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Are reduced quinones necessarily involved in the antitumour activity of quinone drugs?

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Summary Several studies have shown that the antitumour quinones, adriamycin, mitomycin C, mitozantrone and 3,6-diaziridinyl-2,5-bis (carboethoxyamino)-1,4-benzoquinone (AZQ) can form semiquinone radicals *in vitro*. The biophysical chemistry data for these reactions and the properties of the radical quinones are presented together with the proposed mechanisms for cytotoxicity. An attempt has been made to correlate the available *in vitro* data for the quinones with each other and with the available *in vivo* data. As a consequence of these correlations a number of conclusions have been proposed regarding the techniques of investigation and the evidence for the role of reduced quinones in antitumour activity.

The National Cancer Institute (NCI) published a report in 1974 on the screening of fifteen hundred quinones for antitumour activity (Driscoll *et al.*, 1974). These quinones were tested on one or more of five animal tumours or for cytotoxicity towards KB cells. The total number of quinones tested to date exceeds two thousand. One of the objectives of the NCI study was to determine if there were any structure-activity relationships within the twelve major structural groups which ranged from simple benzoquinones to multiple heteroatom quinones. However, one of the main conclusions from this study was that the most active compounds were mitomycin C, the 3,6-diaziridinylbenzoquinones with 2,5 alkylamino substituents, adriamycin and daunomycin. The structures of the above drugs, together with those of mitozantrone and 3,6-diaziridinyl-2,5-bis (carboethoxy-amino)-1,4-benzoquinone (AZQ), are shown in Figure 1. Interestingly, mitomycin C and adriamycin were already being used in the clinic in 1974.

It can be seen from Figure 1 that the only apparent similarity in these structures is that they all contain a quinone group! Mitomycin C and adriamycin are secondary metabolites isolated from streptomyces strains. Mitozantrone and AZQ are synthetic and represent the most active analogues synthesised to date.

A considerable amount of research has gone into elucidating the molecular mechanisms of action of these antitumour quinones. It is evident that several mechanisms

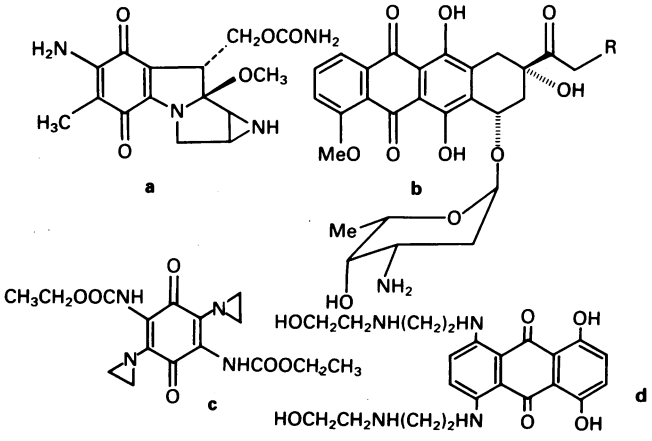

Figure 1 Structures of quinones: A = mitomycin C, B = adriamycin (R = OH), daunomycin (R = H), C = 3,6-diaziridinyl-2,5-bis-carboethoxyamino)-1,4-benzoquinone(AZQ), D = mitozantrone.

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are possible and a single mechanism cannot fully explain all of the cytotoxic effects.

The object of this short review is to evaluate the evidence on the involvement of reduced quinones in cytotoxicity. Where a particular mechanism has been proposed, attempts have been made to compare the implications of this mechanism on the other quinones. Although hundreds of publications have been produced in the last ten years on the role of reduced quinones in toxicity, the limited space restricts reference citation. However, emphasis has been made to ensure that the selection is unbiased. Unfortunately this restricts the number of definite conclusions that can be made.

Enzymic reduction

Quinones can be reduced by purified enzyme systems to form semiquinones ($Q^{\cdot -}$) and/or hydroquinones (QH_2). No specific quinone reductase has been isolated but certain NADH or NADPH enzymes can divert electron donation to a quinone.

$$Q + NAD(P)H + H^+ \rightarrow QH_2 + NAD(P)^+ \quad (1)$$

$$2Q + NAD(P)H \rightarrow 2Q^{\cdot -} + NAD(P)^+ + H^+ \quad (2)$$

Microsomal NADPH-cytochrome P-450 reductase, mitochondrial NADH dehydrogenase and cytosolic xanthine oxidase can all function as quinone reductases. It has also been demonstrated that a reductase in the nucleus can also reduce quinones (Bachur *et al.*, 1982). As reducing enzymes do not have to be selective, it is possible that other systems are awaiting discovery.

The one-electron reduction potentials of the antitumour quinones as measured by pulse radiolysis, are listed in Table 1. In view of the nature of reducing enzymes (e.g., non-reversibility, substrate specificity, association and multiple redox sites) it is not feasible to determine the reduction potentials of these systems. However thermodynamically the enzymes are expected to reduce the quinones in order of ease: AZQ > mitomycin C > adriamycin > mitozantrone. A good correlation has been observed between the one-electron reduction potentials and their ability to be reduced by NADPH cytochrome P-450 reductase (Svingen & Powis, 1981).* Similarly many reports have shown the relative difficulty of enzymically reducing mitozantrone as opposed

*Although we have not used the $E(Q/Q^{\cdot -})$ values from this publication (for criticism see Land *et al.*, 1983) we have little reason to exclude the enzyme data.

to adriamycin in purified enzyme systems (e.g., Doroshow & Davies, 1983) and even in human hepatocytes (Basra *et al.*, 1985). It can be shown using the values in Table I, that if the quinones were to come into equilibrium with the enzymes, then for the same concentration of enzyme and quinone, the concentration of $Q^{\cdot -}$ at equilibrium would be more than 2,000 times greater for adriamycin than for mitozantrone.

There is no clear evidence from clinical studies that the antineoplastic activity of these drugs is necessarily brought about by enzymic reduction (although in some cases the circumstantial evidence is accumulating). The main criticism of the *in vitro* studies is that detection of semiquinone radicals from enzymic reduction requires very high concentrations of the quinone. This is due to the relatively slow rates of formation of $Q^{\cdot -}$ by the enzyme and their much faster rates of decay:

$$Q \xrightarrow{k_1} Q^{\cdot -} \quad (3)$$

$$2Q^{\cdot -} + 2H^+ \xrightarrow{k_2} QH_2 + Q \quad (4)$$

The steady state concentration of the semiquinone radical is: $[Q^{\cdot -}]_S = \sqrt{(k_1[Q]/k_2)}$. Since the rate for formation of $Q^{\cdot -}$, as measured by NAD(P)H oxidation, are typically nmol min^{-1} mg^{-1} protein and the rate constants for disproportionation are typically $10^7 - 10^9\,M^{-1}s^{-1}$, it is not surprising that it is difficult to detect semiquinone radical intermediates at physiologically relevant drug concentrations. Free radical concentrations as low as 10^{-8} M can be detected using ESR, but even with this sensitive technique drug concentrations of $>50\,\mu M$ have had to be used to detect $Q^{\cdot -}$ in an enzyme system (e.g. Basra *et al.*, 1985; Gutierrez *et al.*, 1982). This concentration of adriamycin or mitozantrone represents at least a hundred fold increase over that required to produce a toxic response in a sensitive tumour cloning system (e.g. Cowan *et al.*, 1983). It has been proposed (Basra *et al.*, 1985) that high concentrations of drug may actually be achieved *in vivo* as the anthracyclines strongly bind to DNA and hence could accumulate in the nucleus. Unfortunately, there are still conflicting views on the possibility of DNA-bound drugs being enzymically reduced (e.g. Rowley & Halliwell, 1983; Kalyanaraman *et al.*, 1980).

Oxygen, lipid peroxidation, glutathione

From the reduction potentials in Table I and the one-electron reduction potential of the couple $E(O_2/O_2^{\cdot -}) = -155$ mV (Ilan *et al.*, 1976), it is possible to calculate the equilibrium constants for the reaction:

$$Q^{\cdot -} + O_2 \rightleftharpoons Q + O_2^{\cdot -} \quad (5)$$

These values are listed in Table I together with the forward and back rate constants of the equilibria based on direct measurements from pulse radiolysis (from $k(O_2^{\cdot -} + Q)$ for AZQ and $k(Q^{\cdot -} + O_2)$ for the other quinones) and the equilibrium constants.

All of the quinones lead to an increased oxygen consumption when reduced either by purified systems or by cell extracts from the heart or liver (e.g. Basra *et al.*, 1985; Kharasch & Novak, 1982; Doroshow & Davies, 1983). Interestingly, using the same systems, adriamycin has been shown to consume more oxygen (nM O_2 min^{-1} mg^{-1} protein) than mitozantrone (e.g. Basra *et al.*, 1985) whereas AZQ appears to consume more oxygen than adriamycin (e.g. Gutierrez *et al.*, 1982). This must be a reflection of the rate of semiquinone radical production rather than the position or rates of the equilibria with oxygen. It is also noteworthy that the ESR signal from AZQ semiquinones have also been detected in aerobic solutions (Gutierrez *et al.*, 1985). This would not be possible if the potential of AZQ was much more negative than -70 mV.

The increase in cellular superoxide radical production by these quinones is believed to overcome the cellular antioxidant capability of the cell including those provided by superoxide dismutase, catalase and the glutathione redox cycle. The main damaging species is the hydroxyl radical ($^{\cdot}OH$) which can be formed in a Fenton type reaction:

$$Q \rightarrow Q^{\cdot -} \quad (3)$$

$$Q^{\cdot -} + O_2 \rightarrow Q + O_2^{\cdot -} \quad (5a)$$

$$O_2^{\cdot -} + M^{(n+1)+} \rightarrow O_2 + M^{n+} \quad (6)$$

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + {}^{\cdot}OH + OH^- \quad (7)$$

The production of $^{\cdot}OH$ is more efficient under hypoxic conditions due to the direct reduction of the metal by $Q^{\cdot -}$ (Gutteridge *et al.*, 1984; Butler *et al.*, 1985):

$$Q^{\cdot -} + M^{(n+1)+} \rightarrow Q + M^{n+} \quad (8)$$

and possibly:

$$Q^{\cdot -} + H_2O_2 \rightarrow Q + {}^{\cdot}OH + OH^- \quad (9)$$

Reaction (9) is thermodynamically possible (Koppenol & Butler, 1985) and adriamycin/daunomycin radicals have been shown to react with rate constants of $10^4 - 10^5\,M^{-1}s^{-1}$ (Kalyanaraman *et al.*, 1984).

The production of $^{\cdot}OH$ by semiquinone radicals has been implicated by several workers as the main free radical mechanism for biological damage. The hydroxyl radical is strongly oxidising ($E(^{\cdot}OH, H^+/H_2O)_7 = 2.18$ V (Koppenol & Butler, 1985)) and can undergo $-H$ abstraction or $-OH$ addition with all biological components. There is therefore little point in listing the numerous publications that simply show that if $^{\cdot}OH$ is produced, it leads to biological damage.

A large proportion of the work on free radical production by quinones has been concerned with lipid peroxidation. The formation of lipid peroxide in membranes is one mechanism for cytotoxicity that could be related to antitumour activity and can certainly be related to anthracycline cardiotoxicity. The cardiotoxicity is cumulative and is presented by the rupture of the mitochondrial and sarcoplasmic reticulum

Table I One-electron reduction potential and oxygen equilibrium data found at pH 7 by pulse radiolysis for the quinones investigated

Quinone	$E(Q/Q^{\cdot -})/mV$	$k(Q^{\cdot -} + O_2)/10^8\,M^{-1}s^{-1}$	$k(O_2^- + Q)/10^6\,M^{-1}s^{-1}$	K[a]
AZQ	-70 ± 5	0.33 ± 0.3	905 ± 5	0.036
Mitomycin C	-310 ± 5	2.2 ± 0.2	0.52 ± 0.05	423
Adriamycin	-328 ± 5[b]	3.0 ± 0.2	0.35 ± 0.02	855
Mitozantrone	-527 ± 5	5.1 ± 0.5	0.0002	2.02×10^6

[a]Equilibrium constant, K, is for the reaction $Q^{\cdot -} + O_2 \rightleftharpoons Q + O_2^{\cdot -}$; [b] (Land *et al.*, 1983).

membranes. Adriamycin *in vitro* promotes lipid peroxidation (e.g. Kharasch & Novak, 1982) and this is demonstrated *in vivo* by an increase in rat heart lipid peroxides (Mimnaugh *et al.*, 1983). Various attempts have been made to modify this cardiotoxicity. These include the use of vitamin E or glutathione inhibitors and redox inactivated anthracyclines. Mitozantrone is generally considered to be less cardiotoxic than adriamycin and produces fewer lipid peroxides *in vitro* (e.g. Doroshow & Davies, 1983) and may even inhibit adriamycin induced peroxidation (Kharasch & Novak, 1982). This is consistent with the reduction potentials in Table I.

It has also been shown that methylene blue (MB) ameliorates adriamycin cardiotoxicity. It is proposed that MB is preferentially reduced in the heart and thus prevents lipid peroxidation (Hrushesky *et al.*, 1985). It is interesting that MB was used in this study as it is easily reduced and the resulting radical should be incapable of forming $O_2^{\bullet -}$, $E(MB/MB^{\bullet}) = 0.011$ V. As the antitumour activity of adriamycin was not affected by MB, it implies that this activity is not due to free radicals.

The glutathione redox cycle can protect against free radical damage. Inhibition of these enzymes in the presence of a quinone should therefore enhance radical damage, if the quinone functions by a free radical mechanism. Several studies have demonstrated this effect (e.g. Babson *et al.*, 1981). However, a more recent *in vitro* study (Capranico *et al.*, 1986) shows that depletion of glutathione has little effect on the cytotoxicity, mutagenicity or DNA damage, caused by adriamycin. These anomalies were attributed to the different concentrations of drug used. Once again, this is another example of where the biochemical/chemical estimates of damage have been assessed at concentrations which were more than 100 times that required to produce a physiologically relevant effect.

Bioreductive alkylation

This term was first used to describe a series of mechanisms by which a drug can be reduced to a reactive intermediate which can undergo nucleophilic addition (Moore, 1977). Quinone methides may be formed from semiquinones or hydroquinones of quinone drugs. A general, simplified mechanism is shown in Figure 2.

The hydroquinone of adriamycin *in vitro* undergoes loss of the daunosamine. The resulting methide can react with a proton to form 7-deoxyadriamycinone (Kleyer & Koch, 1984 and references therein). This process has been directly observed using pulse-radiolysis (Land *et al.*, 1985). It may be irrelevant whether the sugar is lost at the semiquinone or hydroquinone stage as $Q^{\bullet -}$ and $QH_2^{\bullet}$ can be in equilibrium (Land *et al.*, 1985). Quinone methide formation by anthracyclines has been extensively studied (for an excellent explanation and references see, Abdella & Fisher, 1985). To summarise briefly, quinone methide reactions occur over time scales of several seconds and are complex.

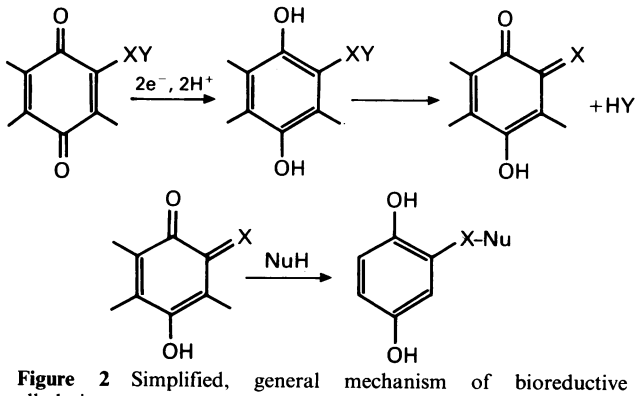

Figure 2 Simplified, general mechanism of bioreductive alkylation.

The hydroquinone of mitozantrone could undergo a type of bioreductive alkylation due to the 5,8 hydroxyethylamino groups. However, this has not been demonstrated. A HPLC study of the metabolites in urine has shown that mono- and dicarboxylic acid derivatives are formed (Ehninger *et al.*, 1985). It is difficult to envisage these metabolites being formed as a consequence of reduction.

Mitomycin C undergoes a pH dependent aziridine ring opening process upon reduction and several metabolites can be separated using HPLC. It has been proposed that the modifications occur at the $Q^{\bullet -}$ stage (Pan *et al.*, 1984) but pulse radiolysis and steady state irradiation studies show that rearrangements occur after $Q^{\bullet -}$ forms QH_2 by disproportionation (Hoey *et al.*, submitted). Mitomycin C is known to be a potent DNA alkylator (e.g. Dorr *et al.*, 1985 and references therein) and studies with polynucleotides show that the main site of attack is the N^2 position of guanine. However, the nucleotides must be present during reduction, showing that it is an intermediate and not a product that alkylates (Tomasz *et al.*, 1974).

AZQ undergoes acid assisted ring opening to form secondary amines. The isolated amines do not cross-link DNA but if DNA is present during the reaction, then cross-links do occur (King *et al.*, 1984). AZQ also produces cross-links in isolated nuclei at pH 7.0 in the presence of a reducing agent (Szmigiero & Kohn, 1984). This is reminiscent of mitomycin C, which only produces aziridine ring opening at pH 7 when reduced.

Unfortunately, bioreductive alkylation reactions occur as a consequence of intramolecular processes which are normally slow. Figure 3 shows two pulse radiolysis traces from our laboratory which illustrate the time scales over which hydroquinones undergo these reactions. Semiquinones and hydroquinones normally react with oxygen several orders of magnitude faster than those processes (see Table I) and

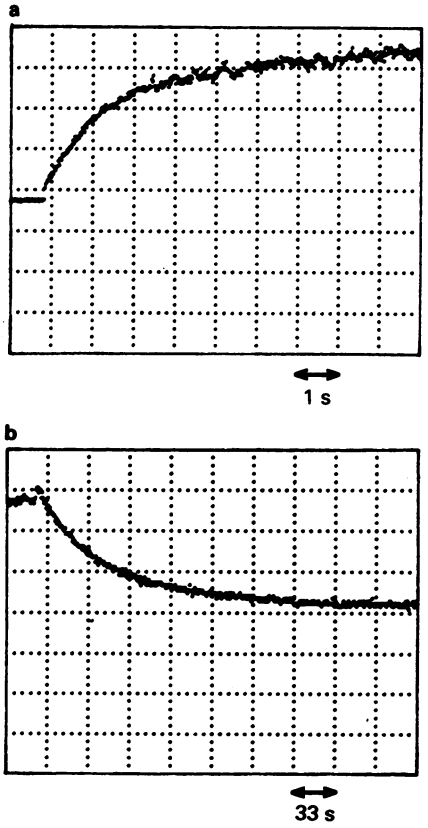

Figure 3 Pulse radiolysis of intramolecular rearrangement reactions: A: Reaction resulting in the loss of daunosamine from adriamycin, $\lambda = 475$ nm. B: Formation of a mitosene from mitomycin C, $\lambda = 510$ nm. The quinones were reduced to the hydroquinone using formate radicals.

hence minute traces of oxygen *in vivo* should prevent bioreductive alkylation. The importance of this mechanism can therefore be evaluated by the sensitivity of cells to the action of the drug in hypoxia. The results from hypoxic studies vary greatly throughout the literature, perhaps reflecting the concentrations of oxygen deemed as hypoxic by these different studies. Thus, there are several problems associated with correlating this data but it appears that adriamycin is not a hypoxic chemosensitizer in the strict sense whereas mitomycin C is (Kennedy *et al.*, 1983). Mitomycin C also shows a marked pH dependence in its cross-linking ability towards EMT6 cells (Kennedy *et al.*, 1985). The rate of intramolecular rearrangement of reduced mitomycin C has also been shown to be similarly pH dependent (Hoey *et al.*, submitted). Collectively these results show that there is reasonable evidence that mitomycin C undergoes bioreductive alkylation whereas adriamycin does not.

The semiquinone radical of AZQ can be in equilibrium with oxygen and superoxide radicals. The intracellular concentration of $Q^{\cdot -}$ and presumably, QH_2 could therefore depend on several factors including the activity of superoxide dismutase (Butler & Hoey, 1986). Unfortunately, at present, there is insufficient evidence available to make a firm judgement on the hypoxic chemosensitization of AZQ.

Sites of damage

The damaging or killing of cells by the drugs have been attributed mainly to membranal or DNA-related effects. The DNA effects can be summarized as (i) covalent and/or non covalent binding (ii) strand breaks and, (iii) disturbance of DNA repair systems. As a consequence of these effects, the cell can produce chromosomal aberrations, SCE's and chromatid breakages. If $^{\cdot}OH$ radicals are produced from reduction of the drugs, then all of these effects could occur since ionizing radiation can also cause these types of damage. A large proportion of the research into semiquinone radicals appears to assume that these are the only reasonable mechanisms of DNA damage. However binding to DNA could lead to perturbations in the DNA conformation which could be recognized by the repair enzymes such as polymerases or topoisomerases. The apparent DNA damage observed *in vitro* could be produced by the actions of these enzymes. A mechanism of this type has been used to explain why components which are not expected to be redox active such as the acridine derivative, AMSA or ellipticine can apparently produce single and double strand breaks.

One of the few definite conclusions to be found in this review is that mitozantrone should produce few, if any, free radicals. Yet mitozantrone apparently produces changes in the cell cycle (Traganos *et al.*, 1980) and forms SCE's and chromosomal aberrations in CHO cells (Nishio *et al.*, 1982). If this is considered together with the fact that mitozantrone affects the topoisomerase activity in human breast cancer cells (Crespi *et al.*, 1986), then free radicals may play no part in DNA damage. A similar argument can be derived for adriamycin except adriamycin does have the possibility to form some free radicals. It is unlikely that mitomycin C or AZQ can function simply by this mechanism as they do not efficiently bind to DNA unless activated.

Most of the work on membrane damage has been done on adriamycin and has been related to its cardiotoxicity. Concentrations of adriamycin as low as 0.1 μM can affect membrane properties of sarcoma 180, ascities cells (Murphree *et al.*, 1976). Another effect on membranes could be related to the high specific binding of adriamycin to cardiolipin. Although normally found in mitochondrial membranes, cardiolipin is also observed in other cellular membranes of transformed cells (Tritton *et al.*, 1978). Mitomycin C induces lipid peroxidation in mouse and rat pulmonary microsomes (a side effect of mitomycin C is pulmonary toxicity) and can be inhibited by SOD, catalase and chelators (Trush *et al.*, 1982). However, it is generally believed that lipid peroxidation is not involved in the antitumour activity of mitomycin C. AZQ has a high lipid solubility and a low ionization and hence could partition more easily into membranes but the other similarities between AZQ and mitomycin C would suggest that lipid peroxidation is not important for antitumour activity.

Conclusions

As stated previously, it is difficult to make any firm conclusions about the role of reduced quinones in the action of antitumour drugs. However, the hundreds of publications on quinone drugs demonstrate that an attempt should be made. Our conclusions are therefore:

1. The techniques and manipulations that are available at present are not sensitive enough to be able to state unequivocally that physiologically relevant concentrations of drugs give rise to free radicals which are involved in the antitumour activity. This statement may not be true for the cardiotoxicity or the pulmonary toxicity of the drugs.
2. Adriamycin, mitomycin C and AZQ could all be reduced *in vivo* to form semiquinone radicals. Mitozantrone is expected to produce few, if any, free radicals.
3. The available data suggests that the production of $O_2^{\cdot -}$ radicals by adriamycin and mitomycin C is not necessarily involved in the antitumour activity but could be involved in cardiotoxicity or pulmonary toxicity.
4. Adriamycin, mitomycin C and AZQ can undergo bioreductive alkylation reactions. These reactions are probably necessary for the action of mitomycin C and AZQ.
5. Bioreductive alkylation by mitomycin C should only occur under hypoxic conditions. This may not necessarily be true for AZQ.

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Discussion

Alper: When you talk of drugs being antitumour drugs, do you mean that they are drugs which kill or stop cells proliferating, because that's all that matters if you are trying to treat patients with cancer. You are not really talking about them being toxic to cycling cells? Second, if these are antitumour drugs are they preferentially toxic to cancer cells and not to normal cells?

Butler: The answers to your two questions are Yes and Yes.

Alper: So you are really concerned only with what basically matters and how these antitumour drugs work in cycling cells?

Butler: In a sense. Adriamycin is an established antitumour drug and does stop cells in certain parts of the cycle. I was just interested to show that it probably is not a free radical mechanism on the available data that we have got so far.

Adams: Can I just clear up this point about whether oxygen is involved or not in the toxicity? You referred to experiments in which cells were exposed to adriamycin in acute hypoxia, be they plateau phase or exponential phase, and there was no difference whatsoever in the cell killing efficiency of that drug, to when oxygen was present. Now people tend to invoke OH radicals produced by the reactions of oxygen. So in the absence of oxygen those kind of experiments, which have since been repeated, suggest to me that oxygen is not involved in the cytotoxic effect of adriamycin, be it cardiotoxicity or anti-tumour.

Butler: Cardiotoxicity – I am not so sure, because of the three pieces of evidence I have discussed.

Adams: I am talking about via oxygen. Oxygen is not necessary for adriamycin to kill or damage cells from the experiments so far done *in vitro*. It is important because you