75, 76]. Interestingly, the hydroquinone product of the reduction of AZQ by DT-diaphorase may auto-oxidize in air and the ensuing redox cycling may also contribute to the cytotoxic and genotoxic effects of this drug [76].

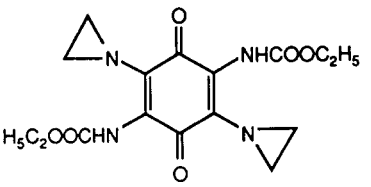
In vitro reduction of AZQ to a product which introduces DNA interstrand cross-links has been demonstrated using an agarose gel technique [77]. This cross-linking is facilitated at low pH [77]. In addition, it has been suggested that DT-diaphorase may bioactivate AZQ analogues to sequence-selective alkylating species and that this effect is a function of the chemistry of the substrate [78]. AZQ has been shown to undergo obligatory two-electron reduction by DT-diaphorase from MCF-7 human breast carcinoma cells [76], HT-29 human colon carcinoma cells [75] and rat liver [75]. Studies with two human colon carcinoma cells lines have shown that cells containing at least 300-fold higher levels of DT-diaphorase (HT-29 cells) may be around 2-fold more sensitive to AZQ than cells which express virtually no activity (BE cells). This occurs despite the fact that the sensitive cells exhibit higher levels of reduced glutathione and GSTs [75], both of which may be involved in cellular defense mechanisms against AZQ [65]. The differences in the levels of these additional cytoprotective mechanisms may explain why there is not an even greater difference in sensitivity between the two cell lines, given the disparity in DT-diaphorase levels. Additionally, the involvement of alternative bioreductive enzymes cannot be excluded.

Mitomycin C (MMC)

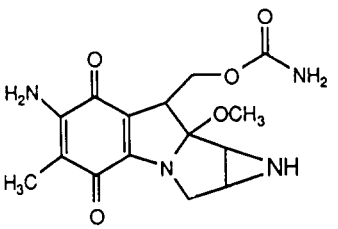
MMC is a naturally occurring antibiotic which exhibits activity against a variety of animal and human tumours [79]. It is used quite widely in human gastrointestinal and bladder cancer therapy [79]. The precise mechanisms responsible for the anti-tumour effects of MMC are unclear, although bioreductive activation to genotoxic metabolites appears to be a prerequisite for its activity [80, 81]. Chemical reduction and acid-mediated degradation of MMC have also been shown to activate the drug to a reactive form which can cross-link adjacent guanines at the N^2 position in the minor groove of DNA via the 1 and 10 positions of the MMC molecule [82, 83]. As for AZQ, generation of a highly reactive bifunctional alkylating metabolite may occur through one- or two-electron reduction of the quinone moiety [81, 84]. The one-electron reduced semiquinone intermediate may give rise to reactive oxygen species through interactions with molecular oxygen, which could account for some of the activity demonstrated in normoxic tumour cells [85]. MMC displays modest preferential toxicity towards hypoxic as opposed to aerobic (or normoxic) tumour cells *in vitro* and *in vivo* [86–88]. Cellular reductases which reduce MMC



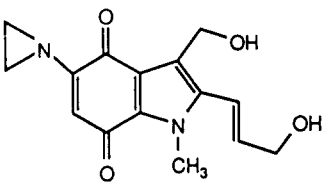
Menadione



Diaziquone (AZQ)

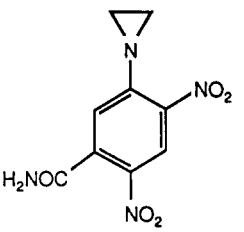


Mitomycin C



EO9

SR 4233



CB 1954

Fig. 1. Chemical structures of the prototype quinone menadione and several anticancer drugs which have been shown to be substrates for DT-diaphorase.

under anaerobic conditions include cytochrome P450 reductase [84, 89], xanthine oxidase [89] and DT-diaphorase [90]. However, the role of DT-diaphorase in the reductive bioactivation of MMC has been the subject of much controversy [91].

Early studies by Sartorelli and co-workers [84, 92, 93] relied on the use of dicoumarol as a specific inhibitor of DT-diaphorase to probe for the role of this enzyme in the cellular toxicity of the drug. Dicoumarol was shown to inhibit the cytotoxicity of MMC in EMT-6 mouse mammary tumour cells, which suggested a role for DT-diaphorase in the bioreductive activation of this drug. However, under hypoxic conditions, MMC cytotoxicity was increased by dicoumarol in these same cells [84, 92, 93]. At this time, the authors concluded reasonably that DT-diaphorase activates MMC in air but detoxifies the drug under hypoxic conditions. However, problems associated with the use of dicoumarol as a cellular diagnostic inhibitor of DT-diaphorase have been highlighted recently [91, 94]. Indeed, when one considers that dicoumarol has been shown to exert a plethora of effects on the cell, including the uncoupling of mitochondrial respiration [4] and inhibition of UDP-glucuronyl transferases [95] and xanthine oxidase [96], results obtained in studies relying solely on this line of evidence should be interpreted with extreme caution.

Subsequent, unsuccessful attempts to demonstrate that MMC was a substrate for purified DT-diaphorase from rat liver [97], human kidney [98] and DT-diaphorase-fortified preparations from human HT-29 colon carcinoma cells and rat Walker 256 mammary tumour cells [91] disputed the role of DT-diaphorase in the metabolism of MMC [91]. However, more recently, MMC has been shown to be a substrate for DT-diaphorase purified from both rat liver [99], rat Walker tumour [100] and HT-29

human colon cancer cells [99]. The metabolism of MMC, unlike the majority of other DT-diaphorase substrates, is markedly pH dependent, being readily apparent only at pH < 7 [99]. This marked pH dependence in DT-diaphorase-mediated metabolism has also been demonstrated for porfiromycin [101], an MMC analogue, and has been explained in terms of the ambivalent nature of the quinone methides formed upon reduction of these drugs [90, 101, 102]. The quinone methide of MMC can act as an electrophile at pH 7.8 and may alkylate the enzyme, resulting in a loss of catalytic activity. However, at pH 5.8 it can be protonated giving rise to 2,7-diaminomitosene, thereby preserving enzyme activity. The pharmacological relevance of bioreductive activation at such low pH levels remains to be established. However, recent studies with a more sensitive spectrophotometric assay, showed that MMC was reduced, albeit at a low rate and with a high K_m (around 1 mM), by rat Walker tumour DT-diaphorase at pH 7.8 [100]. Perhaps a relatively small number of DT-diaphorase-activated molecules, resulting in potent DNA cross-links, would be sufficient to account for the cytotoxicity of MMC. But it is likely that other reductases which may exhibit a higher affinity for MMC are also involved [103]. In contrast to AZQ, the hydroquinone of MMC does not readily auto-oxidize [99] and hence redox cycling is unlikely to accompany reduction of MMC by DT-diaphorase in normoxic tissues.

Studies with HT-29 and BE cells have demonstrated that the cells expressing higher levels of DT-diaphorase were some 6-fold more sensitive to the toxic effects of MMC [99]. Furthermore, HT-29 cells were also more susceptible to the development of MMC-induced DNA inter-strand cross-links, which could be reduced markedly by dicoumarol [99]. These data are in agreement with previous studies conducted in L5178Y/HBM10 lymphoblasts, which possess 24-fold higher levels of DT-diaphorase than the parent line L5178Y [104]. These cells, although deemed to be "quinone resistant" having been induced by exposure to hydrolysed benzoquinone mustard [104], were found to be highly sensitive to MMC [104] and also trenimon (a therapeutically obsolete aziridinylbenzoquinone which is an excellent substrate for DT-diaphorase) [105]. These results are consistent with the hypothesis that DT-diaphorase bioactivates both these anticancer agents under aerobic conditions. Several studies employing more molecular approaches have supported this hypothesis and have demonstrated that a loss of DT-diaphorase activity in Chinese hamster ovary cells [103] and non-transformed human skin fibroblasts from a cancer-prone family [106, 107] may render these cells resistant to the cytotoxic effects of MMC and its analogues, such as the methylated derivative porfiromycin. Furthermore, it has been suggested that there may be a causal link between decreased levels of DT-diaphorase and the predisposition of such individuals to cancer, presumably because these subjects have a diminished capacity to detoxify and eliminate potential carcinogens [108].

Indoloquinone EO9

EO9 is the lead compound in a series of indoloquinones synthesized by Oostveen and Speckamp [109]. The drug is structurally related to the quinone antitumour antibiotic MMC and is about to enter clinical trial as an anticancer agent with the EORTC in Europe on the basis of its distinct antitumour profile and lack of myleosuppression. Recent data from our laboratory have shown that EO9 is metabolized by DT-diaphorase in rat Walker 256 mammary tumour cells and human HT-29 colon carcinoma cells [110]. The rat enzyme reduced EO9 at a much faster rate than the human enzyme, and the respective apparent K_m values (around 3 and 15 μM) suggested that EO9 may have a reduced affinity for the human enzyme [110]. Using a highly purified DT-diaphorase preparation from the rat Walker 256 tumour cell line, EO9 was found to undergo reductive bioactivation under aerobic conditions *in vitro* to a reactive species which produced single-strand breaks in pBR 322 plasmid DNA [110]. Our recent data have also shown that EO9 causes DNA cross-links in Walker tumour cells (Baily SM, Knox RJ and Workman P, unpublished observations). In contrast to MMC, the metabolism of EO9 is not pH dependent in the range 5.8 to 7.8 [110]. Moreover, the V_{max} for the Walker enzyme is 20–30 times higher than for MMC and only 3–4 times lower than that for menadione [110].

These findings suggest that tumours rich in DT-diaphorase may be particularly sensitive and therefore represent attractive targets for EO9. Indeed, our own recent unpublished data (with Dr. J. Plumb) have shown that EO9 is around 25-fold more toxic towards DT-diaphorase-rich HT-29 human colon carcinoma cells compared to enzyme-deficient BE cells. In addition, when the DT-diaphorase levels were compared in two transplantable mouse colon adenocarcinomas growing as solid tumours in their syngeneic hosts, it was found that the tumour more sensitive to EO9 expressed around 15 times the level of DT-diaphorase in the resistant tumour, whereas the activities of cytochrome P450 reductase were identical [111]. However, further studies are required to elucidate the relative roles of one-electron versus two-electron donating enzymes in the bioactivation and detoxication of this novel anticancer agent.

Dinitrophenylaziridine CB 1954

CB 1954 was synthesized by Kahn and Ross [112, 113] at the Chester Beatty Institute, U.K., as one of a series of anti-tumour nitrophenylaziridine analogues. It proved to be highly potent and selective in its effects against the Walker 256 rat tumour line [112–114] but comparatively inactive against a series of other tumours originally tested [114, 115]. The demonstration of the remarkable and selective activity of CB 1954 towards the Walker tumour [112–114] initiated extensive studies on the mechanism of action of this agent [52, 116, 117]. Although CB 1954 is, chemically, a monofunctional alkylating agent by virtue of its aziridine ring, Knox and colleagues have shown that the selectivity of action of CB 1954 is a consequence of its ability to undergo aerobic nitroreduction by DT-diaphorase to a bifunctionally reactive species [52, 117].

DT-diaphorase in Walker cells has been shown to

reduce the nitro group in the 4-position of CB 1954 to the hydroxylamine derivative [52]. The product, 5 - (aziridin - 1 - yl) - 4 - hydroxylamino - 2 - nitrobenzamide, is a highly toxic metabolite which reacts bifunctionally in cells and induces DNA–DNA interstrand cross-links [52, 117]. Further studies demonstrated that DT-diaphorase-rich rat cell lines other than the Walker 256 line were sensitive to CB 1954 but human cells were comparatively resistant, even though they expressed significant levels of DT-diaphorase [118]. The species differences in sensitivity to CB 1954 may be explained by inequalities in the kinetics of CB 1954 reduction by the human and rat forms of DT-diaphorase; both forms are capable of reducing CB 1954 to the hydroxylamine but the rate of reduction by the human enzyme is very much lower [118].

Interestingly, all cell types so far examined appear to exhibit a similar sensitivity to the hydroxylamine of CB 1954 [118]. Moreover, this compound has been shown to induce DNA–DNA interstrand cross-links in cells but not in naked DNA, suggesting that a further activation step may be required to convert the hydroxylamine to the proximal reactive species [117]. The situation thus appears analogous to that observed with a wide range of chemical toxins, including 4-nitroquinoline 1-oxide and *N*-acetylaminofluorene, whereby the respective hydroxylamine is relatively chemically inert *per se* and requires further metabolism by phase II (conjugation) enzymes to elicit its cytotoxic or mutagenic effects [119, 120]. Indeed, a recent report by Knox and co-workers [121] has shown that the hydroxylamine of CB 1954 can be activated non-enzymatically through a chemical reaction with acetyl-coenzyme A and other thioesters. The ultimate DNA reactive species was not defined conclusively but it seems likely that it is 4-(*N*-acetoxy)-5-(aziridin-1-yl)-2-nitrobenzamide or a further derivative of this compound.

As a direct result of these detailed enzymological studies, Sunters and co-workers [122] and Connors [123] have suggested that CB 1954 may be a useful drug for *a*ntibody *d*irected *e*nzyme *p*ro-drug *t*herapy or ADEPT. These authors are looking at the potential for using CB 1954 in combination with an immunoconjugate comprising Walker rat DT-diaphorase and an anti-carcino-embryonic antigen monoclonal antibody in the ADEPT system against colon cancer [122]. In this context, the relatively poor performance of human DT-diaphorase towards CB 1954 is a significant advantage for selective chemotherapy.

*Benzotriazine di-*N*-oxide SR* 4233 (*WIN* 59075)

SR 4233 (WIN 59075; 3-amino-1,2,4-benzotriazine-1,4-oxide) is the lead compound in a series of highly selective hypoxic cytotoxins, the benzotriazine di-*N*-oxides, which exhibit improved efficacy and increased selectivity compared with their predecessors, the mitomycins and nitroimidazoles [124]. The compound is reduced preferentially under anaerobic conditions to both two- and four-electron reduction products [125, 126], neither of which is toxic to cells under aerobic or hypoxic conditions [125, 127]. Consequently, it has been proposed that a one-electron reduced species, by definition a free radical intermediate, is the most likely damaging metabolite [125, 128, 129]. The principal reductases which catalyse this reductive bioactivation are cytochrome P450 and NADPH:cytochrome P450 reductase [126, 130].

More recent data from our laboratory have shown that SR 4233 may be metabolized by DT-diaphorase from the Walker 256 rat carcinoma to both two- and four-electron reduced products [127, 131]. Detailed characterization of the kinetics of this reduction demonstrated that SR 4233, like mitomycin C and CB 1954, does not appear to be a particularly good substrate for the purified Walker enzyme, the K_m and V_{max} being 1.2 mM and 8.6 nmol/min/μg protein, respectively [131]. However, the direct, obligatory two-, four- and, possibly, six-electron reduction of SR 4233 would bypass the formation of the one-electron reduced drug free radical, and DT-diaphorase may therefore provide an important cellular defense against this novel hypoxic cytotoxin.

Enzyme-directed bioreductive drug development

We have proposed that the selectivity of bioreductive anticancer agents could be enhanced not only by tumour hypoxia, but also by increased expression of key reductase enzymes in cancer cells [66, 100]. The relative role of DT-diaphorase and other enzymes in the activation of cancer drugs under normoxic versus hypoxic conditions, remains unclear, and is clearly complicated for mitomycin C [91, 99]. Nevertheless, we have further suggested that even greater selectivity could be achieved by tailor-making drugs to be activated specifically by DT-diaphorase or indeed other appropriate reductase enzymes [66].

DT-diaphorase from various sources is available in crystalline form [52, 132]. It has not yet been possible to obtain a high resolution X-ray structure although preliminary data have been reported [133]. Current efforts are therefore concentrating on a medicinal chemistry approach, i.e. determining the effects of manipulating various structural features of drug molecules on their ability to act as substrates for DT-diaphorase. These results can then be correlated with cytotoxicity data in DT-diaphorase rich and deficient cell lines, with and without enzyme inducers or inhibitors. Construction of an appropriate structure–activity data base might then allow a more rational drug design approach to be implemented. Such studies are in progress with analogues of AZQ, EO9, MMC and CB 1954.

The effect of replacing the carbamoyl ester ethyl groups in AZQ with alternative alkyl moieties has been studied by two groups. Work in our laboratory has shown that the order of reduction rates for the purified Walker rat tumour enzyme was di-*n*-butyl > di-methyl > di-*iso*-butyl > di-*n*-propyl = di-ethyl > di-*iso*-propyl > di-*sec*-butyl [134]. The order of cytotoxic potency in Walker cells was very comparable: di-*n*-butyl > di-methyl > di-ethyl > di-*n*-propyl > di-*iso*-butyl > di-*sec*-butyl > di-*iso*-propyl. Although the exact order was slightly different, a similar overall relationship was found between reduction by purified rat liver enzyme and cytotoxicity in HT-29 human colon carcinoma cells [135].

For EO9 analogues, replacement of the aziridine group with methyl aziridine markedly decreased both Walker enzyme reduction rate and cytotoxicity [134]. Aziridine ring opening to the hydroxyethylamino derivative essentially eliminated both properties. Replacement of aziridine by methoxy reduced the rate of DT-diaphorase metabolism only slightly, but lowered cytotoxicity considerably. Thus, the aziridine appears to contribute to cell killing by regulating DT-diaphorase activation and also through an enzyme-independent effect (presumably DNA cross-linking). For both the indoloquinone and the AZQ analogues, it appears that the rate of reduction is not a simple function of redox potential, or "electron affinity", and further studies are required to elucidate the major contributing factor(s). Structural variation also affects the pH-dependent metabolism of mitomycins by rat liver DT-diaphorase. Thus, mitomycins A and B which contain a 7-methoxy group were reduced at both pH 7.8 and 5.8, whereas mitomycin C was an inhibitor at neutral pH [101].

Major effects of structural modification on reduction by the Walker rat and human HepG2 cell DT-diaphorase have also been noted with CB 1954 analogues. As with EO9, the aziridine ring-opened derivative was a very inefficient substrate. However, no clearly predictable structure–activity relationship is yet emerging.

The observation that both CB 1954 and EO9 are much poorer substrates for the human vis-a-vis the rodent form may give a useful guide to the design of human-specific drugs [100, 118, 134]. This difference, which explains why human cells that express equivalent DT-diaphorase activity are much less sensitive to CB 1954, is probably related to the charge at amino acid 124 within the proposed substrate binding region [118]. This information could prove useful to new drug design. In fact, derivatives of both CB 1954 and EO9 with much improved performance for the human enzyme have now been found by screening against the latter enzyme [118, 134].

Future perspectives

A steady accumulation of data testifies to the increased expression of DT-diaphorase in tumour tissues from experimental animal models and patients. The regulatory mechanisms controlling the level of expression of DT-diaphorase genes are becoming clearer. The much higher levels of NQO_1 mRNA seen in human hepatocarcinoma [57] correlate with similar results in rodent models, where hypomethylation is a major factor. However, much further work needs to be done to characterize the molecular basis for changes in DT-diaphorase which occur in human cancers.

The continuing reports of high levels of expression of DT-diaphorase in human tumours strengthen the case for increasing efforts to design improved anticancer drugs for activation by the enzyme. Results with AZQ, MMC, EO9 and CB 1954 support the view that high DT-diaphorase levels can confer a selective tumour cell killing by these agents, although other sensitivity and resistance genes are certainly involved in the overall pattern of response.

We are not yet in a position to design improved bioreductive drugs from first principles. The ongoing elucidation of structure–activity relationships for bioreduction and cytotoxicity are giving us a hint of some of the molecular features which are either advantageous or disadvantageous. We are now aware that bioreduction can lead to pH-dependent enzyme inactivation. Indeed, those interested in designing irreversible inhibitors of enzymes would see some of our currently available agents as excellent candidates for mechanism-based inhibitors, and it is perhaps surprising that this is not a bigger problem.

In the cancer clinic, use of DT-diaphorase-activated drugs is currently restricted to MMC and AZQ. The balance of opinion suggests that high enzyme activity is likely to be a predictive factor. However, the pharmacological significance for MMC is undermined by the low pH requirement. Current thinking is moving away from an acidic intracellular milieu in tumours, favouring instead the view that the low pH is restricted to the extracellular fluid. Recent magnetic resonance spectroscopy studies suggest that human brain tumours have a slightly alkaline pH. However, local intracellular acidic environments may exist. It is probably advantageous that activation of AZQ and the developmental indoloquinone EO9 is not pH dependent within the normal physiological range.

Very few data are available as regards the role of DT-diaphorase in the control of tumour response *in vivo*. The recent correlation between EO9 response and enzyme activity in mouse colon carcinomas [110] has suggested the potential value of further work in this area. Moreover, we recommend clinical studies correlating tumour response to EO9 with DT-diaphorase activity. Similar clinical studies with AZQ in brain tumours might be considered.

One of the factors which may complicate any simple relationship between DT-diaphorase activity and drug sensitivity is the participation of additional bioreductive enzymes. This requires greater attention, including *in vivo* mechanistic studies. The relative involvement of one-electron donating enzymes needs to be established, especially, for example, the degree of competition between DT-diaphorase and cytochrome P450 reductase.

Another controversial issue is the comparative role of DT-diaphorase under hypoxic versus normoxic conditions. The classical work from Sartorelli and co-workers [84, 92, 93] suggested that the enzyme bioactivates mitomycin C under hypoxia but is protective in air. Similar studies need to be performed with other bioreductive agents. As mentioned earlier, the above experiments were highly dependent on the use of dicoumarol as a specific DT-diaphorase inhibitor. However, because of its lack of specificity, additional model systems should be used in future studies. These include naturally occurring high and low DT-diaphorase expressing lines (for example, HT-29 and BE cells); cell lines treated with inducers of DT-diaphorase; cells with induced resistance which express altered enzyme levels; and low DT-diaphorase expressing lines which have been transfected with the relevant gene under the control of the appropriate promoter.

It is also essential to continue studies aimed to

elucidate the mechanism of resistance to bioreductive anticancer agents. Of particular interest would be the possibility of selecting for cells with down-regulated, mutated or deleted DT-diaphorase. This could be conducted with MMC, AZQ, EO9 and CB 1954. However, a previous study with MMC resistance revealed a down-regulation of P450 reductase with no change in DT-diaphorase [136]. Connections with multidrug resistance also require clarification.

Should clinical resistance to certain bioreductive drugs prove to involve reduced expression of DT-diaphorase, we can imagine an attractive strategy to exploit the new enzyme profile. This would involve treating the resistant tumour with an agent which is detoxified by DT-diaphorase. At the moment, the best potential candidate for this would be the benzotriazine-di-*N*-oxide SR 4233. The converse might also apply. Thus, an agent like SR 4233 might induce resistance via up-regulation of DT-diaphorase and one could then treat the resistant tumour with DT-diaphorase-activated drugs. Possibilities also exist for selective induction or inhibition of DT-diaphorase in tumour versus normal cells. Another neglected area is the involvement of DT-diaphorase in the sensitivity or resistance of normal tissues to bioreductive agents. Such analyses may decipher why MMC is myelosuppressive while the indoloquinone EO9 is not.

As in other areas of biochemical pharmacology and toxicology, much work remains to be conducted on the roles of DT-diaphorase in cancer chemotherapy. It seems likely that the next few years will see further substantial advances in our understanding of the mechanism of drug action, together with the results of clinical studies correlating enzyme activities with drug response and the design of improved agents for activation by the human forms.

Note added in proof. A recent study shows that the anthraquinone-based antitumour agents mitoxantrone, daunorubicin and ametantrone are substrates for rat liver DT-diaphorase; the reaction can generate superoxide anions, hydrogen peroxide and hydroxyl radicals, probably by slow auto-oxidation of the corresponding hydroquinones [137].

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REFERENCES

1. Ernster L and Navazio F, Soluble diaphorases in animal tissues. *Acta Chem Scand* **12**: 595, 1958.
2. Ernster L, Ljunggren M and Danielson L, Purification and some properties of a highly dicoumarol-sensitive liver diaphorase. *Biochem Biophys Res Commun* **2**: 88–92, 1960.
3. Ernster L, Danielson L and Ljunggren M, DT-diaphorase. I. Purification from the soluble fraction of rat-liver cytoplasm and properties. *Biochim Biophys Acta* **58**: 171–188, 1962.
4. Ernster L, DT-diaphorase: A historical review. *Chem Scripta* **27A**: 1–13, 1987.
5. Wallin R, Gebhardt O and Prydz H, NAD(P)H dehydrogenase and its role in the vitamin K (2-methyl - 3 - phytyl - 1,4 - naphthaquinone) - dependent carboxylation reaction. *Biochem J* **169**: 95–101, 1978.
6. Suttie JW, Vitamin K-dependent carboxylase. *Annu Rev Biochem* **54**: 459–477, 1985.
7. Wallin R, Ramnels SR and Martin LF, DT-diaphorase and vitamin K-dependent carboxylases in liver and lung microsomes and in macrophages and type II endothelial cells isolated from rat lung. *Chem Scripta* **27A**: 193–202, 1987.
8. Lind C, Hochstein P and Ernster L, DT-diaphorase: Properties, reaction mechanism, metabolic function. A progress report. In: *Oxidases and Related Redox Systems* (Eds. King TE, Mason HS and Morrison M), pp. 321–347. Pergamon Press, Oxford, 1982.
9. Danielson L, Ernster L and Ljynggren M, Selective extraction of DT-diaphorase from mitochondria and microsomes. *Acta Chem Scand* **14**: 1837–1838, 1960.
10. Conover TE and Ernster L, DT-diaphorase. IV. Coupling of extramitochondrial reduced pyridine nucleotide oxidation to mitochondrial respiratory chain. *Biochim Biophys Acta* **67**: 268–280, 1963.
11. Edlund C, Elhammer A and Dallner G, Distribution of newly synthesised DT-diaphorase in rat liver. *Biosci Rep* **2**: 861–865, 1982.
12. Martius C, Quinone reductases. *Enzymes* **7**: 517–532, 1963.
13. Spencer SR, Wilczak CA and Talalay P, Induction of glutathione transferases and NAD(P):quinone reductase by fumaric acid derivatives in rodent cells and tissues. *Cancer Res* **51**: 7871–7875, 1990.
14. Schlager JJ and Powis G, Cytosolic NAD(P)H:(quinone-acceptor) oxidoreductase in human normal and tumour tissue: Effects of cigarette smoking and alcohol. *Int J Cancer* **45**: 403–409, 1990.
15. Lind C, Cadenas E, Hochstein P and Ernster L, DT-diaphorase: Purification, properties and function. *Methods Enzymol* **186**: 287–301, 1990.
16. Prochaska HJ and Talalay P, Purification and characterization of two isofunctional forms of NAD(P)H:quinone reductase from mouse liver. *J Biol Chem* **261**: 1372–1378, 1986.
17. Höjeberg B, Blomberg K, Stenberg S and Lind C, Biospecific adsorption of hepatic DT-diaphorase on immobilized dicoumarol. I. Purification of cytosolic DT-diaphorase from control and 3-methyl-cholanthrene-treated rats. *Arch Biochem Biophys* **207**: 205–216, 1981.
18. Segura-Aguilar JE and Lind C, Isolation and characterisation of DT diaphorase enzymes from rat liver. *Chem Scripta* **27A**: 37–41, 1987.
19. Edwards YH, Potter J and Hopkinson DA, Human FAD-dependent NAD(P)H diaphorase. *Biochem J* **187**: 429–436, 1980.
20. Shaw PM, Reiss A, Adesnik M, Nebert DW, Schembri J and Jaiswal AK, The human dioxin-inducible NAD(P)H:quinone oxidoreductase cDNA-encoded protein expressed in COS-1 cells is identical to diaphorase 4. *Eur J Biochem* **195**: 171–176, 1991.
21. Benatti V, Guida L, DeFlora A and Hochstein P, The effect of encapsulated DT diaphorase on the sensitivity of human red blood cells to menadione. *Chem Scripta* **27A**: 169–171, 1987.
22. Jaiswal AK, McBride OW, Adesnik M and Nebert DW, Human dioxin-inducible cytosolic NAD(P)H:menadione oxidoreductase. cDNA sequence and localization of gene to chromosome 16. *J Biol Chem* **263**: 13572–13578, 1988.
23. Robertson JA, Chen H and Nebert DW, NAD(P)H:menadione oxidoreductase. Novel purification of enzyme, cDNA and complete amino acid sequence and gene regulation. *J Biol Chem* **261**: 15794–15799, 1986.
24. Jaiswal AK, Burnett P, Adesnik M and McBride OW, Nucleotide and deduced amino acid sequence of a human cDNA (NQO_2) corresponding to a second

member of the NAD(P)H:quinone oxidoreductase gene family. Extensive polymorphism at the NQO_2 gene locus on chromosome 6. *Biochemistry* **29**: 1899–1906, 1990.
25. Ernster L, DT-diaphorase. *Methods Enzymol* **10**: 309–317, 1967.
26. Forrest GL, Qian J, Ma J-X, Kaplan WD, Akman S, Doroshow J and Chen S, Rat liver NAD(P)H:quinone oxidoreductase: cDNA expression and site-directed mutagenesis. *Biochem Biophys Res Commun* **169**: 1087–1093, 1990.
27. Siest G, Batt M, Fournel-Gigleaux S, Galteau MM, Wellman-Bednawska M, Minn A and Costesee AA, Induction of plasma and tissue enzymes by drugs: Significance in toxicological studies. *Xenobiotica* **18** (Suppl 1): 21–34, 1988.
28. Okey AB, Roberts, EA, Harper PA and Denison MS, Induction of drug-metabolizing enzymes: Mechanisms and consequences. *Clin Biochem* **19**: 132–141, 1986.
29. Williams JB, Lu AYH, Cameron RG and Pickett CB, Rat liver NAD(P)H-quinone reductase. *J Biol Chem* **261**: 5524–5528, 1986.
30. De Long MJ, Prochaska HJ and Talalay P, Induction of NAD(P)H:quinone reductase in murine hepatoma cells by phenolic antioxidants, azo dyes and other chemoprotectors: A model system for the study of anticarcinogens. *Proc Natl Acad Sci USA* **83**: 787–791, 1986.
31. Talalay P and Prochaska HJ, Mechanisms of induction of NAD(P)H:quinone reductase. *Chem Scripta* **27A**: 61–66, 1987.
32. Prochaska HJ and Talalay P, Regulatory mechanisms of monofunctional and bifunctional anticarcinogenic enzyme inducers in murine liver. *Cancer Res* **48**: 4776–4782, 1988.
33. Poland A and Knutson JC, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* **22**: 517–554, 1982.
34. Whitlock JP Jr, Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu Rev Pharmacol Toxicol* **30**: 251–277, 1990.
35. Talalay P, De Long MJ and Prochaska HJ, Identification of a common signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proc Natl Acad Sci USA* **85**: 8261–8265, 1988.
36. Talalay P, Mechanisms of induction of enzymes that protect against chemical carcinogenesis. *Adv Enzyme Regul* **28**: 149–159, 1989.
37. Spencer SR, Xue L, Klenz EM and Talalay P, The potency of inducers of NAD(P)H:(quinone acceptor) oxidoreductase parallels their efficiency as substrates for glutathione transferases. Structural and electronic correlations. *Biochem J* **273**: 711–717, 1991.
38. Bayney RM and Pickett CB, Regulation and sequence analysis of rat liver DT diaphorase mRNA. *Chem Scripta* **27A**: 89–94, 1987.
39. Favreau LV and Pickett CB, Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic compounds and phenolic antioxidants. *J Biol Chem* **266**: 4556–4561, 1991.
40. Rushmore TH, Morton MR and Pickett CB, The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem* **266**: 11632–11639, 1991.
41. Storz G, Tartaglia LA, Farr SB and Ames BN, Bacterial defenses against oxidative stress. *Trends Genet* **6**: 363–368, 1990.
42. Miller EC and Miller JA, Mechanism of chemical carcinogenesis: Nature of proximate carcinogens and interactions with macromolecules. *Pharmacol Rev* **18**: 805–838, 1966.
43. Cerutti PA and Trump BF, Inflammation and oxidative stress in carcinogenesis. *Cancer Cells* **3**: 1–7, 1991.
44. Sheratt AJ, Banet DE and Prough RA, Glucocorticoid regulation of polycyclic aromatic hydrocarbon induction of cytochrome P450IA1, glutathione-*S*-transferases, and NAD(P)H:quinone oxidoreductase in cultured fetal rat hepatocytes. *Mol Pharmacol* **37**: 198–205, 1990.
45. Farber E, Resistance phenotype in the initiation and promotion of chemical hepatocarcinogenesis. *Chem Scripta* **27A**: 131–133, 1987.
46. Pickett CB, Structure and regulation of glutathione-*S*-transferase genes. *Essays Biochem* **23**: 116–143, 1987.
47. Talcott RE, Rosenblum M and Levin VA, A possible role of DT-diaphorase in the bioactivation of antitumour quinones. *Biochem Biophys Res Commun* **111**: 346–351, 1983.
48. Schor NA, Ogawa K, Lee G and Farber E, The use of DT-diaphorase for the detection of foci of early neoplastic transformation in rat liver. *Cancer Lett* **5**: 167–171, 1978.
49. Tsuda H, Marked increases in DT diaphorase activity in malignantly transformed Syrian hamster fibroblastic cells. *Cancer Lett* **56**: 189–195, 1991.
50. Beyer RE, Segura-Aguilar JE and Ernster L, The anticancer enzyme DT diaphorase is induced selectively in liver during ascites hepatoma growth. *Anticancer Res* **8**: 233–238, 1988.
51. Berger MS, Talcott RE, Rosenblum ML, Silva M, AliOsman F and Smith MT, Use of quinones in brain-tumor therapy: Preliminary results of preclinical laboratory investigations. *J Toxicol Environ Health* **16**: 713–719, 1985.
52. Knox RJ, Boland MP, Friedlos F, Coles B, Southan C and Roberts JJ, The nitroreductase enzyme in Walker cells that activates 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is a form of NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2). *Biochem Pharmacol* **37**: 4671–4677, 1988.
53. Beyer RE, Segura-Aguilar J, Lind C and Castro VM, DT diaphorase activity in various cells in culture with emphasis on induction in ascites hepatoma cells. *Chem Scripta* **27A**: 145–150, 1987.
54. Becker FF and Stout DL, A constitutive deficiency in the monooxygenase system of spontaneous mouse liver tumors. *Carcinogenesis* **5**: 785–788, 1984.
55. Koudstaal J, Makkink B and Overdiep SH, Enzyme histochemical pattern in human tumours–II. Oxidoreductases in carcinoma of the colon and the breast. *Eur J Cancer* **11**: 111–115, 1975.
56. Schor NA and Cornelisse CJ, Biochemical and quantitative histochemical study of reduced pyridine nucleotide dehydrogenation by human colon carcinomas. *Cancer Res* **43**: 4850–4855, 1983.
57. Cresteil T and Jaiswal AK, High levels of expression of the NAD(P)H:quinone oxidoreductase (NQO_1) gene in tumor cells compared to normal cells of the same origin. *Biochem Pharmacol* **42**: 1021–1027, 1991.
58. Segura-Aguilar J, Cortes-Vizcaino V, Llombart-Bosch A, Ernster L, Monsalve E and Romero FJ, The levels of quinone reductases, superoxide dismutase and glutathione-related enzymatic activities in diethylstilbestrol-induced carcinogenesis in the kidney of male Syrian golden hamsters. *Carcinogenesis* **11**: 1727–1732, 1990.
59. de Waziers I, Cugnenc PH, Berger A, Leroux J-P and Beaune PH, Drug-metabolizing enzyme

expression in human normal, peritumoral and tumoral colorectal tissue samples. *Carcinogenesis* **12**: 905–909, 1991.
60. Ehlen T and Dubeau L, Loss of heterozygosity on chromosomal segments 3p, 6q and 11p in human ovarian carcinomas. *Oncogene* **5**: 219–223, 1990.
61. Vogelstein B, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura Y and White R, Allelotypes of colorectal carcinomas. *Science* **244**: 207–211, 1989.
62. Sato T, Tanigami A, Yamakawa K, Akiyama F, Kasumi F, Sakamoto G and Nakamura Y, Allelotype of breast cancer: Cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res* **50**: 7184–7189, 1990.
63. Nowell PC, How many human cancer genes? *J Natl Cancer Inst* **83**: 1061–1064, 1991.
64. Smith MT, Eastmond DA and DiMonte D, The activation and detoxication of quinones by DT-diaphorase. *Chem Scripta* **27A**: 105–107, 1987.
65. Ross D, Siegel D, Gibson NW, Pacheco D, Thomas DJ, Reasor M and Weirda D, Activation and deactivation of quinones catalysed by DT-diaphorase. Evidence for bioreductive activation of diaziquone (AZQ) in human tumour cells and detoxification of benzene metabolites in bone marrow stroma. *Free Radic Res Commun* **8**: 373–381, 1990.
66. Workman P and Walton MI, Enzyme-directed bioreductive drug development. In: *Selective Activation of Drugs by Redox Processes* (Eds. Adams GE, Breccia A, Wardman P and Fielden EM), pp. 173–191. Plenum Press, New York, 1990.
67. Khan AH and Driscoll JS, Potential central nervous system antitumor agents. Aziridinylbenzoquinones. 1. *J Med Chem* **19**: 313–317, 1976.
68. Chou F, Khan AH and Driscoll JS, Potential central nervous system antitumor agents. Aziridinylbenzoquinones. 2. *J Med Chem* **19**: 1302–1308, 1976.
69. Driscoll JS, Hazard GF Jr, Wood HB Jr and Goldin A, Structure-antitumor activity relationships among quinone derivatives. *Cancer Chemother Rep* (Part 2) **4**: 1–362, 1974.
70. Schold SC Jr, Friedman HS, Bjornsson TD and Falletta JM, Treatment of patients with recurrent primary brain tumors with AZQ. *Neurology* **34**: 615–619, 1984.
71. Lee EJ, Van Echo DA, Egorin MJ, Nayar MSB, Schulman P and Schiffer CA, Diaziquone given as a continuous infusion is an active agent for relapsed adult acute nonlymphocytic leukemia. *Blood* **67**: 182–187, 1986.
72. Gutierrez PL, Mechanisms of bioreductive activation. The example of diaziquone (AZQ). *Free Radic Biol Med* **6**: 405–455, 1989.
73. Mossoba MM, Alizadeh M and Gutierrez P, Mechanism of the reductive activation of diaziquone. *J Pharm Sci* **74**: 1249–1254, 1985.
74. Bachur N, Gordon S and Gee M, A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res* **38**: 1745–1752, 1978.
75. Siegel D, Gibson NW, Preusch PC and Ross D, Metabolism of diaziquone by NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase): Role in diaziquone-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* **50**: 7293–7330, 1990.
76. Fisher GR and Gutierrez PL, The reductive metabolism of diaziquone (AZQ) in the S9 fraction of MCF-7 cells: Free radical formation and NAD(P)H:quinone-acceptor oxidoreductase (DT-diaphorase) activity. *Free Radic Med Biol* **10**: 359–369, 1991.
77. Hartley JA, Berardini M, Ponti M, Gibson NW, Hoey BM and Butler J, DNA crosslinking and sequence selectivity of aziridinylbenzoquinones. *Br J Cancer* **63** (Suppl XIII): 22, 1991.
78. Hartley JA, Berardini M, Butler J, Ross D and Gibson NW, Alteration in DNA crosslinking and sequence selectivity of aziridinylbenzoquinones after enzymatic reduction by DT-diaphorase. *Proc Am Assoc Cancer Res* **32**: 319, 1991.
79. Crooke ST and Bradner WT, Mitomycin C: A review. *Cancer Treat Rev* **3**: 121–139, 1976.
80. Iyer VN and Szybalski W, A molecular mechanism of mitomycin action. Link of complementary DNA strands. *Proc Natl Acad Sci USA* **50**: 355–362, 1963.
81. Powis G, Metabolism and reactions of quinoid anticancer agents. *Pharmacol Ther* **35**: 57–162, 1987.
82. Tomasz M and Lipman R, Alkylation reactions of mitomycin C at acid pH. *J Am Chem Soc* **101**: 6063–6067, 1979.
83. Tomasz M, Lipman R, Chowdary D, Pawlak J, Verdine GL and Nakamishi K, Isolation and structure of a covalent cross-link between mitomycin C and DNA. *Science* **235**: 1204–1208, 1987.
84. Keyes SR, Facasso PM, Heimbrook DC, Rockwell S, Sligar S and Sartorelli AC, Role of NADPH:cytochrome *c* reductase and DT-diaphorase in the biotransformation of mitomycin C. *Cancer Res* **44**: 5638–5643, 1984.
85. Dustre L, Rajagopapan S, Eliot HM, Covey JM and Sinha BK, DNA inter-strand cross-link and free radical formation in a human multi-drug resistant cell line from mitomycin C and its analogues. *Cancer Res* **50**: 648–652, 1990.
86. Kennedy KA, Rockwell S and Sartorelli AC, Preferential activation of mitomycin C to cytotoxic metabolites by hypoxic tumor cells. *Cancer Res* **40**: 2356–2360, 1980.
87. Rauth AM, Mohindra JK and Tannock IF, Activity of mitomycin C for aerobic and hypoxic cells *in vitro* and *in vivo*. *Cancer Res* **45**: 3642–3645, 1983.
88. Rockwell S, Keyes SR and Sartorelli AC, Preclinical studies of porfiromycin as an adjunct to radiotherapy. *Radiat Res* **116**: 100–113, 1988.
89. Pan S-S, Andrews PA, Glover CJ and Bachur NR, Reductive activation of mitomycin C and mitomycin C metabolites catalyzed by NADPH-cytochrome P-450 reductase and xanthine oxidase. *J Biol Chem* **259**: 959–966, 1984.
90. Peterson DW and Fisher J, Autocatalytic quinone methide formation from mitomycin C. *Biochemistry* **25**: 4077–4084, 1986.
91. Workman P, Walton MI, Powis G and Schlager JJ, DT-diaphorase: Questionable role in mitomycin C resistance, but a target for novel bioreductive drugs? *Br J Cancer* **60**: 800–802, 1989.
92. Keyes SR, Rockwell S and Sartorelli AC, Enhancement of mitomycin cytotoxicity to hypoxic tumor cells by dicoumarol *in vivo* and *in vitro*. *Cancer Res* **45**: 213–216, 1985.
93. Keyes SR, Rockwell S and Sartorelli AC, Porfiromycin as a bioreductive alkylating agent with selective toxicity to hypoxic EMT6 tumor cells *in vitro* and *in vivo*. *Cancer Res* **45**: 3642–3645, 1985.
94. Keyes SR, Rockwell S and Sartorelli AC, Modification of the metabolism and cytotoxicity of bioreductive alkylating agents by dicoumarol in aerobic and hypoxic murine tumor cells. *Cancer Res* **49**: 3310–3313, 1989.
95. Segura-Aguilar JE, Barreiro V and Lind C, Dicoumarol-sensitive glucuronidation of benzo(*a*)pyrene metabolites in rat liver microsomes. *Arch Biochem Biophys* **251**: 266–275, 1986.
96. Hajos AKD and Winston GW, Dinitropyrene nitroreductase activity of purified NAD(P)H-quinone oxidoreductase: Role in rat liver cytosol and induction

by Aroclor-1254 treatment. *Carcinogenesis* **12**: 697–702, 1991.
97. Iyer VN and Szybalski W, Mitomycins and porfiromycin: Chemical mechanism of activation and crosslinking of DNA. *Science* **145**: 55–58, 1964.
98. Schlager JJ and Powis G, Mitomycin C is not metabolized by but is an inhibitor of human kidney NAD(P)H:(quinone acceptor)oxidoreductase. *Cancer Chemother Pharmacol* **22**: 126–130, 1988.
99. Siegel D, Gibson NW, Preusch PC and Ross D, Metabolism of mitomycin C by DT-diaphorase: Role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* **50**: 7483–7489, 1990.
100. Walton MI, Suggett N and Workman P, The role of human and rodent DT-diaphorase in the reductive metabolism of hypoxic cell cytotoxins. *Int J Radiat Oncol Biol Phys* **22** 643–648, 1992.
101. Siegel D, Gibson NW, Preusch PC and Ross D, pH-dependent metabolism of mitomycins by DT-diaphorase. *Proc Am Assoc Cancer Res* **32**: 317, 1991.
102. Ross D, Siegel D and Gibson NW, Metabolism and bioreductive activation of mitomycin C by DT-diaphorase. *Biochemistry*, in press.
103. Dulhanty AM and Whitmore GF, Chinese hamster ovary cell lines resistant to mitomycin C under aerobic but not hypoxic conditions are deficient in DT-diaphorase. *Cancer Res* **51**: 1860–1865, 1991.
104. Begleiter A, Robotham E, Lacey G and Leith MK, Increased sensitivity of quinone resistant cells to mitomycin C. *Cancer Lett* **45**: 173–176, 1989.
105. Silva JM and O'Brien PJ, Molecular mechanisms of trenimon-induced cytotoxicity towards resistant L5178Y/HBM10 cells. *Int J Radiat Oncol Biol Phys* **22**: 639–642, 1992.
106. Marshall RS, Paterson MC and Rauth AM, Deficient activation by a human cell strain leads to mitomycin resistance under aerobic but not hypoxic conditions. *Br J Cancer* **59**: 341–346, 1989.
107. Marshall RS, Paterson MC and Rauth AM, Studies on the mechanism of resistance to mitomycin C and porfiromycin in a human cell strain derived from a cancer-prone individual. *Biochem Pharmacol* **41**: 1351–1360, 1991.
108. Marshall RS, Paterson MC and Rauth AM, DT-diaphorase activity and mitomycin C sensitivity in non-transformed cell strains derived from members of a cancer-prone family. *Carcinogenesis* **12**: 1175–1180, 1991.
109. Oostveen EA and Speckamp WN, Mitomycin analogues I. Indoloquinones as (potential) bis-alkylating agents. *Tetrahedron* **43**: 255–262, 1987.
110. Walton MI, Smith PJ and Workman P, The role of NAD(P)H:quinone reductase (EC 1.6.99.2, DT-diaphorase) in the reductive bioactivation of the novel indoloquinone antitumor agent EO9. *Cancer Commun* **3**: 199–206, 1991.
111. Workman P, Walton MI, Bibby MC and Double DA, *In vivo* response of mouse adenocarcinoma of the colon (MAC) tumours to indoloquinone EO9: Correlation with bioreductive enzyme content. *Br J Cancer* **62**: 515–516, 1991.
112. Khan AH and Ross WCJ, Tumour-growth inhibitory nitrophenylaziridines and related compounds: Structure-activity relationships. *Chem Biol Interact* **1**: 27–47, 1969/70.
113. Khan AH and Ross WCJ, Tumour-growth inhibitory nitrophenylaziridines and related compounds: structure–activity relationships. II. *Chem Biol Interact* **4**: 11–22, 1971/72.
114. Cobb LM, Connors TA, Elson LA, Kahn AH, Mitchley BCV, Ross WCJ and Whisson ME, 2,4-Dinitro-5-ethylene iminobenzamide (CB 1954): A potent and selective inhibitor of the growth of the Walker carcinoma 256. *Biochem Pharmacol* **18**: 1519–1527, 1969.
115. Connors TA and Melzack DH, Studies on the mechanism of action of 5-aziridinyl-2,4-dinitrobenzamide (CB 1954), a selective inhibitor of the Walker tumour. *Int J Cancer* **7**: 86–92, 1971.
116. Roberts JJ, Friedlos F and Knox RJ, CB 1954 (2,4-dinitro-5-aziridinyl benzamide) becomes a DNA interstrand crosslinking agent in Walker tumour cells. *Biochem Biophys Res Commun* **140**: 1073–1078, 1986.
117. Knox RJ, Friedlos F, Jarman M and Roberts JJ, A new cytotoxic, DNA interstrand crosslinking agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) by a nitroreductase enzyme in Walker carcinoma cells. *Biochem Pharmacol* **37**: 4661–4669, 1988.
118. Boland MP, Knox RJ and Roberts JJ, The differences in kinetics of rat and human DT diaphorase result in a differential sensitivity of derived cell lines to CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). *Biochem Pharmacol* **41**: 867–875, 1991.
119. Tada M and Tada M, Seryl-tRNA synthetase and activation of the carcinogen 4-nitroquinoline 1-oxide. *Nature* **255**: 510–512, 1975.
120. De Braum JR, Miller EC and Miller JA, *N*-Hydroxy-2-acetylaminofluorene sulfotransferase: Its probable role in carcinogenesis and in protein-(methion-*S*-yl) binding in rat liver. *Cancer Res* **30**: 577–595, 1970.
121. Knox RJ, Friedlos F, Marchbank T and Roberts JJ, Bioactivation of CB 1954: Reaction of the active 4-hydroxylamino derivative with thioesters to form the ultimate DNA-DNA interstrand crosslinking species. *Biochem Pharmacol* **42**: 1691–1697, 1991.
122. Sunters A, Baer J and Bagshawe KD, Cytotoxicity and activation of CB1954 in a human tumour cell line. *Biochem Pharmacol* **41**: 1293–1298, 1991.
123. Connors TA, Antibody-directed enzyme prodrug therapy. *Cancer Cells* **2**: 56–57, 1990.
124. Zeman EM, Brown JM, Lemmon MJ, Hirst VK and Lee WW, SR 4233: A new bioreductive agent with high selective toxicity for mammalian cells. *Int J Radiat Oncol Biol Phys* **12**: 1239–1242, 1986.
125. Baker MA, Zeman EM, Hirst VK and Brown MJ, Metabolism of SR 4233 by Chinese hamster ovary cells: Basis of selective hypoxic cytotoxicity. *Cancer Res* **48**: 5947–5952, 1988.
126. Walton MI and Workman P, Enzymology of the reductive bioactivation of SR 4233: A novel benzotriazine di-*N*-oxide hypoxic cell cytotoxin. *Biochem Pharmacol* **39**: 1735–1742, 1990.
127. Costa AK, Baker MA, Brown JM and Trudell JR, *In vitro* hepatotoxicity of SR 4233 (3-amino-1,2,4-benzotriazine-1,4-dioxide), a hypoxic cytotoxin and potential antitumor agent. *Cancer Res* **49**: 925–929, 1989.
128. Laderoute K, Wardman P and Rauth AM, Molecular mechanisms for the hypoxia-dependent activation of 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233). *Biochem Pharmacol* **37**: 1487–1495, 1988.
129. Laderoute K and Rauth AM, Identification of two major reduction products of the hypoxic cell cytotoxin 3-amino-1,2,4-benzotriazine-1,4-oxide. *Biochem Pharmacol* **35**: 3417–3420, 1986.
130. Walton MI, Wolf CR and Workman P, Molecular enzymology of the reductive bioactivation of hypoxic cell cytotoxins. *Int J Radiat Oncol Biol Phys* **16**: 983–986, 1989.
131. Riley RJ and Workman P, Enzymology of the reduction of the potent benzotriazine di-*N*-oxide hypoxic cell cytotoxin SR 4233 (WIN 59075) by NAD(P)H:(quinone acceptor) oxidoreductase (EC

1,6.99.2) purified from Walker 256 rat tumour cells. *Biochem Pharmacol* **43**: 167–174, 1992.
132. Prochaska HJ and Talalay P, Crystallisation and characterisation of NAD(P)H:quinone reductase from murine liver. *Chem Scripts* **27A**: 43–47, 1987.
133. Amzel LM, Bryant SH, Prochaska HJ and Talalay P, Preliminary crystallographic X-ray data for an NAD(P)H:quinone reductase from mouse liver. *J Biol Chem* **261**: 1379, 1986.
134. Bailey SM, Suggett N and Workman P, Structure–activity relationships for DT-diaphorase reduction of hypoxic cell directed agents: indoloquinones and diaziridinyl benzoquinones. *Int J Radiat Oncol Biol Phys* **22**: 649–654, 1992.
135. Pacheco D, Siegel D, Butler J, Gibson NW and Ross D, The relationship between DT-diaphorase mediated metabolism of AZQ analogs and cytotoxicity in HT-29 human colon carcinoma cells. *Proc Am Assoc Cancer Res* **32**: 321, 1991.
136. Hoban P, Walton MI, Robson C, Godden J, Stratford I, Workman P, Harris A and Hickson I, Decreased NADPH: cytochrome P450 reductase activity and impaired drug activation in a mammalian cell line resistant to mitomycin C under aerobic but not hypoxic conditions. *Cancer Res* **50**: 4692–4697, 1990.
137. Fisher GR, Gutierrez PL, Oldcorne MA and Patterson LH, NAD(P)H (quinone acceptor) oxidoreductase (DT-diaphorase)-mediated two-electron reduction of anthraquinone-based antitumour agents and generation of hydroxyl radicals. *Biochem Pharmacol* **43**: 575–585, 1992.