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THE METABOLISM OF QUINONE-CONTAINING ALKYLATING AGENTS: FREE RADICAL PRODUCTION AND MEASUREMENT

Peter L. Gutierrez

The University of Maryland Greenebaum Cancer Center, University of Maryland School of Medicine, 655 W. Baltimore Street, Baltimore, MD 21201

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1. ABSTRACT

The metabolism of quinone-containing antitumor agents involves enzymatic reduction of the quinone by one or two electrons. This reduction results in the formation of the semiquinone or the hydroquinone of the anticancer drug. The consequence of these enzymatic reductions is that the semiquinone yields its extra electron to oxygen with the formation of superoxide radical anion and the original quinone. This reduction by a reductase followed by oxidation by molecular oxygen (dioxygen) is known as redox-cycling and continues until the system becomes anaerobic. In the case of a two electron reduction, the hydroquinone could become stable, and as such, excreted by the organism in a detoxification pathway. In some cases such as aziridine quinones, the hydroquinone can be oxidized by one electron at a time resulting in the production of superoxide, the semiquinone and the parental quinone. Quinone anticancer agents upon reduction can also set up an equilibrium between the hydroquinone, the parental quinone and the semiquinone which results in a long-lived semiquinone. Depending on the compound, aziridine quinones, for example, this equilibrium is long lasting thus allowing for the detection of the semiquinone under aerobic conditions. This phenomenon is known as comproportionation-disporportionation equilibrium. The series of reviews in this Special Issue address the consequences of bioreduction of quinone alkylators used in the treatment of cancer. In this particular review we are interested in describing the phenomenon of redox-cycling, how it is measured, and the biological consequences of the presence of the semiquinone and the oxygen radicals generated.

2. INTRODUCTION

There is a great number of anticancer agents that contain quinones in their structure (Figure 1). By virtue of their quinones, these agents can undergo a biochemical reduction by one or two electrons that is catalyzed by flavoenzymes in the organism using NADPH as an electron donor. This bioreductive process leads to the semiquinone and subsequent reactions with oxygen, all of which are believed to be responsible for most of the drug's activity (1-3). These findings have found applications in onco-pharmacology, beginning with the concept of bioreductive alkylation suggested by Sartorelli and coworkers (1). The process of reductive activation is the first step in the process of redox cycling because bioreduction leads to the semiquinone of the anticancer agent which, under aerobic conditions, is oxidized by molecular oxygen to the parent compound, a process that results in the concomitant production of superoxide radical anion (O_2^-). The formation of O_2^- is the beginning of a cascade that generates H_2O_2 and hydroxyl radicals ($^{\cdot}OH$), generally referred to as reactive oxygen species (ROS) (4, 5). To our knowledge, all quinone-containing antitumor agents, some of which are depicted in Figure 1, undergo redox cycling at different rates. This review addresses the phenomenon of redox cycling in detail, shows how it is measured, discusses the

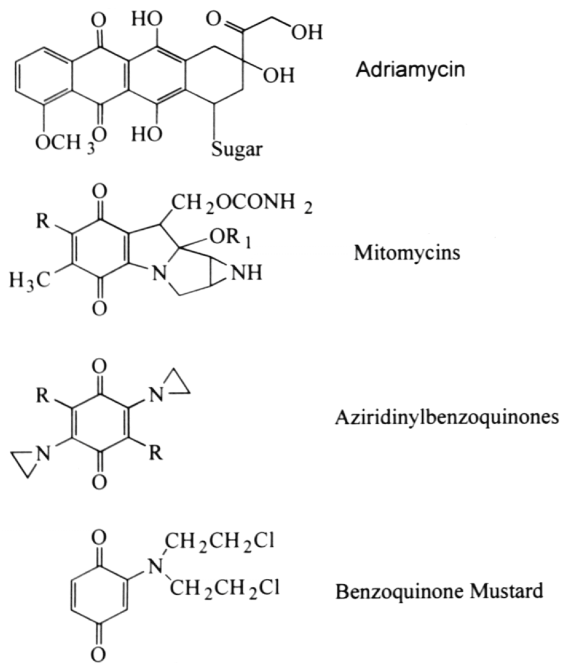


Figure 1. Chemical structure of some quinone anticancer agents used in the clinic.

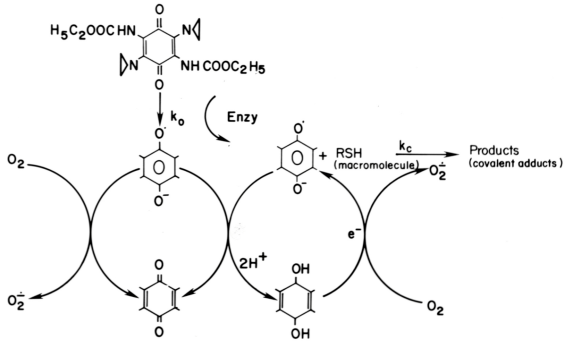


Figure 2. Redox cycling of diaziquone (AZQ) initiated by a one electron reduction. Also depicted are subsequent reactions between two semiquinone molecules, and subsequent pathways. (From reference 6, used by permission)

fate of the reduced species and the reactive oxygen species thus generated, and shows the effect that these redox products have on biological systems. This Special Issue concentrates on quinone alkylators, but in this review we will mention other important quinone-containing anticancer agents as we explain the redox cycling of these compounds.

3. WHAT REDOX CYCLING IS AND HOW TO MEASURE IT

The enzymes involved in redox cycling are, in general, flavoenzymes that use NAD(P)H as an electron source. The enzymes mediate the transfer of electrons to the quinone of the anticancer agent chemically reducing it to the semiquinone. Subsequent electron transfer to oxygen from the semiquinone results in the formation of $O_2^{\cdot-}$. This redox cycle continues until the system becomes anaerobic at which time the oxygen radical production decreases and the semiquinone begins to accumulate to detectable levels (Figure 2, left hand side).

The superoxide anion participates in a cascade facilitated by metal ions that results in the formation of H_2O_2 and hydroxyl radical ($^{\cdot}OH$). The bioreduction of diaziquone (AZQ, structure shown in Figure 2), and subsequent generation of ROS can be summarized by the following reactions:

$$AZQ + NADPH \rightarrow AZQ^{\cdot-} + NADP^{+} \quad 1$$
$$AZQH_2 + AZQ \rightarrow 2AZQ^{\cdot}H \quad 2$$
$$AZQ^{\cdot-} + O_2 \rightarrow AZQ + O_2^{\cdot-} \quad 3$$
$$2\,O_2^{\cdot-} + 2H^{+} \rightarrow H_2O_2 + O_2 \quad 4$$
$$M^{n+1} + O_2^{\cdot-} \rightarrow M^{n} + O_2 \quad 5$$
$$M^{n} + O_2^{\cdot-} + 2H^{+} \rightarrow M^{n+1} + H_2O_2 \quad 6$$
$$M^{n} + H_2O_2 \rightarrow M^{n+1} + {}^{\cdot}OH + {}^{-}OH \quad 7$$

A more complicated cycle for reduction and oxidation of a quinone anticancer agent can occur when the quinone is reduced by two electrons to the hydroquinone. Some hydroquinones, such as that of AZQ, can then be oxidized one electron at a time with the formation of the semiquinone and the parental compound respectively. Reduction by two electrons in general leads to detoxification, but not in the case of AZQ and its analogs. Two AZQ semiquinones can also disproportionate to form the parental compound and the hydroquinone. This creates an equilibrium (reaction 2, Figure 2) that renders the semiquinone rather stable and, depending on the compound, it can be detected under aerobic conditions, as is the case with AZQ. Diaziquone is one of a family of aziridinylquinones with anti-neoplastic activity (Figure 1), that have, in addition to redox properties, alkylating activity that is enhanced by a two electron reduction (7). The semiquinone of AZQ can be detected under aerobic conditions, whereas the anthracycline Adriamycin (Figure 1) is detectable only under anaerobic conditions. This is due to the ease with which AZQ and not Adriamycin undergoes reaction 2.

The redox cycling of AZQ mediated by whole cells is depicted in Figure 3. Here one can clearly see that the decrease of hydroxyl radicals trapped by DMPO (5,5-dimethyl-1-pyrroline-1-oxide) (DMPO-OH adducts, see Section 3.3.4) is accompanied by the increase in the AZQ semiquinone measured by electron paramagnetic resonance (EPR) (see Section 3.3.1) as the cell suspension becomes anaerobic.

Thus, there are four basic measurements that can be made which together will document the presence of redox cycling. These are: 1) the rate of oxidation of NADPH; 2) the measurement of oxygen consumption; 3) the detection of the semiquinone; and 4) the detection of reactive oxygen species (i.e. H_2O_2 and oxygen radicals).

3.1. Oxidation of NAD(P)H

The rate of oxidation of NAD(P)H is readily detected

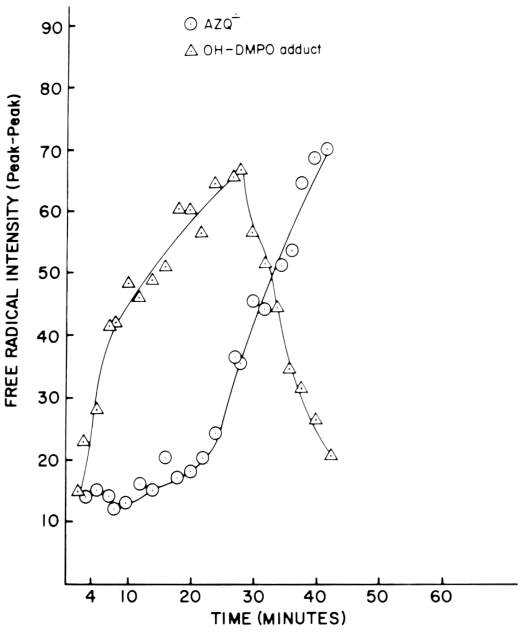


Figure 3. Time course of AZQ semiquinone and hydroxyl radical formation detected by Electron Paramagnetic Resonance (EPR) The signals were generated by murine leukemia cells P388 (10^7 cells/ml) in suspension exposed to 1mM AZQ. (Adapted from reference 8)

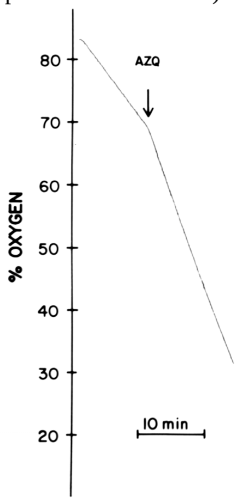


Figure 4. Oxygen consumption rate of whole murine P388 leukemia cells (10^7 cells/ml) in the presence of 1 mM AZQ.

and quantified at 340 nm with an extinction coefficient of 6.24 mM^{-1}. Table 1 shows the oxidation rates of NADPH during the redox cycling of Adriamycin in the presence of cytochrome c reductase. The effect of Vitamin E is shown as well as the positive control Menadione which competes with Adriamycin for electrons. The effect of the spin trap DMPO is also shown at concentrations where the semiquinone was quenched.

3.2. Oxygen Consumption Measurements

Oxygen consumption measurements are generally made with a Clark-type electrode in a Yellow Springs biological oxygen monitor. Rigorous measurements which include the determination of maximum pH and best cofactors for a particular enzyme result in V_{max} and K_M values. Measurements can also be performed in whole cells. Figure 4 shows one such measurement using a Yellow Springs instrument where whole cells were treated with AZQ eliciting the same redox cycle shown in Figure 3. The units of oxygen utilization are in either percent oxygen consumed /min/mg protein or quantified to nmoles/min/mg protein (10).

3.3. Detection of the Semiquinone and Reactive Oxygen Species

3.3.1 Semiquinone Detection

The semiquinone is detected and quantified by EPR. The EPR spectra of the semiquinone depends on the particular anticancer drug as well as experimental conditions. For instance, for Adriamycin or daunorubicin, one observes a singlet in aqueous biological systems (11-14), where the signal is relatively weak, but as organic solvents are added or reduction is achieved chemically, additional hyperfine lines are detected. These additional lines are sometimes enough to obtain EPR parameters such as hyperfine constants which allow to determine the electron's distribution in the molecule and thus characterize the free radical (15, 16). Similarly, the AZQ semiquinone consists of 5 EPR lines in aqueous solvents or biological incubations (Figure 5, second spectrum), but when reduced electrochemically in organic solvents, one obtains 11 EPR lines which allow for the complete characterization of this semiquinone (6, 17).

3.3.2. Superoxide Detection

The superoxide radical anion can be detected by spectrophotometric methods, such as the reduction of acetylated cytochrome c (19), and other colorimetric assays described by McCord (20), or by the EPR method of spin trapping. This latter method employs nitrones or nitroso compounds (spin traps) to scavenge transient radicals and thus produce more persistent nitroxyl radicals (spin adducts). The intensity and pattern of the EPR spectrum of the nitroxyl radical can then provide some information about the magnitude and identity of the trapped radical. In the case of $O_2^{\cdot-}$ the EPR spectrum is very distinctive as can be seen in Figure 6 for the reduction of AZQ by the enzymes contained in the S9 cellular fraction from the cancer cell line MCF-7 in the presence of the metal chelator DETAPAC. Adding superoxide dismutase to the system abrogates the spectrum of the trapped $O_2^{\cdot-}$. The dismutation of $O_2^{\cdot-}$ by superoxide dismutase (reaction 4) or catalyzed by metal ions (reactions 5 and 6) leads to H_2O_2 which can also be measured during redox cycling. The further decomposition of H_2O_2 leads to the formation of $^{\cdot}OH$ (reaction 7). Reactions 4, 5 and 6 are known as Fenton chemistry.

3.3.3. Hydrogen Peroxide Quantification

Hydrogen peroxide can by quantified by colorimetric assays such as the one described by Jiang (22). Table 2 shows the rate of H_2O_2 production that 20 microM AZQ and 5 micro M DZQ (R= H in the aziridinylbenzoquinone structure of Figure 1) can generate in microsomes isolated from MCF-7 cells supplemented with NADPH (23).

Table 1. Oxidation of NADPH in the presence of Adriamycin and cytochrome c reductase: effect of inhibitors*

Substrate or Inhibitor	NADPH Oxidized (micro Moles/min)
Adriamycin (1 mM)	51.5
Menadione (0.6 mM)	82.0
Vit E (2 mM)	3.2
DMPO (320 mM)	4.8
Enzyme and NADPH	0.0

*Modified from reference 9

Table 2. H_2O_2 production by AZQ and DZQ in icrosomes from human breast cancer cells MCF-7*

Drug	Conditions	nmole/mg/hr (H_2O_2)
AZQ	20 microM	640 ± 40
	20 microM + Catalase	32.5 ± 5.8
DZQ	5 microM	700 ± 0
	5 microM + catalase	25 ± 5.8

*****Modified from reference 23.

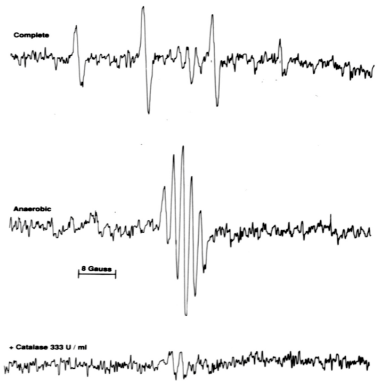


Figure 5. Electron Paramagnetic Resonance spectra recorded from a system of purified rat liver cytochrome c reductase (0.51 mg/ml), AZQ (200 microM), NADPH (500 microM) and DMPO (100 mM)

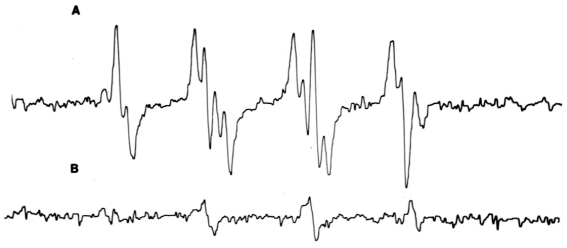


Figure 6. Electron Paramagnetic Resonance spectra of: A) AZQ (200 micro M), NADPH (500 micro M) and S9 cell fraction from MCF-7 cells in the presence of the metal chelator DETAPC (3 mM) and DMPO (100 mM) B) Same as A), except for 400 U/ml superoxide dismutase. (Modified from reference 21)

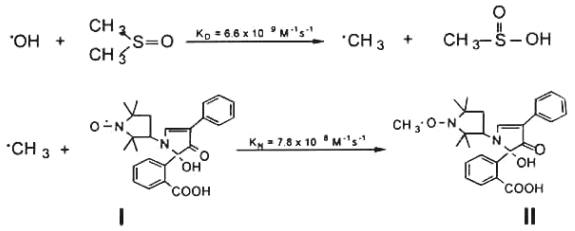
3.3.4. Hydroxyl Radical Detection and Quantification

Hydroxyl radicals which arise from H_2O_2 via Fenton chemistry (reaction 7) can also be detected by EPR using a variety of spin traps. For DMPO, the DMPO-OH adduct gives rise to a quartet of EPR lines with relative height ratios of 1:2:2:1 and hyperfine splittings (distance between the lines in gauss) of typically $A_N=A_H= 15$ Gauss (24). An example of the trapping of ·OH during the redox cycling of AZQ as it is reduced by cytochrome c reductase in the presence of NADPH is seen in Figure 5, first spectrum. The complete system under aerobic conditions yields the typical DMPO-OH adduct EPR quartet. Under anaerobic conditions, only the 5-line EPR signal from the semiquinone of AZQ can be observed. Catalase, which breaks down H_2O_2, abrogates the EPR signal from the DMPO-OH adduct (Figure 5 last spectrum) indicating the role of this oxidant on the formation of ·OH (reaction 7).

There are other methods that rely on the reactivity of ·OH radicals for their detection such as aromatic hydroxylation. This particular method uses high performance liquid chromatography (HPLC) to detect the hydroxylated products arising from the reaction of ·OH with salicylic or benzoic acids (e.g. 25).

Recently we have described a very sensitive and reliable method to quantify hydroxyl radicals in the nM range (26-28) This method employs the rapid reaction between ·OH and dimethylsulfoxide (DMSO) to produce a single carbon-centered radical (the methyl radical), which then reacts with a fluorescamine-derivatized nitroxide (I, Scheme I) to produce the stable O-methylhydroxylamine product (II, Scheme I). The O-methylhydroxylamine is then separated by reversed phase HPLC and quantified fluorometrically with an estimated detection limit of ~ 250 fmoles (26-28). Detailed HPLC methodology to quantify ·OH as well as procedures for the synthesis of I and II are given elsewhere (26,27). It suffices to say at this point that it was necessary to establish the conditions under which ·OH could be determined quantitatively. This involved determining the dependence of the formation rate of II on the concentration of DMSO, and also determining the dependence of formation rate of II on the concentration of I, taking into account the competition of oxygen for the methyl radical. This last radical is the one that reacts with I to produce II, the compound detected by HPLC (Scheme I). The concentration of II reflects the concentration of ·OH as shown in Scheme 1. Thus the rate of formation of II was demonstrated to be related to the rate of formation of ·OH by multiplying the former by a factor of 2.8 ±0.4 (26, 27).

This method was successfully applied to explore the production of ·OH in cells treated with AZQ concentrations as low as 20 micro M (28). The results from these experiments showed very interesting data. First, we were able to show that at concentrations of 20 microM and 200 microM AZQ, whole cells (mouse epidermal cells JB6 clone 41) were able to produce ·OH at rates of 0.071±0.01 and 0.84 ±0.05 nMs^{-}, respectively (28). This is significant because we can show that redox cycling occurs at concentrations of AZQ near to the range used in the clinic. For instance, AZQ is administered as a single dose at concentrations of 10 to 25 mg/ m^2 body area (29). In the case of 10 mg/ m^2 body area AZQ, this dose results in a plasma concentration of 1.7 microg/mL (4.7 microM) 2-3 minutes after drug infusion. Second, we found that ·OH is produced both extra- and intra-cellularly in the presence of AZQ indicating that this compound is being reduced in both



Scheme 1. Reactiions involved in the detection of hydroxyl radicals by the production of O-methylhydroxylamine (II) detected by HPLC.

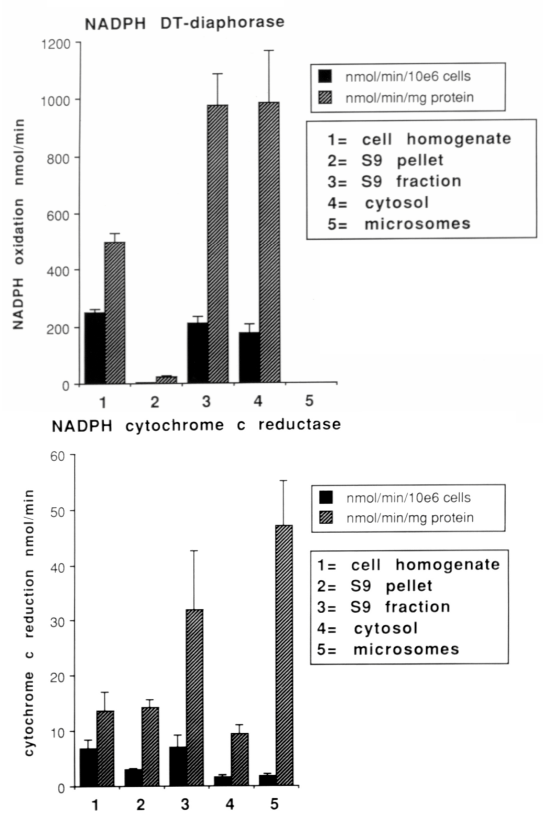


Figure 7. Relative activities of DT-Diaphorase and cytochrome c reductase in MCF-7 cell fractions.

Reactions involved in the detection of hydroxyl radicals by the production of O-methylhydroxylamine (II) detected by HPLC locales, thus acting as an internal and external source of superoxide and hydrogen peroxide. The incorporation of AZQ into the cells is consistent with the incorporation of ^{14}C-labeled AZQ in experiments detecting the accumulation/efflux of this compound in whole cells (30). Lastly, the role of metal ions is very important in many respects; for instance, one finds that superoxide acts to reduce oxidized trace metals, which then react with H_2O_2 to form ·OH (reactions 5 and 7). The significant increase in the rate of ·OH formation in the presence of added Fe-EDTA and a significant decrease in the presence of metal chelator DPTA is consistent with this finding. Further studies on Fe with this methodology have revealed that in the redox cycling of quinones, metal ions are a limiting factor in the production of ·OH. For instance, in the case of AZQ, ·OH production rates are linear up to 60 micro M in a reaction catalyzed by cytochrome c reductase in the presence of NADPH. In plots of ·OH production as a function of AZQ concentration, there is a curvature after 60microM AZQ suggesting that ·OH formation rates would eventually reach a plateau at high AZQ concentrations. This curvature was observed both in the presence and absence of enzyme, but disappeared when exogenous Fe(III)-EDTA was added (31). Thus, levels and locations of redox active metal ions can control the quantity of ·OH formed and therefore mediate oxidative stress induced by quinone anticancer agents upon redox cycling.

4. ENZYME SYSTEMS

As mentioned earlier, the metabolism of quinones in biological systems involves a one or two electron reduction. Some enzymes that catalyze a single electron reduction include: NADPH-cytochrome P450 reductase (E.C. 1.6.2.3) (32,33), NADH-cytochrome b_5 reductase (E.C. 1.6.2.2) (33), NADH:ubiquinone oxidoreductase (E.C. 1.6.5.3), and ferredoxin-$NADP^+$ reductase (E.C. 1.6.7.1) (34). A two electron reduction is catalyzed by NAD(P)H quinone acceptor oxidoreductase (E.C. 1.6.99.2)(NQOR, DT-Diaphorase) (34-36). There are also enzymes that catalyze simultaneously the reduction of quinones by one- and two-electrons, for example, oxygen oxidoreductase (E.C. 1.2.3.2) (37), NADH:lipoamide oxidoreductase (E.C. 1.6.4.3) (38) and xanthine dehydrogenase (39,409). Xanthine oxidase, is also able to reduce anthracyclines with the production of free radicals (41).

When studying redox cycling in whole cells and cell fractions it is important to ascertain which enzyme may be involved in the reduction of the quinone anticancer agent in question. Above is listed a wide variety of enzymes that can reduce/activate a particular drug and levels of these enzymes can vary with cell line. There is a whole area of research dealing with the enzymology of bioreduction (see Hodnick and Beall in this Special Issue). In the case of aziridine-quinones, two enzymes are important, namely, NADPH cytochrome c reductase (e.g. 14,32,42,43) and DT-Diaphorase (e.g. 21, 35,44-56). Figure 7 shows the activity of these two enzymes in different cell fractions from MCF-7 cells. As expected, DT-Diaphorase showed most of the activity in the cytosol and S9 supernatant fractions, whereas cytochrome c reductase showed most activity in microsomes and some in the S9 fraction. (S9 fraction contains both, cytosol and microsomes). That DT-Diaphorase is important in the bioactivation of aziridine-quinones is demonstrated by the diminished cytotoxicity of AZQ in BE human colon cancer cells which lack the enzyme (e.g. 53).

5. FATE OF THE REDOX CYCLING PRODUCTS

The fate of the reduced products semiquinone, hydroquinone and reactive oxygen species is important, for they are implicated in the toxicity/antitumor activity of quinones. In the case of the Adriamycin semiquinone,

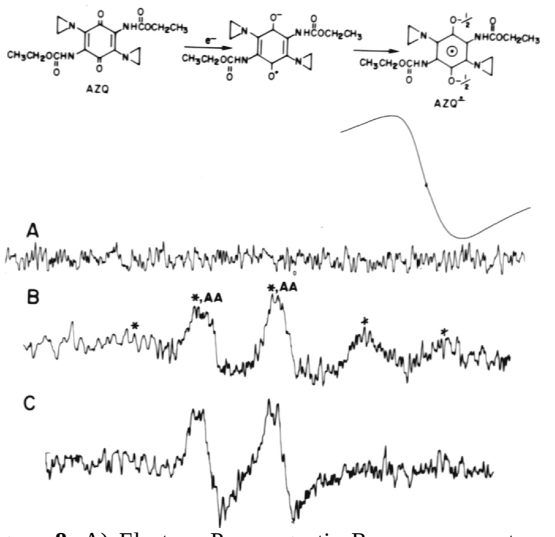


Figure 8. A) Electron Paramagnetic Resonance spectrum of red blood cells. B) EPR spectrum after treating the cells in (A) with 1 mM AZQ; a decrease in AZQ semiquinone (*) is indicated with a concomitant increase in ascorbyl radical (AA) C) Same as (B) except 16 minutes after adding AZQ. (Adapted from reference 58).

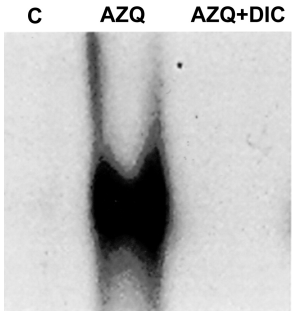


Figure 9. Effect of DT-Diaphorase on the induction of p53 in human MCF-7 breast cancer cells exposed to 20 micro M AZQ for 18 hours and its inhibition in cells exposed to 50 micro M dicumarol for 15 minutes prior to adding AZQ. (From reference 23, used by permission)

experiments have shown that the EPR shape of the singlet line change from the freely rotating molecule into a more restricted motion as the insoluble aglycone is formed and precipitates out of solution (11,57). Simultaneous quantitative measurements of aglycone production and Adriamycin semiquinone show a correlation suggesting that this semiquinone is involved in the cleavage of the glycosidic bond to form Adriamycin aglycone (9).

In the case of aziridine-quinones, the semiquinone of AZQ is quenched by ascorbic acid after AZQ's reduction in blood (Figure 8) (58,59). Thus in blood, the effects of oxidative stress caused by the redox cycling of AZQ may be minimized. The fate of the semiquinone and ROS is also intimately related to the damage that theses species can inflict to the cell. This will be discussed in the next section.

6. EFFECTS OF REDOX PRODUCTS ON BIOLOGICAL SYSTEMS

Most of the currently used antineoplastic agents elicit their effects by directly damaging cellular DNA or through mechanisms involving the inhibition of DNA synthesis, the mitotic apparatus, or topoisomerases (60). The variability observed in the effectiveness of chemotherapeutic agents probably reflects cell-type specific responses to DNA damaging agents.

6.1. Damage to DNA

In general, anthracyclines and other planar anticancer agents (Figure 1) tend to intercalate into DNA. To date there is no evidence that the semiquinone of these compounds is involved in this process, but it can generate ROS very close to DNA. On the other hand, quinone alkylators in general, as well as those whose alkylating properties are enhanced by enzymatic or chemical reduction, tend to cross link DNA and synthetic oligos at 5'-GN_nC or 5'-GC sites as in the case of aziridinyl-benzoquinones (61-64). For details see Hargreaves in this Special Issue.

Reactive oxygen species generated during the redox cycling of AZQ and DZQ can result in the formation of strand breaks (35, 65-67). The $^{\cdot}OH$ in particular can lead to the formation of the mutagenic lesion 8-OHdG in MCF-7 cells treated with H_2O_2 or AZQ (Table 3).

6.2.Gene Activation by Redox Cycling Aziridine Quinones

The generation of ROS during the redox cycling of aziridine quinones implicates these compounds in all the oxidative stress-related events that take place in the cell, for example, signal transduction, gene activation and DNA damage. For more detail on the effects of these quinones on gene activation and cell cycle, the reader is referred to the Cadenas, Review VII of this Special Issue. Here we will only mention the tumor suppressor gene p53. In general, DNA damaging agents were shown to increase p53 in part through an increase in the half life of this protein (68,69). There is evidence that AZQ, DZQ, and MeDZQ (R=methyl in aziridinylquinone structure of Figure 1) are able to increase p53 levels in MCF-7 cells, and that this induction depends on the drug concentration and on the time of exposure (23). Furthermore, this induction is also dependent on the bioreductive activation of these agents by DT-Diaphorase (Figure 9).

Redox cycling accounted for part of the p53 induction process, but alkylation, which is enhanced by reduction, also has an important role. This was ascertained by measuring the levels of p53 in MCF-7 cells after treatment with thiotepa, a compound that has three aziridines but no quinone moiety, or treatment with p-benzoquinone (p-BQ). Thiotepa was able to induce p53 at 50 micro M for 5 hr, while p-BQ had but a faint induction band at the same concentration and exposure time (23). Thus, the quinone and aziridine moieties act in concert to induce p53 in MCF-7 cells. Another very important result

Table 3. Levels of 8-OhdG in DNA from MCF-7 cells*

Treatment	Molar Ratio of 8-OHdG/10^5 dG
MCF-7 + 100 micro M AZQ, 4 hrs	7.6
MCF-7 + 100 micro M H_2O_2, 4 hrs	13.1
MCF-7 + catalase + 100 micro M H_2O_2	1.5
MCF-7 cells	1.0

*Gutierrez, P. and Sura, T. (unpublished data).

is that these aziridine quinones induced apoptosis that is independent of the p53 status of the cells (23).

7. PERSPECTIVE

Redox cycling is fundamental to the metabolism of quinone alkylating agents used in the treatment of cancer. The consequences of redox cycling may change with cell line, cellular pH, oxygen concentration, and with the type of tumor. Thus this process is at the core of drug development, gene activation, the enzymology of bioreduction, and cytotoxic and genotoxic effects, all of which are addressed in this Special Issue. In addition, the role of ubiquitous metal ions is important and can control not only the amount of hydroxyl radicals generated, but the site of their generation as well.

8. ACKNOWLEDGMENTS

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9. REFERENCES

1. Lin, A. J., Cosby, L. A., C. W. Shansky & A.C. Sartorelli: Potential bioreductive alkylating agents. 1. Benzoquinone derivatives. *J Med Chem* 15, 1247-1252 (1972)

2. Wilson, I. Wardman, P., T.S. Lin & A.C. Sartorelli: One-electron reduction of 2- and 6-methyl-1,4-naphthoquinone bioreductive alkylating agents. *J Med Chem* 29, 1381-1384 (1986)

3. Abdella, R. J. & J. Fisher: A chemical prespective on the anthracycline antitumor antibiotics. *Environ Health Prespec* 64, 3-18 (1985)

4. Sato, S. Iwaizumi, M., K. Handa & Y. Tamura: Electron spin resonance study on the mode of generation of free radicals of daunomycin, adriamycin and carboquone in NAD(P)H-microsome system. *Gann* 68, 603-608 (1977)

5. Bachur, N.R., S. L. Gordon & M.V. Gee: A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res* 38, 1745-1750 (1978)

6. P. L. Gutierrez: Mechanisms of bioreductive activation. The example of diaziquone (AZQ) *Free Radic Biol Med* 6, 406-445 (1989)

7. Fisher, G R., J. Donis & P. L. Gutierrez:, Reductive metabolism of diaziquone (AZQ) in the S9 fraction of MCF-7 cells. II. Enhancement of the alkylating activity of AZQ by NAD(P)H: Quinone-acceptor oxidoreductase (DT Diaphorase) *Biochem Pharm* 44, 1625-1625 (1992)

8. Gutierrez, P. L., Wilder, P.J., and Biswal, N. *In vitro* multidrug resistance of p388 murine leukemia selected for resistance to diaziquone. *Cancer Commun* (1989)

9. Gutierrez, P.L., M.V. Gee & N. R. Bachur: Kinetics of anthracycline antibiotic free radical formation and reductive glycoside activity. *Arch Biochem Biophys* 223, 68-75 (1983)

10. Kharasch, E. D. & R. F. Novak: Bis(alkylamino anthracenedione antineoplastic agent metabolic activation by NADPH-cytochrome P-450 reductase and NADH dehydrogenase diminished activity relative to anthracyclines. *Arch Biochem Biophys* 224, 682-694 (1983)

11. Kalyanaraman, B., E. Perez-Reyes & R. P. Mason: Spin-trapping and direct electron spin resonance investigations of the redox metabolism of quinone anticancer drugs. *Biochim Biophys Acta* 630, 119-130 (1980)

12. *Kalyanaraman*, B., K. M. Morehouse & R. P. Mason: An electron paramagnetic resonance study of the interactions between the Adriamycin semiquinone, hydrogen peroxide, iron-chelators, and radical scavengers. *Arch Biochem Biophys* 286, 164-170 (1991)

13. *Kalyanaraman*, B., R. C. Sealy, & B. K. Sinha: An electron spin resonance study of the reduction of peroxides by anthracycline semiquinones. *Biochim Biophys Acta 799, 270-275* (1984)

14. Bachur, N.R., Gordon, S.L., M. V. Gee,& H. Konh: NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. *Proc. Natl. Acad. Sci. USA* 76, 954-957 (1979)

15. Lown, J. W., & H.-S. Chen: Electron paramagnetic resonance characterization and conformation of daunorubicin semiquinone intermediate implicated in anthracycline metabolism, cardiotoxicity, and anticancer action. *Can J Chem* 59, 3212-3217 (1981)

16. Schreiber, J., Mottley, C., Sinha, B. K., Kalyanaraman & R. P. Mason: One-electron reduction of daunomycin, daunomycinone, and 7-deoxydaunomycinone by the xanthine/xanthine oxidase system: Detection of semiquinone free radicals by electron spin resonance. *J Am Chem Soc* 109, 348-351 (1987)

17. Gutierrez, P. L., Fox, B. M., M. M. Mossoba & N. R. Bachur: Free radicals in quinone-containing antitumor agents. Electrochemical reduction of diaziquone (2,5-diaziridine-3,6-bis(carboethoxyamino)-1,4-benzoquinone) and two analogues. *Mol Pharmacol* 26, 582-586 (1984)

18. Fisher, G. R., J. Donis & P.L. Gutierrez: Free radical formation and DNA strand breakage during metabolism of diaziquone by NAD(P)H quinone-acceptor oxidoreductase (DT-diaphorase) and NADPH cytochrome c reductase. *Free Radic Biol Med* 11, 597-607 (1991)

19. Azzi, A., C. Montecucco & C. Richter: The use of acetylated ferricytochrome c for the detection of superoxide radicals produced in biological membranes. *Biochem Biophys Res Commun* 65, 597-607 (1975)

20. J M McCord, J D Crapo & I Fridovich: Superoxide Dismutases Assays: A Review of Methodology, In: Superoxide and Superoxide Dismutase. Eds: Michelson A M, McCord J M, & Fridovich I, Academic Press, London, New York, San Francisco (1977)

21. Fisher & P. L. Gutierrez: The redutive 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 Biol Med* 10, 359-370 (1991)

22. Jiang, Z. Y., A. C. S. Wollard & S. P. Wolff: Hydrogen peroxide production during experimental protein glycation, *FEBS Lett* 268, 69-71 (1990)

23. Ngo, E.O., Nutter, L. M., T. Sura & P. L. Gutierrez: Induction of p53 by the concerted actions of aziridine and quinone moieties of diaziquone, *Chem Res Toxicol.* 11, 360-368 (1998)

24. G.R. Buettner: Spin trapping: ESR parameters of spin adducts, *Free Radic Biol Med* 3, 259-303 (1987)

25. Floyd, R.A., J. J. Watson & P. K. Wong: Sensitive assay of hydroxyl free radical formation utilizing high pressure liquid chromatography with electrochemical detection phenol and salicylate hydroxylation productions. *Biochim Biophys Acta* 10, 221-235 (1984)

26. Li, B., P. L. Gutierrez & N.V. Blough: Trace determination of hydroxyl radical in biological systems. *Anal Biochem* 69, 4295-4302 (1998)

27. Li, B., P. L. Gutierrez & N.V. Blough: Trace determination of hydroxyl radical using fluorescence detec*tion. Methods Enzymol* 300, 202-216 (1999)

28. Li, B., Gutierrez, P. L., P. Amstad & N. V. Blough: Hydroxyl radical production by mouse epidermal cell lines in the presence of quinone anti-cancer compounds. *Chem Res Toxicol* 12, 1042-1049 (1999)

29. Schilsky, R. L., Kelley, J. A., Ihde, D. C., Howser, D. M., R. S. Cordes & R. C. Young: Phase I trial and pharmacokinetics of aziridinylbenzoquinone (NSC 182986) in humans. *Cancer Res* 42, 1582-1586 (1982)

30. Egorin, M., Fox, B. M., Siegel, J. F., Gutierrez, P.L., Friedman, R. D., & N. R. Bachur: Cellular pharmacology in murine and human leukemic cell lines of diaziqiuone (NSC182986) *Cancer Res* 45, 992-999 (1985)

31. B. Li: Trace detection of hydroxyl radicals in biological systems. Doctoral Dissertation, University of Maryland College Park (1998)

32. Komiyama, T., T. Kikuchi & Y. Sugiura: Generation of hydroxylradical by anticancer quinone drugs, carbazilquinone, mitomycin C, aclacinomycin A and adriamycin, in the presence of NADPH-cytochrome P-450 reductase. *Biochem Pharmacol* 31, 3651-3656 (1982)

33. T. Iyanagi, & I. Yamazaki: One electron-transfer reactions in biochemical systems. III. One -electron reduction of quinones by microsomal flavin enzymes. *Biochim Biophys Acta* 172, 370-381 (1969)

34. T. Iyanagi, & I. Yamazaki: One-electron reductions in biochemical systems. V. Difference in the mechanism of quinone reduction by the NADH dehydrogenase and the NAD(P)H dehydrogenase (DT-diaphorase) *Biochem Biophys Acta* 216: 282-294 (1970)

35. Gibson, N.W., Hartley, J.A., Butler, J., D. Siegel, & D. Ross: Relationship between DT-diaphorase-mediated metabolism of a series of aziridinylbenzoquinones and DNA damage and cytotoxicity. *Mol Pharm* 42, 531-536 (1992)

36. Nutter, L.M., Ngo, E.O., G.R. Fisher, & P.L. Gutierrez: DNA strand scission and free radical production in menadione-treated cells: Correlation with cytotoxicity and role of NAD(P)H:quinone acceptor oxidoreductase. J *Biol Chem* 267, 2474-2479 (1992)

37. M. Nakamura, & I. Yamazaki: One electron transfer reactions in biochemical systems. VII. Changes in electron outlets in milk xanthine oxidase. *Biochim Biophys Acta* 327, 247-256, (1973)

38. M. Nakamura, & I. Yamazaki: One-electron transfer reactions in biochemical systems. VI. Changes in electron transfer mechanism of lipoamide dehydrogenase by modification of sulfhydryl groups. *Biochim Biophys Acta* 267, 249-257, (1972)

39. I. Yamazaki: Free radicals in enzyme-substrate reactions. In: Pryor, W.A., ed. Volume III *Free Radicals in Biology* New York: Academic Press, 183-215, (1977)

40. D.L. Gustafson & C.A. Pritsos: Enhancement of xanthine dehydrogenase mediated mitomycin C metabolism by dicumarol. *Cancer Res* 52, 6936-6939 (1992)

41. S.-S. Pan, & N.R. Bachur: Xanthine oxidase catalyzed reductive cleavage of anthracycline antibiotics and free radical formation. *Mol Pharmacol* 17, 95-99 (1979)

42. Gutierrez, P.L., R.D. Friedman & N.R. Bachur: Biochemical activation of AZQ [3,6-Diaziridinyl-2,5-

bis(carboethoxyamino)-1,4-benzoquinone] to its free radical species. *Cancer Treat Rep* 66, 339-342 (1982)

43. Keys, S.R., Francasso, P.M., Heimbrook, D.C., Rockwell, S., S.G. Silger, & A.C. Sartorelli A.C: Role of NADPH:cytochrome c reductase and DT-diaphorase in the biotransformation of mitomycin C. *Cancer Res* 44, 5638-5643 (1984)

44. I. D. Ordoñez, & E. Cadenas: Thiol oxidation coupled to DT-diaphorase-catalysed reduction of diaziquone. Reductive and oxidative pathways of diaziquone semiquinone modulated by glutathione & superoxide dismutase, *Biochem. J* 286, 481-490 (1992)

45. Beall, H.D., Mulcahy, R.M., Siegel, D., Traver, R.D., N.W. Gibson, & D. Ross, D.: Metabolism of bioreductive antitumour compounds by purified rat and human DT-diaphorase. *Cancer Res* 54, 3196-3201 (1994)

46. Plumb, J.A., M. Gerritsen, & P. Workman: DT-diaphorase protects cells from the hypoxic cytotoxicity of indoloquinone E09. *Br J Cancer* 70, 1136-1146 (1994)

47. Siegel, D., Gibson, N.W., P.C. Preusch, & D. Ross: Metabolism of mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* 50, 7483-7489, (1990)

48. Siegel, D., Beall, H., Senekowitsch, C., Kasai, M., Arai H., N.W. Gibson, & Ross, D.: Bioreductive activation of mitomycin C by DT-diaphorase. *Biochemistry* 31, 7879-7885, (1992)

49. Bailey, S.M., Lewis, A.D., Know, R.J., Patterson, L.H., G.R. Fisher, & P. Workman: Reduction of the indoloquinone anticancer drug EO9 by purified DT-diaphorase" A detailed kinetic study and analysis of metabolites. *Biochem Pharmacol* 56, 613-621, (1998)

50. Workman, P., Walton, M.I., M.C. Bibby, & D.A. Double: *In vitro* response of mouse adenocarcinoma of the colon (MAC) tumors to indoloquinone EO9: Correlation with bioreductive enzyme content. *Br J Cancer* 62, 515-516, (1991)

51. Ross, D., Siegel, D., Gibson, N.W., Pacheco, D., Thomas, D.J., M. Reasor, & D. Weirda: 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.

52. G.R. Fisher & P.L. Gutierrez: 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.

53. Siegel, D., Gibson, N.W., P.C. Preusch, & D. Ross: Metabolism of diaziquone by NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorse): Role in diaziquone-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* 50, 7293-7300, (1990)

54. Gibson, N.W., Hartley, J.A., Butler, J., D. Siegel, & D. Ross: Relationship between DT-diaporase-mediated metabolism of a series of aziridinylbenzoquinones and DNA damage and cytotoxicity. *Mol Pharm* 42, 531-536, 1992.

55. Fisher, G.R., J. Donis, & P.L. Gutierrez: Reductive metabolism of diaziquone (AZQ) in the S9 fraction of MCF-7 cells. II. Enhancement of the alkylating activity of AZQ by NAD(P)H:quinone-acceptor oxidoreductase (DT diaphorase) *Biochem Pharmacol* 44, 1625-1625, (1992)

56. G.R. Fisher, & P.L. Gutierrez: Free radical formation and DNA strand breakage during metabolism of diaziquone by NAD(P)H quinone-acceptor oxidoreductase (DT-diaphorase) and NADPH cytochrome c reductase. *Free Radic Biol Med* 11, 597-607 (1991)

57. Pan, et al., P. Gutierrez, & N. R. Bachur: Flavo-protein catalysis of anthracycline antibiotic reductive cleavage, free radical formation and free radical interconversions. *Fed Proc* 39, 310 (1980)

58. Gutierrez, P., Egorin, M., T. Davis & N. Bachur: The effects of ascorbic acid on biologically obtained diaziquone free radicals, *Biochem Pharmacol* 34, 2394-2397 (1985)

59. P.L. Gutierrez: The influence of ascorbic acid on the free radical metabolism of xenobiotics: The example of diaziquone, *Drug Metab Rev* 19, 319-343 (1988)

60. L.H. Hartwell & M.B. Kastan: Cell cycle control and cancer. *Science* 266, 1821-1828 (1994)

61. Alley, S.C., K.A. Brameld, & P.B. Hopkins: DNA interstrand cross-linking by 2.5-Bis (1-aziridinyl)-1,4-benzoquinone: Nucleotide sequence preferences and covalent structure of the dG-todG cross-links at 5'-d(GN_nC) in synthetic oligonucleotide duplexes. *J Am Chem Soc* 116, 2734-2741 (1994)

62. Berardini, M.D., Souhami, R.L., Lee, C.S., Gibson, N.W., J. Butler, & J. A. Hartley: Two structurally related diaziridinylbenzoquinones preferentially cross-link DNA at different sites upon reduction with DT-diaphorase. *Biochemistry* 32, 3306-3312 (1993)

63. Hartley, J.A., Berardini, M., Ponti, M., Gibson, N.W., Thompson, A. S., Thurston, D.E., B.M. Hoey, & J. Butler: DNA cross-linking and sequence selectivity of aziridinyl-benzoquinones: A unique reaction at 5'-GC-3' Sequences with 2,5-diaziridinyl-1,4-benzoquinone upon reduction. *Biochemistry* 30, 11719-11724 (1991)

64. Lee, C.-S., Hartley, J.A., Berardini, M.D., Butler, J., Siegel, D., D. Ross & N.W. Gibson: Alteration in DNA cross-linking and sequence selectivity of a series of aziridinyl-benzoquinones after enzymatic reduction by DT-diaphorase, *Biochemistry* 31, 3019-3025 (1992)

65. Szmigiero, L., Erickson, L.C., R.A. Ewig & K.W. Kohn: DNA strand scission and corsslinking by diaziridinyl benzoquinone (Diaziquone) in human cells and relation to cell killing, *Cancer Res* 44, 4447-4454 (1984)

66. King, C.L., W.N. Hittelman, & T.L. Loo: Induction of DNA strand breaks and cross-links by 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone in Chinese hamster ovary cells, *Cancer Res* 44, 5634-5637 (1984)

67. Gutierrez, P.L., Biswal, S., R. Nardino, & N. Biswal: Reductive activation of diaziquone and possible involvement of free radicals and the hydroquinone dianion. *Cancer Res* 46, 5779-5785 (1986)

68. Kastan, M.B., Onyekwere, O., Sidransky, D., B. Vogelstein, & R.W.: Craig: Participation of p53 protein in the cellular response to DNA damage, *Cancer Res* 51, 6304-6311 (1991)

69. Tishler, R B., Calderwood, S.K., C.N. Coleman, & B. D. Price: Increases in sequence specific DNA binding by p53 following treatment with chemotherapeutic and DNA-damaging agents. *Cancer Res* 53, 2212-2216 (1993)

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Send correspondence to: Dr Peter L. Gutierrez, The University of Maryland Greenebaum Cancer Center, University of Maryland, School of Medicine, 655 W. Baltimore Street, Baltimore, MD 21201, Tel: 410-328-3685, Fax: 410-328-6559, E-mail: pgutierr@umgcc.umaryland.edu

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