Induction of p21 Mediated by Reactive Oxygen Species Formed during the Metabolism of Aziridinylbenzoquinones by HCT116 Cells*

Xiaobo Qiu, Henry Jay Forman, Axel H. Schönthal, and Enrique Cadenas§

From the Department of Molecular Pharmacology and Toxicology, School of Pharmacy and the ‡Department of Microbiology, School of Medicine, University of Southern California, Los Angeles, California 90033

Aziridinylbenzoquinones are a group of antitumor agents that elicit cytotoxicity by generating either alkylating intermediates or reactive oxygen species. The mechanism of toxicity may not always, however, involve profound damage of cellular constituents, but may involve a cytostatic effect through interference with the cell cycle. In this context, we have examined the induction of the cell cycle inhibitor p21 (WAF1, CIP1, or sd1), whose overexpression suppresses the growth of various tumor cells, in human tumor cells metabolizing 3,6-diazaaziridinyl-1,4-benzoquinones (DZQ) and its C2,C5-substituted derivatives: 2,5-bis-(carboethoxyamino) (AZQ) and 2,5-bis-(I-hydroxyethylamino) (BZQ).

Both DZQ and AZQ were effectively activated by HCT116 human colonic carcinoma cells; the activation of the former involved largely a dicoumarol-sensitive activity, whereas that of the latter appeared to be accomplished primarily by one-electron transfer reductases. BZQ was not a substrate for the dicoumarol-sensitive enzyme in HCT116 cells. Cellular activation of the first two quinones was associated with formation of oxygen-centered radicals as detected by EPR in conjunction with the spin trap 5,5′-dimethyl-1-pyrroline-N-oxide. The redox transitions of DZQ involved hydroxyl radical formation and were strongly inhibited by catalase, whereas those of AZQ showed a strong superoxide anion component sensitive to superoxide dismutase. These signals were suppressed by N-acetylcysteine with concomitant production of a thyl radical adduct. This suggests an effective electron transfer between the thiol and free radicals formed during the activation of these quinones.

DZQ and AZQ induced significantly the expression of p21 in HCT116 cells, but a 10-fold higher concentration of AZQ was required to achieve the level of induction elicited by DZQ. BZQ had little effect on p21 expression. p21 induction at both mRNA and protein levels correlated with the inhibition of either cyclin-dependent kinase activity or cell proliferation. p21 induction elicited by the above quinones was inhibited by N-acetylcysteine, whereas the non-sulfur analog, N-acetylasaline, was without effect. Catalase and superoxide dismutase did not effect p21 induction by aziridinylbenzoquinones in HCT116 cells, thus suggesting that extracellular sources of oxygen radicals generated by plasma-membrane reductases have no influence in the expression of this gene. Hydrogen peroxide, a product of quinone redox cycling, elicited an increase of p21 mRNA levels in HCT116 and K562 human chronic myelogenous leukemia cells. The latter lacks p53, one of the activators of p21 transcription, thus suggesting that p21 expression can be accomplished in a p53-independent manner in these cells.

This study suggests that p21 induction is mediated by an increase in the cellular steady-state concentration of oxygen radicals and that the greater effectiveness in p21 induction by DZQ may be related to its efficient metabolism by NAD(P)H:quinone oxidoreductase activity in HCT116 cells.

The paper was aimed at gathering information on the metabolism of aziridinylbenzoquinones.
lecular mechanisms inherent in the activation of p21 during the metabolism of several aziridinylbenzoquinones by HCT116 human colon carcinoma cells. A comprehensive approach to elucidate this mechanism(s) involved (a) characterization of the cellular activation of these quinones, (b) measurement of the ensuing steady-state concentration of oxygen radicals, and (c) establishment of a relationship between the latter and expression of p21 at mRNA and protein levels. The cellular effects of these quinones are expected to be an expression of both their functional group chemistry and mode of activation. The former led us to investigate aziridinylbenzoquinones with different substitution patterns at C2 and C5: 3,6-diaziridinyl-1,4-benzoquinone (DZQ), 2,5-bis-(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone (AZQ),1 and 2,5-bis-(2-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone (BZQ) (see Structures 1–3). The latter is also of interest because most cancer cells and preneoplastic nodules, among them HCT116 human colon carcinoma cells, overexpress NAD(P)H:quinone oxidoreductase (DT-diaphorase), a two-electron transfer flavoprotein (25) with salient features in quinone metabolism.

MATERIALS AND METHODS

Cell Culture and Treatments—Human colonic carcinoma HCT116 cells (ATCC CCL247) and human chronic myelogenous leukemia K562 cells (lacking p53; ATCC CCL243) were grown in McCoy's 5A medium or RPMI 1640 medium (Sigma) with 10% fetal bovine serum. Human colon carcinoma HCT116 cells, RPMI 1640 medium (Sigma) with 10% fetal bovine serum (Biocell Laboratories Inc., Rancho Dominguez, CA). Cells were seeded 24 h before drug treatment. All quinones were dissolved in Me2SO. Control cultures were treated with Me2SO alone. In experiments with cells supplemented with N-acetylcyysteine or N-acetylcysteine, the pH values of these compounds were adjusted to 7.4 before the addition. Cell viability was determined by trypan blue exclusion. Cell numbers were counted in hemacytometer by light microscopy.

RNA Isolation and Northern Blot Analysis—Extraction of total RNA (20 μg/lane) from HCT116 cells and Northern blot analysis were carried out by standard procedures (26). Human p21 probe was prepared by the polymerase chain reaction as described (27) using human genomic DNA from HCT116 cells. Human GAPDH cDNA was used as control probe to determine the relative amount of RNA loaded. GAPDH cDNA was prepared from the plasmid with human GAPDH cDNA insert in Escherichia coli (ATCC 57090) by using a plasmid purification kit (Qiagen Inc., Chatsworth, CA).

Immunoblot Analysis—5 × 10⁶ cells were lysed in radioimmune precipitation buffer as described (28) and the amount of protein was determined using the biinchinoninic acid protein assay (Pierce). 50 μg of total protein was loaded on a 12% SDS-polyacrylamide gel. After electrophoresis at 120 V for 5–6 h, the protein was transferred to a nitrocellulose membrane and incubated for 3 h at room temperature with monoclonal p21 antibody or purified p34cdc2 antibody (PharMingen, San Diego, CA). The levels of antigens were analyzed by sensitized chemiluminescence (Amersham Corp.). Histone H1 Kinase Assay—the cell lysate was prepared as described for immunoblot analysis. 150 μg of total protein was subjected to immunoprecipitation with edc2 or cyclin A antibody (PharMingen). The immunocomplexes were bound to protein A-agarose, washed three times with radioimmune precipitation buffer and twice with kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 1 mM dithiothreitol). 25 μl of kinase buffer containing 4 μg of histone H1, 30 μM ATP, and 5 μCi of (γ-32P)ATP (3000 Ci mmol⁻¹) was added and incubated at room temperature for 15 min. The reaction products were analyzed on a 12% acrylamide gel, quantified by autoradiography (Instant Image Electon Autoradiography; Packard Instrument Co.), and exposed to a Kodak X-AR film.

EPR Spectroscopy—HCT116 cells in exponential growth were harvested by trypsinizing, washed twice in phosphate-buffered saline (PBS, pH 7.4), sonicated for 1 min, and then centrifuged at 10,000 × g for 5 min at 4°C to obtain the homogenates. Protein was determined by the Lowry method (29). Cell homogenates (2.17 ± 0.03 mg of protein/ml) were mixed with 80 μM DMPO, 2% DMSO, 1 mM NADH, and 100 μM quinone in the absence or presence of 20 μM dicoumarol. EPR spectra were recorded on a Bruker ECS106 spectrometer with the following settings: microwave frequency, 9.80 GHz; microwave power, 20 mW; sweepwidth, 100 G; receivergain, 1; time constant, 1.3 s; sweep rate, 20 G/min.

Absorption Spectroscopy—HCT116 cell homogenates were prepared as described above. NAD(P)H:quinone oxidoreductase activity was measured by cytochrome c reduction (ε = 21 mM⁻¹ cm⁻¹) as described (30). The reaction mixture contained 77 μM cytochrome c, 200 μM NADH, 50 mM Tris-HCl, pH 7.5, 0.7 mg/ml of bovine serum albumin, 0.1% DMSO, and 10 μM quinone in the absence or presence of cell homogenate (1–10 μg of protein/ml).

RESULTS

Cellular Activation of Aziridinylbenzoquinones—NAD(P)H:quinone oxidoreductase, a two-electron flavoprotein overexpressed in most cancer cell lines and preneoplastic tumors is expected to play an important role in the bioactivation of aziri-

**Table 1**

| Quinone | Total + Dicoumarol | Superoxide dismutase | p21 induction<br>nmol/min/mg protein | Control + Dicoumarol | -fold Δ μM quinone |
|---------|-------------------|---------------------|------------------------------------|---------------------|-----------------|
| DZQ     | 10.7 ± 1.43       | 43.3 ± 1.39         | 5.66 ± 1.07                        | 170.1 ± 1.10        | 0.41 ± 0.15     |
| AZQ     | 162.5 ± 1.07      | 153.9 ± 9.4         | 9.4 ± 1.70                         | 153.0 ± 9.2         | 0.41 ± 0.15     |

Values were obtained from assays with different cell homogenate concentrations at fixed quinone concentration per duplicate. Assay conditions as described under “Materials and Methods.”

p21 induction was calculated from at least three independent Northern analyses at the points with the statistically significant induction (p < 0.05) at optimal quinone concentration.
FIG. 1. Formation of oxygen radicals during the metabolism of
DZQ and AZQ by HCT116 cell homogenates. Experiments carried
out with DZQ (left panel) or AZQ (right panel): a, control, cell homoge-
nate (2.17 ± 0.03 mg of protein/ml) in PBS buffer, pH 7.4, was supple-
mented with 100 μM quinone (dissolved in 2% Me₆SO and 80 mM
DMPO; b, simulated spectrum of a corresponding to the DMPO–CH₃
adduct for DZQ or a composite of DMPO–OOH and DMPO–CH₃ ad-
ducts ((DMPO–OOH)/(DMPO–CH₃) = 3.3) for AZQ; c, as in a in the
presence of 0.1 mM superoxide dismutase; d, as in a in the presence of 1
mM catalase; e as in a in the presence of superoxide dismutase and
catalase.

Dicyclonbenzoquinones (25, 31, 32). The specific activity of this
oxidoreductase in HCT116 cells is ~390 nmol/min/mg of protein (measured as the dicoumarol-sensitive reduction of dichlo-
rophenolindophenol) (33).

The dicoumarol-sensitive reduction of DZQ by HCT116 cell
homogenates was 374 nmol/min/mg of protein, a value that
represented 72% of the overall reducing activity of HCT116
cells. That of AZQ was only 8.6 nmol/min/mg of protein, indi-
cating that quinone reductases other than NAD(P)H:quinone
oxidoreductase were largely (~95%) involved in the metabo-
This observation suggests that HO' is indeed formed during the
metabolism of DZQ by HCT116 cells. The faster reaction of HO'
with Me₆SO (to yield CH₃) than with the spin trap itself
explains the prevalence of the DMPO–CH₃ adduct. The occur-
rence of HO' in this system is further supported by the inhib-

This experimental model (18, 35). The effect of N-Acetylcysteine on the
EPR signal observed during the metabolism of AZQ by
HCT116 cells is shown in Fig. 3: a new signal (hyperfine split-
ning constants: \(a^N = 14.2\) and \(a^H = 11.3\) G, \(a^H = 1.25\) G) and methyl (DMPO–CH₃: \(a^N = 16.4\) and \(a^H = 23.52\) G)
radical adducts (34) ((DMPO–OOH)/(DMPO–CH₃) = 3.3) (Fig.
1). The contribution of the former adduct to the overall EPR
signal was abolished by superoxide dismutase. The generation of
HO' by AZQ was ~3-fold lower than that by DZQ, in agree-
ment with the enzymic reduction of the quinones (Table 1).

The differences in the EPR signals obtained during the metab-
olism of DZQ and AZQ by HCT116 cells are an expression of
their functional group chemistry and the type of activation.
AZQ is predominantly reduced by quinone reductases other
than NAD(P)H:quinone oxidoreductase, whereas the latter en-
zyme appears largely responsible for the activation of DZQ
(Table 1). The occurrence of \(O_2^·\) during AZQ metabolism ap-
ppears consistent with a univalent activation of the quinone.
Conversely, the absence of this radical adduct during DZQ
metabolism may be rationalized as a predominant role of
O₂ as a propagating species (oxidizing the hydroquinone to
semiquinone).

BZQ did not produce any detectable oxygen-centered radicals
in the cell homogenates within the limits of our assay pro-
dure, but a strong semiquinone signal was detected in the
absence of the spin trap (Fig. 2a). This quinone itself, in PBS
buffer and in the absence of cell homogenates, produced a
semiquinone signal (Fig. 2b), whose intensity was enhanced
upon addition of NADH (Fig. 2c). It may be surmised that
BZQ is mainly reduced nonenzymically to a "stabilized"
semiquinone.

Effect of N-Acetylcysteine on Free Radical Production by
HCT116 Cells—N-Acetylcysteine is a powerful reductant,
which has been used as an intracellular antioxidant in several
experimental models (18, 35). The effect of N-acetylcysteine on
the EPR signal observed during the metabolism of AZQ by
HCT116 cells is shown in Fig. 3: a new signal (hyperfine split-
ing constants: \(a^N = 15.0\) and \(a^H = 16.8\) G) (36) ascribed to the
N-acetylcysteylnyl radical was obtained. This is consistent with
the one-electron oxidation of the thiol by the free radicals
formed during the redox transitions of AZQ (or DZQ, not
shown) and subsequent trapping of the thyl radical by DMPO
(Reaction 2).

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\text{RSH + HO' → RS' + H}_2\text{O}
\]

Reaction 2

p21 Induction in HCT116 Cells Mediated by Aziridinylben-
zoquinones—p21 induction at mRNA level in HCT116 cells was
measured by Northern blot analysis. Our p21 probe hybridized
with a single band around 2.1 kilobases, which is consistent
with human p21 mRNA (12). Treatment of HCT116 cells with DZQ elicited a concentration- and time-dependent increase of p21 mRNA levels (Fig. 4).

p21 protein level was significantly elevated while the level of p34$^{cd2}$ protein, one of the cyclin-dependent kinases, remained unchanged following the treatment with 0.4 or 1.0 $\mu$M DZQ for 15 h (Fig. 5A). The $cd2$-associated H1 kinase activity was strongly suppressed following the above treatments (Fig. 5B). The histone H1 kinase activities of the cyclin-dependent kinases associated with cyclin A (immunoprecipitated by cyclin A antibody) were also substantially inhibited (Fig. 5C). These results are in agreement with the role of p21 in inhibiting cyclin-dependent kinase activities.

Fig. 6 shows that the increase of p21 mRNA levels elicited by the three quinones differed markedly: DZQ produced the highest response (a 6-fold increase) over a narrow concentration range, possibly determined by the toxicity of the quinone at high doses (see below). AZQ significantly elevated (~3-fold) p21 mRNA levels, but higher concentrations than those used with DZQ were required. Conversely, BZQ produced no obvious dose-dependent response on p21 mRNA.

p21 Induction by Reactive Oxygen Species Formed during Quinone Metabolism—The effect of N-acetylcysteine on free radical production by the above quinones suggested an effective transfer of the radical character from an oxygen-centered radical to a less reactive sulfur-center radical (Reaction 2; Fig. 3). The exposure of HCT116 cells to N-acetylcysteine caused a small increase at constitutive levels of p21 mRNA (Fig. 7A and B). The thiol completely inhibited the DZQ-mediated p21 induction, partially inhibited that mediated by AZQ, and had no effect on cells treated with BZQ (Fig. 7B). The effect elicited by N-acetylcysteine appears to be dependent on its free thiol group, because N-acetylalanine, a non-thiol analog, failed to elicit any significant inhibition of p21 induction (Fig. 8).

To further elucidate if oxyradicals mediate p21 induction by quinones, the HCT116 cells were treated with $H_2O_2$, a membrane-permeable product of quinone redox cycling (37); ~2-fold increase in the amount of p21 mRNA was observed (Fig. 9A). $H_2O_2$ also led to an increase at p21 mRNA level in human chronic myelogenous leukemia K562 cells lacking p53 (one of the activators of p21 transcription) in which p21 mRNA was elevated following the treatment of DZQ (Fig. 9B).

Effect of Superoxide Dismutase and Catalase on p21 Induction—These enzymes, when present in the medium surrounding intact cells, did not have any effect on p21 induction. This suggests that extracellular sources of oxygen radicals generated by plasma membrane reductases have no influence on the expression of this gene.
**p21 Induction and Cell Proliferation**—Cell proliferation was also inhibited by DZQ and AZQ at the same concentrations required to induce p21. Similar to what was observed with p21 induction (Fig. 6), BZQ did not elicit any obvious effect on cell proliferation (Fig. 10A). To determine whether inhibition of cell proliferation was a transient phenomenon, cells were counted at 6, 24, 48, and 72 h following their exposure to 0.4 mM DZQ (Fig. 10B). Cells were still proliferating at the early stage of the exposure. After 24 h, cell proliferation was completely inhibited by this treatment, even when the DZQ in the medium was removed after 6-h treatment. This inhibition lasted at least until 72 h after the treatment. A significant percentage of dead cells were identified only in the continuous presence of DZQ for more than 48 h after the treatment. Thus, at a concentration of 0.4 mM, the effect of DZQ on cell number appeared to be due to a decreased proliferation rather than outright toxicity.

**DISCUSSION**

The role of oxygen free radicals in antitumor quinone reactivity and their implications for cancer cell and systemic cytotoxicity has been randomly established (25, 32). Cytotoxicity elicited by aziridinylbenzoquinones is attributed to the quinone moiety and/or aziridinyl substituents; the former participates in redox cycling processes and oxygen radical production, whereas the latter is essential for the alkylating properties of these compounds. Purified NAD(P)H:quinone oxidoreductase reduced aziridinylbenzoquinones with different efficiency, and the redox transitions of the hydroquinone product were associated with production of oxygen-centered radicals (38). Metab-
AZQ are more positive than that of the O/O₂ couple (E = −156 mV), whereas that of BZQ (E(O₂/O⁻)) = −380 mV) (44) is more negative. The equilibrium constant (K_q) values for Reaction 3 are −10⁻² for DZQ and AZQ and 10⁸ for BZQ: this indicates that the first two quinones can be readily reduced by O₂ (k_HO > k_O₂) and that the facility of a O₂-driven reduction will be DZQ >> AZQ >> BZQ. Although this thermodynamic approach would suggest that (Q₂ > O₂) for DZQ and AZQ, these reactions are kinetically driven upon removal of O₂ from the equilibrium. Accordingly, semiquinones of DZQ or AZQ were not observed with HCT116 cell homogenates, whereas they were evident in less complex systems involving activation of the quinone by the purified enzymes in the absence of superoxide dismutase (38).

The effect elicited by N-acetyl cysteine on the EPR signals (Fig. 3) may be rationalized as the prevalence of a reductive pathway involving oxygen-centered radicals or the semiquinone. In both instances, a low steady-state level of oxyradicals would ensue. The reactivity of N-acetyl cysteine toward different radicals is partly dependent on the reduction potential of the radical: the second order rate constant for the reaction of aziridinylbenzoquinones may be activated to electrophilic species that cause DNA cross-links (42, 43). The cytotoxicity elicited by BZQ is supposed to involve the latter mechanism (31, 45).

In this study, we investigated the bioactivation of several aziridinylbenzoquinones and their ability to induce p21. We have shown that the metabolism of DZQ and AZQ by HCT116 cells was associated with formation of oxyradicals and an elevation of p21 mRNA levels, which correlated with inhibition of cell proliferation. BZQ, which was not a substrate for NAD(P)H:quinone oxidoreductase and which is expected to elicit cytotoxicity by causing DNA cross-linking, had little, if any, effect on cell proliferation and only slightly induced p21 in HCT116 cells.

Understanding the biochemical pathways leading to expression of p21 during quinone metabolism by cancer cells requires careful consideration of their bioactivation and functional group chemistry, as well as variations among different cell lines.

HCT116 cells are endowed with a high activity of NAD(P)H:quinone oxidoreductase (33). This enzyme reduces DZQ and AZQ (31, 38) (Table 1), but not BZQ. DZQ was largely reduced by a dicoumarol-sensitive activity associated with HO’ production. AZQ, on the other hand, was primarily reduced by one-electron transfer flavins (dicoumarol-insensitive activities) leading to a high production of O₂⁻ (Table 1 and Fig. 1).

The one-electron reduction potential (E(Q/Q⁻)) of DZQ and
cancer drug development. In this study we have established a correlation between an increased steady-state concentration of oxylipids and induction of p21 in HCT116 cells. These results suggest that the greater effectiveness in p21 induction by DQZ might be related to its efficient metabolism by NAD(P)H:quinone reductase. Although H$_2$O$_2$, a long-lived species, appears to be important as a cellular signal leading to p21 expression, it could be hypothesized that far more reactive and short-lived species, such as HO', may be a more immediate trigger for this response. This concept is supported by, on the one hand, the strong induction of p21 during the metabolism of DQZ, whose redox transitions produced mainly HO' and, on the other hand, the inhibitory effect of N-acylcycteine that reacts at diffusion-controlled rates with HO'. The occurrence of transition metals on the vicinity of molecules engaged in the regulation of the cell cycle machinery may be of utmost importance for the generation of this species in a site-specific manner.

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