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One- and Two-Electron-Mediated Reduction of Quinones: Enzymology and Toxicological Implications

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7.1. Enzymology of Quinone Reduction

Quinones can undergo one- or two-electron reductions to generate semiquinone and hydroquinone derivatives, respectively (Gutierrez 2000; Butler 1998; Ross et al. 1996; O'Brien 1991; Powis 1987). The importance of these reactions from a toxicological perspective depends on the chemical properties of the semiquinone and hydroquinone, the oxygen tension, and the ability of the organ system or cell to remove or metabolize the semiquinone or hydroquinone generated. This review will focus on the enzymology and toxicological implications associated with both one- and two-electron-mediated reduction of quinones.

In the following sections, enzymes associated with either one- or two-electron reductions of quinones (Figure 7.1) will be briefly summarized.

7.1.1. Cytochrome P450 Reductase

NADPH-cytochrome P450 reductase (CPR; NADPH-ferrihemoprotein reductase, EC 1.6.2.4) is the electron-donor flavoprotein for the multi-component monooxygenase system, in which reducing equivalents from NADPH ultimately are transferred to molecular oxygen, found in the endoplasmic reticulum (microsomes) of most eukaryotic cells (Shen, Sem and Kasper 1999). Fundamentally, the monooxygenase system consists of CPR and one of many cytochrome P450 isozymes (Williams and Kamin 1962; Phillips and Langdon 1962), involved in the metabolism of many

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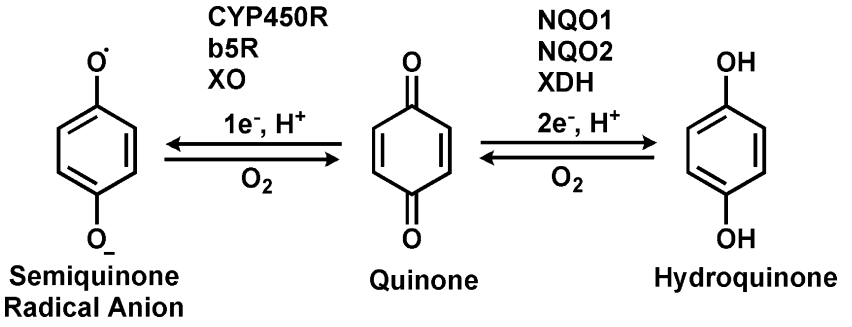

Figure 7.1 The generation of semiquinone and hydroquinone intermediates from one- or two-electron reduction of quinones.

drugs, dietary substances, the synthesis of steroid hormones, and other extracellular lipid signaling molecules. Consistent with its many functions in the cell, CPR is a widely expressed protein, present at some level in all tissues examined. It is most abundant in the liver, where the cytochrome P450 system is highly expressed. Mechanistically, CPR accepts a pair of electrons from NADPH as a hydride ion, with flavinadenine dinucleotide (FAD) and flavin mononucleotide (FMN) being the point of entry and exit, respectively, and transfers these electrons to cytochrome P450. Cytochromes P450, in turn, utilizes these reducing equivalents for the hydroxylation of a variety of substrates. The redox potentials of each flavin half-reaction in the native enzyme have been determined by potentiometric titrations (Iyanagi, Makino and Mason 1974; Vermilion and Coon 1978). The enzyme cycles between $1e^-$ and $3e^-$ reduced levels (or $2e^-$ and $4e^-$), with the one-electron reduced semiquinone of the FMN being the highest oxidation state during catalytic turnover (Masters et al. 1965; Backes and Reker-Backes 1988).

The ability of CPR to reduce quinones to the semiquinone radical is related to their one-electron reduction potential. The analysis of a large number of quinones demonstrated a correlation between their rate of reduction by CPR and their one-electron reduction potential (Butler and Hoey 1993). This correlation, however, is true for quinones with one-electron reduction potentials between –400 mV and –165 mV, while quinones with reduction potentials more positive than –165 mV underwent predominantly two-electron reduction by NADPH directly without the participation of the enzyme (Butler and Hoey 1993). In another study, the rate of reduction of a series of quinones increased as the one-electron redox couple was increased up to a limiting value of >–100 mV (Nemeikaite-Ceniene et al. 2003).

7.1.2. Cytochrome b_5 Reductase

NADH-cytochrome b_5 reductase (b5R, NADH-methemoglobin reductase, EC 1.6.2.2) and cytochrome b_5 are integral membrane proteins with cytosolic active domains and have been implicated as components of a number of systems, where essentially, b5R catalyzes the two-electron transfer from NADH to cytochrome b_5 through the enzyme-bound FAD

cofactor (Strittmatter 1965; Iyanagi 1977). In human erythrocytes, these proteins are components of the major methemoglobin reducing system (Hultquist and Passon 1971; Schwartz and Jaffe 1978). The confusion over the nomenclature of b5R as a diaphorase related to NQO1 has recently been clarified (Vasiliou, Ross and Nebert 2006).

While there has not been an extensive study published on the relationship between the one-electron reduction potential and the rate of reduction by b5R there is evidence using a limited number of quinones suggesting that the higher the one-electron reduction potential the slower the rate of reduction by b5R (Powis and Appel 1980). These studies also demonstrated that there was no correlation between the octanol/H_2O partition coefficient of a series of quinones and their rate of reduction by b5R (Powis and Appel 1980).

7.1.3. Xanthine Oxidoreductase (Xanthine Dehydrogenase, Xanthine Oxidase)

Xanthine oxidoreductase (XOR), a member of the molybdenum hydroxylase flavoprotein family, exists in two interconvertable forms, xanthine dehydrogenase (XDH, EC 1.1.1.204) and xanthine oxidase (XO, EC 1.1.3.22) (Amaya et al. 1990; Della Corte et al. 1969). The functional distinction between XDH and XO is the preference of each for the reducing substrate. Xanthine oxidase uses molecular oxygen efficiently, as an electron acceptor, reducing molecular oxygen by a single electron, but has negligible reactivity with NAD^+. Xanthine dehydrogenase however prefers NAD^+ to molecular oxygen, reducing NAD^+ by a direct two-electron reduction, but is able to use the latter in the course of turnover (Hille and Nishino 1995). Xanthine dehydrogenase isolated from mammalian sources can be either reversibly converted to XO by sulfhydryl modification or irreversibly converted to XO by limited proteolysis (Harris, Sanders and Massey 1999; Amaya et al. 1990; Battelli, Lorenzoni and Stripe 1973; Nishino 1994). Physiologically, XOR is the key rate-limiting enzyme in the catabolism of purines, both XDH and XO catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid, which introduces reducing equivalents into the enzyme. The reoxidation of the molybdenum center occurs via electron transfer to the two iron–sulfur redox-active centers of the enzyme and ultimately to the FAD-site where electrons are removed from the enzyme by reaction with NAD^+ in the case of XDH or with molecular oxygen for XO (Hille 1996; Hille and Massey 1981).

In mammalian tissues XOR exists mainly in the XDH form; however, when O_2 levels decrease, due to an insult, such as ischemia, XDH may be converted to XO which is considered to be important in the pathogenesis of ischemia/reperfusion injury (McCord 1985). During reperfusion, when O_2 levels return to normal, reduction of oxygen by XO yields the superoxide anion, O_2^-, and hydrogen peroxide, H_2O_2, (Terada et al. 1991; Brown et al. 1988) and other reactive-oxygen species (ROS). These reactive-oxygen metabolites have been implicated in diseases characterized by ischemia/reperfusion injury (Linas, Whittenburg and Repine 1990; Sussman and Bulkley 1990; Petrone et al. 1980).

Xanthine oxidase is a one-electron and XDH is a two-electron transfer enzyme and therefore either the semiquinone and hydroquinone forms of the quinone drug can be formed (Figure 7.1). Anthracyclines such as doxorubicin, daunomycin, and marcellomycin are reduced by XOR to the corresponding semiquinone under aerobic conditions (Pan and Bachur 1980). Mitomycin C is reduced by XOR under aerobic conditions to the semiquinone radical with consequent formation of ROS (Pan et al. 1984). Conversely, anaerobic conditions lead to the formation of 2,7-diaminomitosene, a DNA alkylating metabolite (Gustafson and Pritsos 1992). The activation of a number of quinone drugs, such as mitomycin C, by one-electron reduction by XO or two-electron reduction by XDH, at the FAD site, has also been reported (Maliepaard et al. 1995; Pritsos and Gustafson 1994; Komiyama, Kikuchi and Sugiura 1986; Pan et al. 1984).

The relationship between the quinone one-electron reduction potential and the rate of reduction by XO under a nitrogen atmosphere and in air has been studied using a series of 2,5- *bis*(1-aziridinyl)-1,4-benzoquinone analogs. Under nitrogen, the rate of reduction of these quinones by XO correlated with the one-electron reduction potential as well as steric factors (Lusthof et al. 1990). However, when similar studies were performed in air, substantially higher quinone concentrations (>25 μM) were required presumably to outcompete O_2 for electrons donated by XO (Lusthof et al. 1990).

7.1.4. NAD(P)H:quinone Oxidoreductase 1

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, EC 1.6.99.2) is a homodimeric flavoprotein that catalyzes two-electron reduction of a wide range of substrates (Lind et al. 1990). In addition, NQO1 has also been shown to catalyze the four-electron reduction of methyl red (Wu et al. 1997; Chen, Hwang and Deng 1993). The enzyme is characterized by its ability to utilize either NADH or NADPH as reducing cofactors and inhibition by the anticoagulant dicumarol (Hollander and Ernster 1975). Recently, a number of indolequinone-containing mechanism-based inhibitors such as ES936 and MAC220 have been developed and these compounds have demonstrated potent and specific inactivation of NQO1 without affecting the activities of other reductases including NQO2 (Dehn et al. 2003; Siegel et al. unpublished data). The enzymatic mechanism of quinone reduction proceeds through a "ping-pong" type mechanism, whereby NAD(P)H binds to the enzyme, reduces the protein-bound flavin, and leaves the protein prior to substrate binding and subsequent reduction (Li et al. 1995). NQO1 displays a wide, species-specific substrate spectrum, including quinones and their glutathione metabolites, quinoneimines, and several azo- and nitroaromatic compounds. The four-electron reduction of nitroaromatic compounds to the hydroxylamine derivatives has been proposed to occur through the cooperative behavior of the two subunits (Cenas et al. 2001).

Expression of NQO1 protein has been detected primarily in epithelial cells in tissues such as lung, breast, and colon as well as in vascular endothelium and adipocytes (Siegel and Ross 2000). Humans, unlike rodents, dogs, and monkeys, do not express high levels of NQO1 in the normal liver

(Strassburg et al. 2002; Siegel and Ross 2000), but humans do express high levels of NQO1 in hepatic tumors (Cresteil and Jaiswal 1991). A similar finding has been seen in the pancreas which has very low NQO1 expression in normal pancreatic tissue but NQO1 expression increases significantly as the tissue becomes neoplastic (Lyn-Cook et al. 2006; Dehn et al. unpublished data). In addition to pancreatic cancer, there are high levels of NQO1 in most epithelial-derived solid tumors (Siegel and Ross 2000; Schlager and Powis 1990) and this has made NQO1 an attractive target for the bioactivation of many quinone-based antitumor drugs (see below). NQO1 is highly inducible and NQO1 protein levels in tissues can be influenced by a number of environmental and dietary factors (Munday, Smith and Munday 1999a; Sreerama, Hedge and Sladek 1995; De Long, Prochaska and Talalay 1986). A major factor governing the levels of NQO1 protein expression in tissues is the NQO1*2 polymorphism. The NQO1*2 polymorphism has been characterized as a C-to-T base pair substitution at position 609 of the human cDNA that results in a proline-to-serine amino acid substitution at position 187 in the mutant NQO1 protein (Traver et al. 1997) Genotype–phenotype studies have shown that individuals homozygous for the NQO1*2 polymorphism are deficient in NQO1 due to enhanced ubiquitination and proteasomal degradation of the mutant protein (Siegel et al. 2001). In humans the absence of NQO1 has been linked to increased rates of cancer, most notably leukemias (Ross and Siegel 2004). This is supported by studies using NQO1 knockout mice that demonstrated increased bone marrow myeloid hyperplasia in NQO1-deficient mice compared to wild-type mice (Iskander and Jaiswal 2005). NQO1 knockout mice were also more susceptible to menadione-induced toxicities confirming a role for NQO1 in quinone detoxification (Radjendirane et al. 1998).

The rate at which a substrate is reduced by NQO1 cannot be predicted by the one-electron reduction potential. Studies using a series of substituted aziridinylbenzoquinones found no correlation between the one-electron reduction potential and the ability to undergo reduction by NQO1 (Nemeikaite-Ceniene et al. 2003; Gibson et al. 1992), while another study found no correlation between the half-wave reduction potentials of quinone epoxides and hydroxy-, methyl-, methoxy-, and glutathionyl-substituted naphthoquinones and their rate of reduction by NQO1 (Buffinton et al. 1989; Brunmark et al. 1988). In addition, there was no correlation between the two-electron reduction potential and rate of reduction of a series of aziridinylbenzoquinone by NQO1 (Hargreaves et al. 1999).

7.1.5. NRH:quinone Oxidoreductase 2

NRH:quinone oxidoreductase 2 (NQO2, EC 1.10.99.2) is a cytosolic homodimeric flavoprotein that demonstrates 49% amino acid homology with NQO1 (Jaiswal et al. 1990). An update of the NQO gene family has recently been published (Vasiliou, Ross and Nebert 2006). The major difference between the two enzymes is a 43-amino-acid deletion in the C terminus of NQO2 (Jaiswal et al. 1990). NQO2 carries out the direct two- and four-electron reduction of substrates similar to NQO1; however,

NQO2 is more efficient at catalyzing the four-electron reduction of methyl red compared to NQO1 (Wu et al. 1997). A major difference between NQO1 and NQO2 is their ability to utilize reduced pyridine nucleotide cofactors. NQO2 cannot utilize NADPH as a reducing cofactor and NADH is a very poor substrate for NQO2 (Wu et al. 1997). NQO2, however, can efficiently utilize reduced ribosyl- and *N*-alkyldihydronicotinamides as reducing cofactors, including dihydronictinamide riboside (NRH, Knox et al. 2000). NRH can be synthesized from NADH by enzymatic cleavage using phosphodiesterases and phosphatases (Friedlos et al. 1992). Inhibitors of NQO1 such as dicumarol, cibacron blue, ES936, and MAC220 do not inhibit NQO2 (Wu et al. 1997; Siegel and Ross unpublished data); however, NQO2 activity can be inhibited by polyphenolic compounds such as quercetin and resveratrol (Buryanovskyy et al. 2004; Wang et al. 2004; Wu et al. 1997). NQO2 has also been shown to interact with antimalarial quinolines such as primaquine, quinacrine, and chloroquine. Interestingly, it has been shown that primaquine binds to the oxidized form of NQO2 while quinacrine and chloroquine bind to the reduced form of NQO2 (Kwiek, Haystead and Rudolph 2004). Another notable feature of NQO2 is that it can bind melatonin, and NQO2 has been characterized as the melatonin MT_3 binding site (Nosjean et al. 2000). NQO2 is expressed in many human tissues most notably in human liver (Strassburg et al. 2002; Jaiswal 1994), testis (Long and Jaiswal 2000) and red blood cells (Graves et al. 2002). NQO2 activity or protein expression has been detected in many human tumor cells including K562 human leukemia cells and human melanoma cell lines (Hsieh et al. 2005; Buryanovskyy et al. 2004). As observed previously in studies with NQO1 knockout mice, studies using NQO2 knockout mice also demonstrated increased bone marrow myeloid hyperplasia in NQO2-deficient mice compared to wild-type mice (Iskander and Jaiswal 2005; Long et al. 2002). Unlike NQO1 knockout mice, NQO2-deficient animals demonstrated significantly less menadione-induced hepatic toxicity suggesting a positive role for NQO2 in menadione-induced toxicities (Long et al. 2002).

7.1.6. Mitochondrial Reductases

Mitochondria contain a number of enzymes capable of quinone metabolism. NADH:ubiquinone oxidoreductase (complex I) is believed to play an important role in quinone toxicity. This enzyme is a large multisubunit complex consisting of 42 different polypeptide chains including a FMN-containing subunit and six iron–sulfur centers. Complex I catalyzes the transfer of a hydride ion from NADH to FMN through a series of iron–sulfur centers and finally to ubiquinone to generate ubiquinol (Lenaz et al. 2006). NADH: ubiquinone oxidoreductase is responsible for the majority of superoxide generated in mitochondria (Grivennikova and Vinogradov 2006) and NADH :ubiquinone oxidoreductase may play a central role in neurodegenerative diseases such as Parkinson's (Keeney et al. 2006). Another source of quinone reduction in mitochondria is complex II (succinate dehydrogenase). This complex contains five polypeptide chains with one bound FAD and two iron–sulfur centers. Complex II functions by passing electrons from succinate to FAD and then through the iron–sulfur centers to ubiquinone to generate ubiquinol

(Cecchini 2003). Succinate dehydrogenase also catalyzes the oxidation of succinate to fumarate in the Kreb's cycle (Cecchini 2003).

7.1.7. Carbonyl Reductases

Carbonyl reductases (EC 1.1.1.1.81, CBR1, CBR2, CBR3) are monomeric cytosolic enzymes, able to catalyze the NADPH-dependent reduction of a variety of xenobiotic ketones and quinones (Wermuth et al. 1988; Wermuth et al. 1982; Ris and von Wartburg 1981; Wermuth 1981). The human enzyme is a monomer of 277 amino acid residues and was named carbonyl reductase (gene name CBR1) owing to its properties to reduce efficiently various endogenous and xenobiotic carbonyl compounds. Human CBR1 is expressed in a wide variety of tissues, with high levels found in liver, placenta, and the central nervous system (CNS), consistent with a possible protective role against toxic carbonyls (Wirth and Wermuth 1992). The xenobiotic substrates known to be metabolized by CBR1 include *ortho*-quinones derived from polycyclic aromatic hydrocarbons or *para*-quinones, such as menadione (Wermuth et al. 1986), as well as a broad spectrum of xenobiotic carbonyls, such as anthracyclines, metyrapone, or the carcinogen 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (Wermuth 1981; Atalla, Breyer-Pfaff and Maser 2000). The enzyme fulfills an important role in the phase I metabolism of xenobiotics; human CBR1A is the major hepatic reductase of quinones, suggesting a major role in detoxification of these compounds (Wermuth et al. 1986). This is in contrast to the metabolic preference in rat liver, where NQO1 is the major quinone reductase. However, in the absence of superoxide dismutase (SOD), quinone reduction by CBR1 leads to redox cycling, with generation of superoxide anion and semiquinone radicals mediated through one-electron transfer from the reduced hydroquinones to molecular oxygen (Jarabak and Harvey 1993). Thus, CBR1 is an important determinant in the metabolism of quinones; however, a possible protective role against quinone toxicity exerted by CBR1 depends on expression and activity of SOD and further metabolism, reactivity, and excretion of the hydroquinone formed.

7.1.8. Other Enzymes

A wide variety of flavin-containing proteins have been shown to participate in quinone metabolism. Examples include glutathione reductase and thioredoxin reductase, both of which are cytosolic, selenium-containing flavoproteins, catalyzing the reduction of disulfide bonds utilizing NADPH as the reducing cofactor (Mustacich and Powis 2000). Nitric oxide synthase is another flavoprotein that can participate in quinone metabolism. Nitric oxide synthase is a heme-containing flavoprotein whose carboxy terminus demonstrates significant sequence homology with CPR (Iyanagi 2005). Recently, all the three isoforms of nitric oxide synthetase (NOS), whose reductase domains have a high sequence homology with P450 reductase, have also been demonstrated as being capable of one-electron reduction of quinones (Kumagai et al. 1998). Lipoamide dehydrogenase is a member of the pyridine nucleotide disulfide oxidoreductase family. Proteins in this family are homodimers with each subunit containing a

FAD cofactor and a redox active disulfide (Arscott et al. 1997). Lipoamide dehydrogenase is a component of the 2-oxo acid dehydrogenase complexes and is responsible for catalyzing the NAD^+-dependent oxidation of dihydrolipoamide in these complexes. Lipoamide dehydrogenase has been shown to operate in reverse and reduce quinones via a ping-pong mechanism utilizing NADH as the electron donor (Vienozinskis et al. 1990).

7.2. Toxicological Implications of Quinone Reduction

7.2.1. One-Electron Reduction of Quinones and Redox Cycling

The reaction of semiquinones with oxygen to produce superoxide depends on the one-electron reduction potential of the semiquinone radical relative to the oxygen/superoxide couple but may also depend on reaction conditions and removal of products by secondary reactions (Wardman 2001; Gutierrez 2000; Halliwell and Gutteridge 1985; Lusthof et al. 1992). The redox potential of the oxygen/superoxide couple has been estimated at –155 mV (Wardman 2001) and –137 mV (Petlicki and van de Ven 1998). A useful tabulation of redox potentials of biologically relevant molecules and their application can be found in Halliwell and Gutteridge (1999). The semiquinone radical can also disproportionate to form a half mole equivalent of both quinone and hydroquinone, so this reaction represents an alternate fate for the semiquinone radical (Butler 1998). However, many semiquinones do react rapidly with molecular oxygen to produce superoxide. This reaction can initiate and sustain the production of ROS and reactive-nitrogen species (RNS), leading to lipid peroxidation, protein adducts, and DNA modifications, followed by a cascade of altered cellular responses and defense mechanisms (Figure 7.2). Consequently, metabolism of quinones by one-electron reductases has been regarded as a toxification step. The other major point of toxicological relevance with respect to this reaction is its cyclical nature. The interaction of semiquinone with oxygen regenerates the quinone substrate, so under aerobic conditions and in the presence of reductases with sufficient concentrations of reduced pydridine nucleotide cofactors, a cycling reaction can ensue generating large quantities of superoxide radical, hydrogen peroxide, and other aggressive oxygen and nitrogen species formed by downstream reactions (Figure 7.2). This process is referred to as redox or futile cycling (Figure 7.2).

7.2.2. Two-Electron Reduction of Quinones

Two-electron-mediated reduction of quinones is often viewed as a detoxification reaction since it removes an electrophilic quinone from a biological system and bypasses one-electron reduction leading to the production of ROS and oxidative stress. The hydroquinone is generally more water soluble and more easily excreted However, as will be described in subsequent sections, whether the generation of a hydroquinone is truly a detoxification step depends on the stability and pharmacological reactions of the hydroquinone generated.

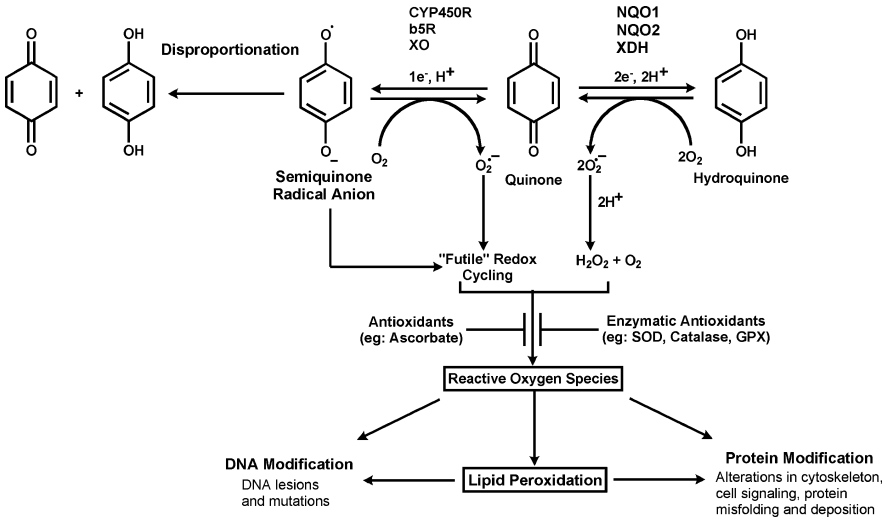

Figure 7.2 Pathways of quinone metabolism and toxicity.

7.2.2.1. Detoxification of Benzene-Derived Quinones

The benzene metabolite hydroquinone can undergo peroxidase-catalyzed oxidation to generate 1,4-benzoquinone, a metabolite that has been shown to directly alkylate DNA and protein (Levay, Ross and Bodell 1993; Thomas et al. 1990; Schlosser and Kalf 1989). Reduction of 1,4-benzoquinone by NQO1, however, regenerates hydroquinone and this reaction prevents the cellular damage induced by 1,4-benzoquinone. The role of the two-electron reductase NQO1 in protection against hydroquinone-induced cytotoxicity in cellular systems is well established. For example, the treatment of KG1A human promyeloblastic cells with hydroquinone resulted in induction of NQO1 and subsequent protection against hydroquinone-induced apoptosis (Moran, Siegel and Ross 1999). Furthermore, the role of NQO1 in protection against benzene toxicity has been studied using NQO1 knockout mice exposed to benzene. In these studies NQO1-deficient mice developed more hematotoxicity when compared to wild-type mice following benzene exposure (Bauer et al. 2003). A role for NQO1 in protection against benzene toxicity in human populations has emerged as well from epidemiological studies that have demonstrated an increased risk for benzene-induced hematotoxicites in individuals lacking NQO1 due to homozygous expression of the NQO1*2 polymorphism (Rothman et al. 1997).

7.2.2.2. Cellular Protection by Generation of Antioxidant Quinones via Two-Electron Reduction

Lipophilic antioxidant quinones such as α-tocopherol quinone and ubiquinone (Figure 7.3) are a special type of quinone that when reduced by two electrons to the corresponding hydroquinone generates stable lipophilic antioxidants that aid in the protection of lipid membranes against peroxidative damage. α-Tocopherolquinone can be generated following oxidation of α-tocopherol (Liebler, Kaysen and Kennedy 1989) and reduction to α-tocopherolhydroquinone has been shown to be carried out by

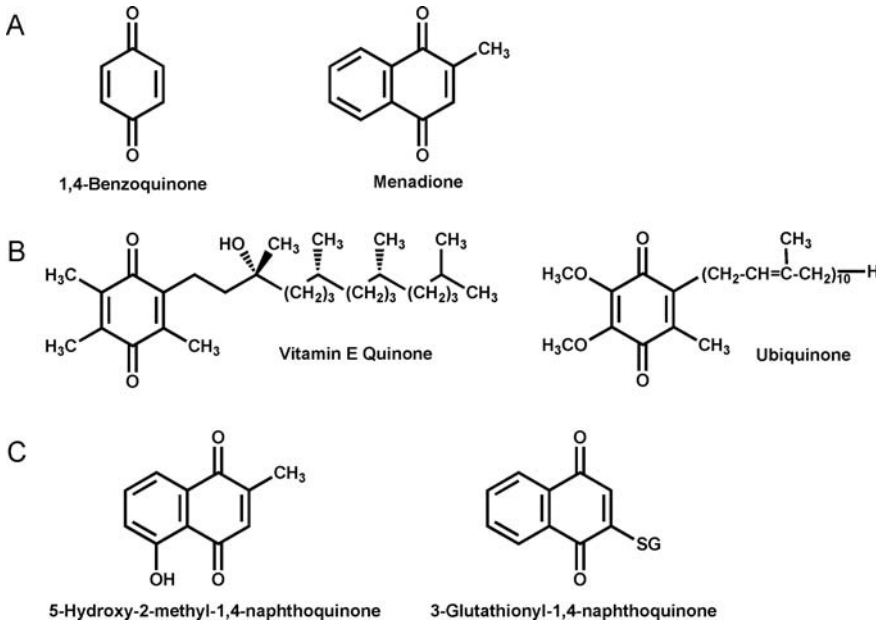

Figure 7.3 The structure of selected quinones that undergo: (**a**) detoxification by two-electron reduction, (**b**) reduction to a more potent hydroquinone antioxidant, and (**c**) activation by two-electron reduction.

NQO1 (Siegel et al. 1997) as well as by complex I of the mitochondrial respiratory chain (Gregor et al. 2006). Microsomal enzymes such as CPR and b5R have also been shown to generate α-tocopherolhydroquinone (Gregor et al. 2006). Besides the role of an electron carrier in the mitochondrial electron transport chain, ubiquinone can also be reduced to a stable hydroquinone and embedded into lipid membranes to protect against lipid peroxidation (Frei, Kim and Ames 1990).

7.2.2.3. Naphthoquinones: Toxification or Detoxification Depending on the Properties of the Hydroquinone

The generation of a hydroquinone metabolite does not always result in quinone detoxification. The two-electron reduction of naphthoquinones by enzymes such as NQO1 can produce hydroquinones that are resistant to autooxidation, but depending on the particular naphthoquinone examined may also produce hydroquinones that rapidly undergo autooxidation generating large quantities of ROS. For napthoquinones like menadione (2-methyl-1,4-naphthoquinone), reduction to a relatively stable hydroquinone metabolite has been shown to be a detoxification pathway by directly competing with one-electron reductases such as CPR. The one-electron reduction of menadione by these flavoproteins generates a semiquinone radical that rapidly reacts with oxygen generating substantial quantities of ROS (Mishin, Pokrovsky and Lyakhovich 1976). In experiments using isolated rat hepatocytes significantly more menadione-induced oxidative damage was observed in cells pretreated with the NQO1 inhibitor dicumarol compared to cells that did not receive the inhibitor (Thor et al.1982). Interestingly, studies in Chinese hamster ovary cell lines engineered to overexpress

a range of NQO1 activities showed that there was a threshold level of NQO1 activity above which offered no greater level of protection against menadione toxicity (De Haan et al. 2002). A role for the two-electron reduction of menadione as a detoxification pathway was further confirmed in studies using NQO1 knockout mice where the absence of NQO1 led to a dramatic decreased in survival following multiple doses of menadione (Radjendirane et al. 1998). Since naphthoquinones have both alkylating and redox activities they have been evaluated as potential antitumor drugs. The anticancer activities of a series of 1,4-naphthoquinones was tested against a series of four human cancer cells lines and in terms of quantitative structure–activity relationships (QSARs) cytotoxicity largely depended upon the hydrophobicity of the naphthoquinone (Verma 2006)

A study using a variety of substituted 1,4-naphthoquinones demonstrated that the rate of autooxidation of hydroquinones formed following reduction by purified NQO1 was influenced to a large degree by the presence of hydroxy and glutathionyl substituents (Buffinton et al. 1989). For example, the rate of menadione autooxidation could be increased significantly by the addition of hydroxy and glutathionyl substituents (Buffinton et al. 1989). Therefore, as a rule the two-electron reduction of naphthoquinones does not always results in detoxification since minor chemical modifications to the naphthoquinone molecule can result in dramatic changes in redox behavior. Other factors that will influence the redox behavior of naphthoquinones include quinone concentration, pH, and the level of superoxide (Munday 2000; Ollinger et al. 1990). Studies by Munday et al. have shown that following reduction by NQO1 the rate of autooxidation for alkyl, alkoxy, hydroxyl, and amino derivatives was decreased in the presence of SOD, while in similar experiments, SOD had no effect on the rate of autooxidation of halogenated compounds (Munday 2000). Since NQO1 has been shown to have superoxide-reducing activity increasing NQO1 levels could also inhibit autooxidation in a manner similar to SOD (Siegel et al. 2004), and in cell-free experiments increasing the concentration of NQO1 was shown to inhibit menadiol autooxidation (Munday 2004). A pivotal role for the two-electron reduction of naphthoquinones by NQO1 in vivo can be seen from studies in rats pretreated with inducers that increase the levels of NQO1 in tissues. As expected, the induction of NQO1 in tissues by BHA decreased the toxicity of menadione but conversely, increasing the tissue levels of NQO1 increased the hemolytic anemia induced by 2-hydroxy-1,4-naphthoquinone (Munday, Smith and Munday 1999b; Munday, Smith and Munday 1998). Thus, prediction of whether NQO1 contributes to activation or deactivation of naphthoquinones requires a detailed knowledge of hydroquinone properties, the cellular context in which it is generated, and other enzyme activities, such as SOD.

7.3. Bioreductive Activation of Antitumor Quinones

The generation of cytotoxic hydroquinones within cancer cells is know as bioreductive activation and has become the basis for the development of a wide variety of both natural and synthetic quinones as chemotherapeutic agents. This type of therapy exploits the high levels of quinone reductases,

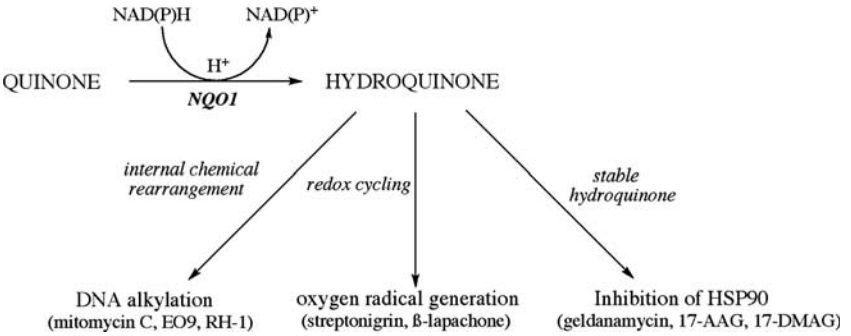

Figure 7.4 The role of two-electron reduction in the bioreductive activation of antitumor quinones.

particularly NQO1, in most solid tumors as a means of targeting drug activation to cancer cells (Ross et al. 1996; Ross et al. 1994; Ross et al. 1993). Following two-electron reduction in cancer cells by enzymes, such as NQO1, antitumor quinones can inflict cellular damage in at least three separate ways (Figure 7.4). Reduction to a hydroquinone can promote intramolecular chemical rearrangements leading to the generation of monofunctional and bifunctional DNA alkylating agents. Secondly, the generation of large quantities of ROS by the intracellular redox cycling of antitumor quinones is another mechanism whereby quinones can exert anticancer activity (Figure 7.4). Finally, the generation of a hydroquinone can have a more pronounced effect against a particular intracellular target and exert an improved therapeutic effect.

7.3.1. Mitomycins

Mitomycin C is inactive until reduced by enzymes such as NQO1 (Siegel et al. 1992), NQO2 (Jamieson et al. 2006; Celli et al. 2006), or XDH (Gustafson and Pritsos 1992). Under hypoxic conditions, XO, CPR, and b5R can also bioactivate mitomycin C (Hodnick and Sartorelli 1993; Pan et al. 1984, see above). A relationship between the reduction potential of a series of mitomycins and cytotoxicity has been observed (Pan and Gonzalez 1990). The bioactivation of mitomycin C by NQO1 has been shown to be pH-dependent where substantially more metabolism and DNA crosslinking were observed as the reaction pH was decreased (Siegel et al. 1992). At higher pH values, mitomycin C was shown to inactivate NQO1 by directly alkylating amino acid residues on the protein (Siegel et al. 1993). Despite mechanism-based inhibition of NQO1 by mitomycin C at high pH values, studies have shown that NQO1 does play an essential role in the bioactivation of mitomycin C in vivo (Fleming et al. 2002; Gan et al. 2001). The *N*-methyl aziridine analogue of MMC, porfiromycin, is a potent antitumor agent that also undergoes pH-dependent metabolism as the result of mechanism-based inhibition of NQO1 in a manner similar to mitomycin C (Siegel et al. 1993). This is in contrast to mitomycin A and B which did not demonstrate mechanism-based inhibition of NQO1 and underwent bioreductive activation by NQO1 under both acidic and basic conditions (Ross et al. 1993).

7.3.2. Diazirdinyl 1,4-benzoquinones

The diaziridinyl 1,4-benzoquinones are another class of bioreductive DNA alkylating quinones that, following metabolism by two-electron reductases, generate the hydroquinone species facilitating aziridinyl ring opening leading to monofunctional and bifunctional DNA alkylation (King, Wong and Loo 1984). AZQ (2,5-diaziridinyl-3,6-*bis*-(carboethoxyamino)-1,4-benzoquinone) was the first of these compounds to enter clinical trials and was designed primarily to cross the blood–brain barrier for the treatment of pediatric brain tumors (Curt et al. 1983). AZQ was shown to be bioactivated rather poorly by NQO1 (Gibson et al. 1992; Siegel et al. 1990) and this led to the design and synthesis of a series of diaziridinyl 1,4-benzoquinones that were more efficiently bioactivated by NQO1 and from these compounds RH-1 (2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1, 4-benzoquinone) has emerged as the lead compound and is currently in clinical trials (Danson et al. 2007; Winski et al. 1998). RH-1 has been shown to undergo very efficient reduction by NQO1 and to induce more DNA crosslinking and cytotoxicity in NQO1-rich cancer cells compared to NQO1-deficient cells (Dehn, Inayat-Hussain and Ross 2005; Dehn, Winski and Ross 2004). It has also been shown using human isogenic cancer cell lines that differ only in the level of NQO1 protein and activity that sensitivity to RH-1 correlated with the level of NQO1 activity. However, there was a threshold level of NQO1 activity and increasing NQO1 activity above this value did not lead to an increase in cytotoxicity (Winski et al. 2001). Studies have also shown that RH-1 can also be reduced by CPR to semiquinone and hydroquinone species leading to both DNA strand breaks and DNA-crosslinking (Nemeikaite-Ceniene et al. 2003). However, studies in human cancer cell lines engineered to overexpress CRP did not demonstrate any significant increase in cytotoxicity following treatment with RH-1 suggesting a limited role for this enzyme in RH-1 bioactivation (Begleiter et al. 2007).

7.3.3. Indolequinones

EO9 is an indolequinone antitumor drug that was developed to undergo bioreductive activation similar to mitomycin C but despite robust antitumor activity against human cancer cell lines with high levels of NQO1 activity EO9 failed to demonstrate a significant antitumor response in clinical trials (Pavlidis et al. 1996; Dirix et al. 1996). The three active centers, the vinylic group at C-2, the hydroxymethyl group at C-3, and the aziridinyl group at C-5 are possibly activated upon reduction of the quinone group of EO9. DNA interstrand crosslinking was observed by indoloquinone following reductive activation with XO (Maliepaard et al. 1995).

A structure/activity study of a series of indolequinones concluded that slight modifications of the indolequinone structure resulted in substantial variations in activation and cytotoxicity after reduction by NQO1 (Swann et al. 2001; Beall et al. 1998). These studies also demonstrated that NQO1 can bioactivate these compounds more efficiently under aerobic conditions while under hypoxia, the one-electron reduction of these indolequinones is an important pathway in bioactivation (Jafar et al. 2003; Naylor et al. 1998).

Indolequinones have also been used to target NQO1 and induce cytotoxicity in human pancreatic cancer cells. A series of indolequinones based on the structure of ES936 (5-methoxy-1,2-dimethyl-3-[(4-phenoxy)methyl]-indole-4,7-dione) demonstrated potent mechanism-based inhibition of NQO1 without affecting the activities of other reductases such as NQO2, CPR, or b5R (Dehn et al. 2003; Siegel et al. unpublished data). Since high levels of cellular NQO1 can scavenge superoxide (Siegel et al. 2004), indolequinones were developed to selectively inhibit NQO1 and increase levels of superoxide leading to increased cell death of pancreatic tumor cells (Reigan et al. 2007; Cullen et al. 2003). However, in studies using a series of indolequinones, despite greater than 95% inactivation of NQO1 in pancreatic cancer cells, there was no correlation between NQO1 inhibition and ability to induce cytotoxicity suggesting that indolequinones may have molecular targets in addition to NQO1 (Reigan et al. 2007).

7.3.4. Streptonigrin

The generation of ROS, and in particular the hydroxyl radical, in close association with DNA can produce large quantities of DNA strand breaks resulting in the loss of genomic integrity and cell death. Streptonigrin is an example of a redox-cycling quinone antitumor antibiotic isolated from cultures of *Streptomyces flocculus* (Marsh, Garretson and Wesel 1961). Streptonigrin has multiple metal binding sites and can interact with a variety of metals to produce streptonigrin-metal-DNA complexes (White 1977). The reduction of the quinone moiety in streptonigrin by either one or two electrons generates the semiquinone radical or hydroquinone, respectively. In the presence of oxygen both forms will rapidly autooxidize to produce ROS and the parent quinone (Beall et al. 1994; Bachur et al. 1979). During autooxidation the presence of metal ions bound to streptonigrin catalyze the generation of hydroxyl radicals via Fenton-type reactions in close proximity to DNA resulting in large quantities of DNA single- and double-strand breaks (Sugiura, Kuwahara and Suzuki 1984). Streptonigrin was shown to be reduced very efficiently by NQO1 to the corresponding hydroquinone in cell-free systems (Beall et al. 1994), and in studies with human isogenic cancer cell lines streptonigrin was more cytotoxic to NQO1-expressing cells compared to NQO1-null cells (Beall et al. 1996).

7.3.5. ß-Lapachone

β-lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2-*b*]-pyran-5,6-dione) is another example of a redox-cycling quinone antitumor drug. β-lapachone was isolated from the bark of the lapacho tree (*Tabebuia avellanedae*) and has been reported to possess a wide range of pharmacological properties, including antiviral, antiparasitic, and antitumor activities (Planchon et al. 1995; Schuerch and Wehrli 1978; Boveris et al. 1978). β-lapachone can inhibit topoisomerase I and prevent DNA repair induced by methylating agents and radiation (Boothman and Pardee 1989; Boorstein and Pardee 1984). However, the treatment of cells with β-lapachone also results in the generation of large quantities of hydrogen peroxide due to

the redox cycling of the quinone. An interesting finding was that in a variety of human cancer cell lines the sensitivity to β-lapachone correlated with intracellular hydrogen peroxide generation; cell lines that generated high levels of hydrogen peroxide were sensitive to β-lapachone while cell lines with low levels of hydrogen peroxide were resistant (Chau et al. 1998). These data suggest that the ability to undergo bioreductive activation to either semiquinone or hydroquinone species and subsequent redox cycling are required for β-lapachone to exert maximal cytotoxicity. In studies using cell sonicates, the addition of β-lapachone resulted in the oxidation of greater than stoichiometric equivalents of reduced pyridine nucleotide cofactors and the rate of cofactor oxidation was dependent upon the activity of NQO1 (Pink et al. 2000). Subsequent studies in cell lines and human xenograft tumors grown in mice have shown that NQO1 is the principle determinant of β-lapachone cytotoxicity (Ough et al. 2005; Pink et al. 2000).

7.3.6. Benzoquinone Hsp90 Inhibitors

A third mechanism whereby the two-electron reduction of quinones to hydroquinones can induce cytotoxicity is through the generation of a stable hydroquinone with unique pharmacological properties (Figure 7.4). An example of this type of bioreductive activation can be seen with the benzoquinone ansamycin class of HSP90 inhibitors which includes geldanamycin, 17-AAG, 17-DMAG, and 17AG. These compounds are characterized by a quinone moiety attached to a large planer macrocyclic ansa bridge structure (Figure 7.5). The benzoquinone ansamycins represent a class of compounds that disrupt multiple pathways involved in tumor cell proliferation (Goetz et al. 2003). It has been shown that these compounds bind to an N-terminal ATP-binding site in heat shock protein 90 (HSP90) preventing ATP binding and hydrolysis (Grenert et al. 1997). The inability of HSP90 to utilize ATP prevents the protein from assisting in the folding and maturation of critical oncogenic and regulatory proteins (Powers and Workman 2006; Goetz et al. 2003). Geldanamycin, the prototype for this class of compounds, is a natural product isolated from cultures of *Streptomyces hygrosopicus* (BeBoer and Dietz 1976). In clinical trials geldanamycin induced unacceptable levels of hepatotoxicity; therefore, a second generation of benzoquinone ansamycin compounds were developed that include 17-AAG and 17-AG. These compounds were selected because they induced less toxicity in animal studies but still retained the ability to inhibit HSP90 (Behrsing et al. 2005; Schulte and Neckers 1998). In studies using a variety of human cancer cell lines treated with 17-AAG there was a positive correlation between 17-AAG sensitivity and NQO1 activity suggesting a role for quinone reduction by NQO1 in the mechanism of action of the benzoquinone ansamycins (Kelland et al. 1999). Subsequent studies using purified recombinant NQO1 demonstrated that the benzoquinone ansamycins could be reduced by NQO1 to their corresponding hydroquinone species (Guo et al. 2005, 2006) and in studies using purified yeast and human HSP90 the hydroquinone species induced substantially greater inhibition of HSP90 when compared to the quinone form (Guo et al. 2005, 2006). Molecular modeling studies also confirmed that when reduced to their corresponding hydroquinones the benzoquinone

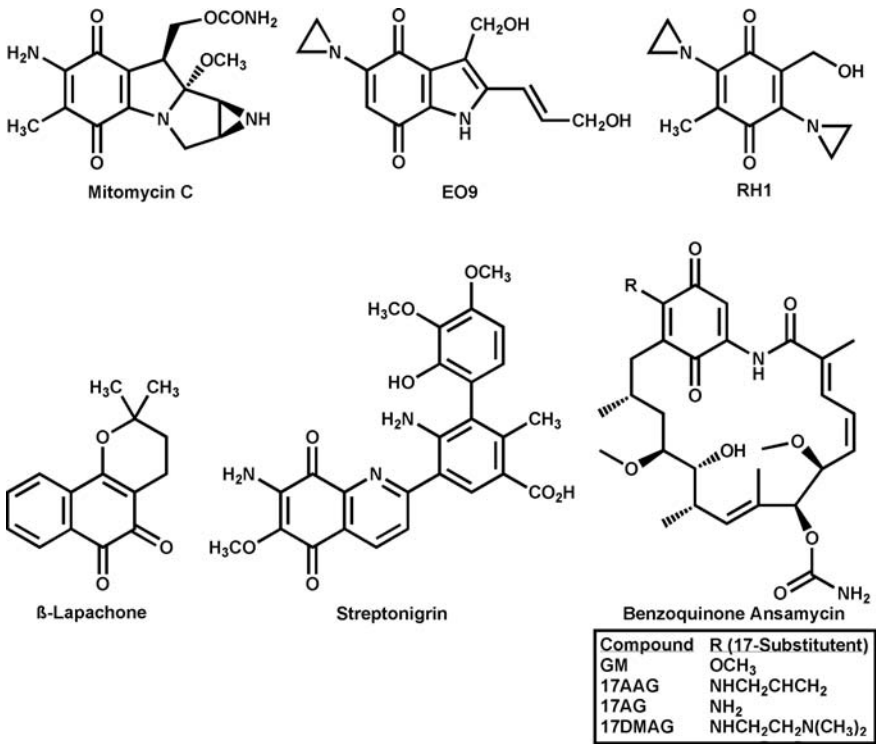

Figure 7.5 The structures of antitumor quinones that undergo bioreductive activation.

ansamycins exhibited a stronger binding interaction with the ATP-binding site in HSP90 (Guo et al. 2005, 2006). In studies using isogenic human cancer cell lines that differ only in the expression of NQO1, the reduction of 17-AAG to the corresponding hydroquinone was seen exclusively in cells expressing NQO1 and formation of the hydroquinone could be inhibited by pretreating the cells with an NQO1 inhibitor (Guo et al. 2005). In addition, these experiments also showed that cells expressing NQO1 were more sensitive to 17-AAG and sensitivity correlated with the marked degradation of HSP90 client proteins (Guo et al. 2005). Recently, the more active and water-soluble hydroquinone of 17-AAG has been formulated into a stable product and is currently in clinical trials (Sydor et al. 2006). While the reduction of benzoquinone ansamycins by NQO1 to their corresponding hydroquinones generates more potent HSP90 inhibitors, one-electron reduction will form an unstable semiquinone radical that in the presence of oxygen may redox cycle back to the quinone-generating ROS. Studies have shown that geldanamycin can undergo CPR-mediated reduction to the semiquinone radical that reacts spontaneously with oxygen to regenerate the quinone and in the process generates superoxide (Dikalov, Landmesser and Harrison 2002; Benchekroun, Myers and Sinha 1994). The ability of geldanamycin to undergo one-electron reduction by enzymes such as CPR in the liver may play a role in the hepatotoxicity observed in patients treated with geldanamycin in clinical trials.

7.3.7. The Case of Hypoxia-Activated Quinone Prodrugs: Therapeutic Exploitation of One-Electron Reduction of Quinines

Despite the association of one-electron cycling of semiquinones with oxidative stress and toxicity, one-electron reduction of quinones with subsequent generation of ROS may be a more desirable outcome from a toxicological perspective than accumulation of potentially toxic semiquinone radicals (Sartorelli 1988). As explained by Wardman, for drug efficacy as a hypoxic cytotoxin, the damaging effect of the reactive product under hypoxia must be greater than the toxicity resulting from oxygen-cycling reactions under aerobic conditions (Wardman 2001). This reaction has been exploited for therapeutic purposes and has been employed in the design of antitumor quinones to kill hypoxic cell fractions in tumors. Quinone analogues were among the first compounds explored as hypoxia-selective tumour-activated prodrugs. After one-electron reduction of a quinone species to the semiquinone radical anion (Belcourt et al. 1996), dependent on the redox potential of the semiquinone, it can be back-oxidized by molecular oxygen in normal well-perfused cells. Under hypoxic conditions, however, the semiquinone radical is not back-oxidized, accumulates, and can lead to cellular damage potentially resulting in hypoxia-selective cytotoxicity (Everett et al. 1998; Sartorelli 1988). For example, the *N*-methyl aziridine analogue of MMC, porfiromycin, is a potent antitumor agent, which shows greater hypoxic selectivity (Belcourt et al. 1996). The reduction of porfiromycin to the semiquinone radical anion can be reversed by molecular oxygen, but under hypoxia this undergoes complex fragmentation to a reactive species that binds to DNA (Figure 7.6) via guanine–guanine crosslinks in the major groove (Pan and Iracki 1988).

Mitomycin C and porfiromycin are naturally occurring prototypes of bioreductive anticancer prodrugs. Since their discovery, various reductively activated prodrugs have been developed based on the mechanism of action revealed by these hypoxically activated cytotoxins. In addition, many hypoxia-targeting hybrid drugs have been designed that incorporate quinones and nitroaromatics (triggers) connected to cytotoxic units (effectors) through linker units. Upon activation, such hybrid drugs are designed to release strong alkylating agents, for example, cyclophosphoramide (Denny 2005).

7.4. Summary

In summary, a wide range of one- and two-electron reductases are capable of metabolizing quinones. Both semiquinones and hydroquinones formed by one- and two-electron reductions, respectively, may be unstable to oxygen and undergo redox-cycling reactions in the presence of adequate oxygen and enzymatic cofactor. Metabolism of quinones to hydroquinone derivatives may result in either toxification or detoxification depending on the properties of the hydroquinone generated, and good examples of both reactions have been documented. Both the one- and two-electron-mediated reduction of quinones have been utilized to target cancers. One-electron reduction has been employed in the targeting of hypoxic fractions in tumors

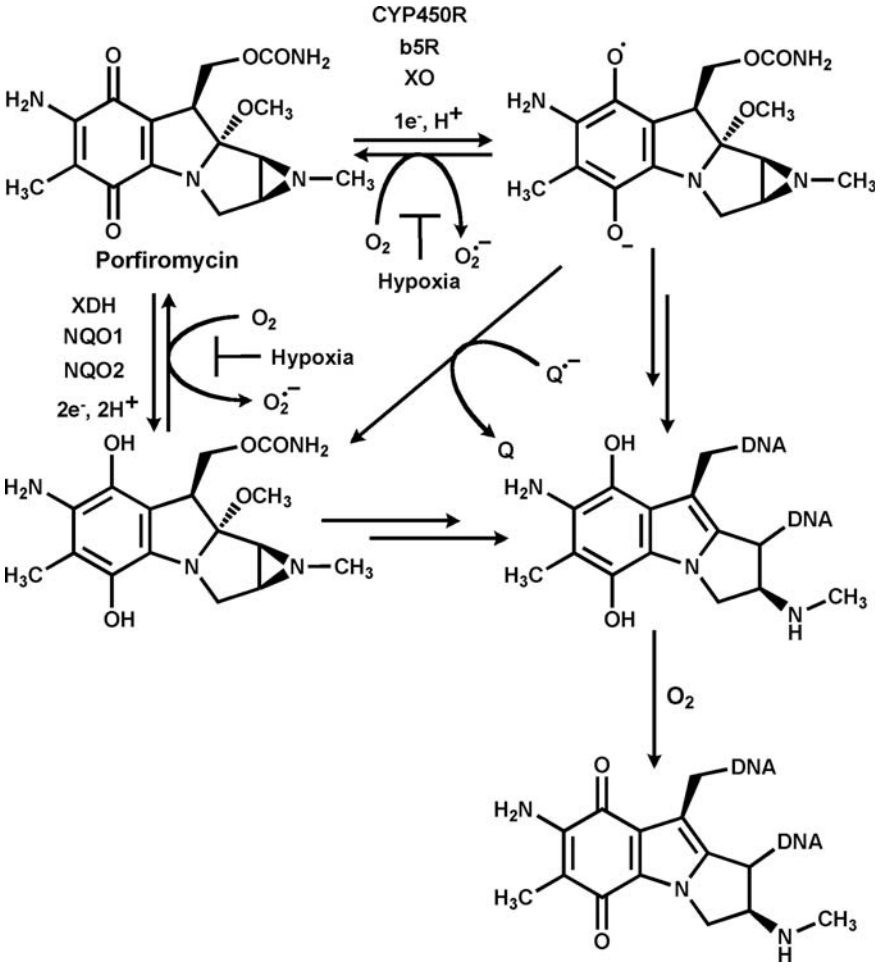

Figure 7.6 Porfiromycin bioactivation and DNA modifications.

while the presence of elevated levels of two-electron reductases such as NQO1 in tumors has led to the design of anticancer quinones that can be activated specifically in neoplasic cells.

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