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COMMENTARY

A Critical Evaluation of the Mechanisms of Action Proposed for the Antitumor Effects of the Anthracycline Antibiotics Adriamycin and Daunorubicin

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ABSTRACT. The mechanisms responsible for the antiproliferative and cytotoxic effects of the anthracycline antibiotics doxorubicin (Adriamycin®) and daunorubicin (daunomycin) have been the subject of considerable controversy. This commentary addresses the potential role of DNA synthesis inhibition, free radical formation and lipid peroxidation, DNA binding and alkylation, DNA cross-linking, interference with DNA strand separation and helicase activity, direct membrane effects, and the initiation of DNA damage via the inhibition of topoisomerase II in the interaction of these drugs with the tumor cell. One premise underlying this analysis is that only studies utilizing drug concentrations that reflect the plasma levels in the patient after either bolus administration or continuous infusion are considered to reflect the basis for drug action in the clinic. The role of free radicals in anthracycline cardiotoxicity is also discussed. BIOCHEM PHARMACOL **57**;7:727–741, 1999.

KEY WORDS. Adriamycin (doxorubicin); daunomycin (daunorubicin); free radical mechanisms; inhibition of topoisomerase II; DNA biosynthesis

The anthracycline antibiotic doxorubicin (Adriamycin®) and its congener, daunorubicin (daunomycin), have been in use for more than 30 years for the treatment of a variety of malignancies. Doxorubicin is used for the treatment of solid tumors such as those arising in the breast, bile ducts, endometrial tissue, the esophagus and liver, osteosarcomas, soft-tissue sarcomas and non-Hodgkin's lymphoma [1], while daunorubicin has utility primarily in acute myeloid leukemia [2]. Despite the extensive and long-standing clinical utilization of these drugs, their mechanism(s) of action is uncertain and has long been the subject of considerable controversy. A number of different mechanisms have been proposed for the cytostatic and cytotoxic actions of these agents. These include intercalation into DNA with consequent inhibition of macromolecular biosynthesis [3–23], free radical formation [24–40] with consequent induction of DNA damage [28–30, 33, 39, 41] or lipid peroxidation [42–45], DNA binding and alkylation [46–53], DNA cross-linking [54, 55], interference with DNA unwinding or DNA strand separation and helicase activity [56–62], direct membrane effects [63–70], and the initiation of DNA damage via the inhibition of topoisomerase II [71–90]. Finally, the anthracyclines have been shown to induce apoptotic cell death [91–95], although this is likely to be the final cellular response to upstream events such as inhibition of topoisomerase II.

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PROPOSED MECHANISMS OF DRUG ACTION

To evaluate the mechanisms of drug action that may be relevant to the clinical effectiveness of these agents, it is necessary to establish the actual drug concentrations that are achieved and/or sustained in patients undergoing treatment. At doses for bolus administration varying between 15 and 90 mg/m^2, the maximal initial plasma concentration detected was approximately 5 μM [96, 97], while the lowest reported concentration was approximately 0.3 μM [98]; generally, initial plasma concentrations fall into the range of 1–2 μM [99–104]. The plasma concentration declines rapidly, falling into the range of 25–250 nM within 1 hr—concentrations similar to those achieved and maintained by continuous infusion [97–101]. Therefore, a fundamental premise underlying this discussion is that while studies involving intact cells utilizing extracellular drug concentrations above 1 or 2 μM may provide information on *potential* mechanisms of drug actions, such studies are unlikely to reflect the mechanism of drug action associated with the clinical utilization of these antineoplastic drugs.

I. Interference with Macromolecular Biosynthesis

INHIBITION OF DNA SYNTHESIS IN THE TUMOR CELL. Some of the earliest studies describing possible mechanisms of action of the anthracyclines relate to the capacity of these drugs to inhibit DNA biosynthesis [3, 4, 10, 15], effects that may be related to DNA intercalation and/or inhibition of DNA polymerase activity [11, 12, 14, 16–18]. As early as 1965, Di Marco *et al.* [3] demonstrated inhibition of both DNA and RNA syntheses in HeLa cells over a concentration range of 0.2 through 2 μM daunorubicin. Similarly, Kim and Kim [7] reported a pronounced effect of Adriamycin on DNA synthesis in HeLa cells with inhibition evident at concentrations as low as 0.02 μM. Bremerskov and Linnemann [5] demonstrated that daunomycin is more effective in inhibiting DNA than RNA biosynthesis in mouse fibroblasts, with a maximal effect on DNA synthesis achieved at approximately 4 μM. In our own work, we have demonstrated that inhibition of DNA synthesis in breast tumor cells and rat hepatoma cells is observed over the concentration range of 0.1 through 5 μM [21, 23].

Studies by a number of investigators have failed to detect effects on DNA synthesis at the lower range of drug concentrations. Dano *et al.* [6] found that daunorubicin concentrations of at least 4 μM were required before effects on DNA synthesis were detected in Ehrlich ascites tumor cells. Studies by Meriwether and Bachur [8] and by Wang *et al.* [9] suggested that a concentration of at least 2 μM doxorubicin was required for inhibition of DNA and RNA syntheses in L1210 cells. Momparler *et al.* [13] also determined that elevated drug concentrations in the range of 2 through 100 μM doxorubicin are required for the inhibition of DNA and RNA syntheses in hamster fibrosarcoma cells, while Siegfried *et al.* [105] reported a dissociation between growth inhibition and interference with DNA synthesis in the S180 mouse sarcoma cell line, particularly at lower drug concentrations. Schott and Robert [22] also reported a dissociation between DNA synthesis inhibition and growth inhibition in drug-sensitive rat glioblastoma cells (although a close correspondence was observed in drug-resistant cells).

In contrast to these reports, Glazer *et al.* [18] reported that in the HT-29 human colon carcinoma cell line, the profile for loss of clonogenicity paralleled that for interference with DNA synthesis over a concentration range of 10^{-8} through 10^{-6} M. Wasserman *et al.* [20] determined that IC_{50} values were similar for growth inhibition and DNA synthesis inhibition in a human myeloid leukemic cell line, while studies from our own laboratory demonstrated a close correspondence between inhibition of DNA synthesis and of cell proliferation in both breast tumor cells [23] and H-35 rat hepatoma cells [21].

These contradictory findings make it difficult to reach a unifying conclusion regarding the involvement of DNA synthesis inhibition in the growth-inhibitory effects of the anthracyclines. It is possible that inhibition of DNA synthesis is an early transient signalling event that is a component of growth arrest related to the function of p53 [106]. There are at least two signalling pathways leading to inhibition of DNA synthesis through the up-regulation of the cyclin-dependent kinase inhibitory protein $p21^{waf1/cip1}$ in response to increased levels of p53 [107]. An elevation in $p21^{waf1/cip1}$ levels can inhibit DNA synthesis through the association of this protein with proliferating cell nuclear antigen (PCNA) [108] as well as through down-regulation of the activity of the transcription factor E2F [109], a protein with binding sites on the promoter regions of genes such as thymidine kinase, thymidine synthetase, dihydrofolate reductase, and DNA polymerase-α [110]. It is therefore feasible that the inhibition of DNA synthesis may relate to a cytostatic (and transient) component of drug action, while other effects of the anthracyclines on the tumor cell such as the inhibition of topoisomerase II (as discussed in a succeeding section) may be more closely associated with lethal effects of the anthracyclines.

II. Role of Free Radicals

The potential involvement of free radical generation in the cytotoxicity of the anthracyclines (both in terms of antitumor effects and cardiotoxicity) is complex and confusing. There is no question that under the appropriate conditions the chemistry of the anthracyclines lends itself to the generation of reactive free radicals [27, 35]. The quinone structure permits Adriamycin and daunorubicin to act as electron acceptors in reactions mediated by oxoreductive enzymes including cytochrome P450 reductase, NADH dehydrogenase, and xanthine oxidase [34, 111–113]. The addition of the free electron converts the quinones to semiquinone free radicals [25, 28], which may induce free-radical injury to DNA [28, 29, 33, 39] of themselves as well as after interaction with molecular oxygen to form superoxides, hydroxyl radicals, and peroxides [25, 27, 35, 37, 38]. The metabolism of the anthracyclines to deoxyaglycone derivatives in animal tumor models [52] and in humans [114] is consistent with reduction of the anthracyclines by one- and two-electron mediated reactions. There is further evidence for free-radical generation mediated through the formation of complexes between Adriamycin and iron [29–32]. The unresolved question is whether free radicals are generated at clinically relevant concentrations of the anthracyclines and at normal (i.e. hypoxic) oxygen tension in the tumor cell and whether such free radicals could be responsible for anthracycline toxicity to the tumor.

DETECTION OF FREE RADICALS. Various free radical species have been detected in cell-free systems at concentrations of Adriamycin between 100 and 300 μM and using techniques such as spin-trapping. Early studies by Winterbourn [115] provided evidence for the production of hydroxyl radicals in a xanthine/xanthine oxidase based experimental system over a concentration range of 30–120 μM.

Bachur *et al.* [25], using electron spin resonance, reported the augmentation of microsomal cytochrome P450-mediated oxygen consumption and semiquinone free radical formation with 500 μM Adriamycin; Graham *et al.* [34] reported similar results using purified cytochrome P450 reductase at 50 μM drug. Using a doxorubicin concentration of 200 μM, Sinha *et al.* [36] reported the production of Adriamycin semiquinone, superoxide anion, and the hydroxyl radical in subcellular fractions from breast tumor cells; the formation of these free radical species was blocked by various scavengers such as SOD,* catalase, DMSO, and glutathione peroxidase.

The generation of semiquinone free radicals of Adriamycin has been shown to result in the cleavage or degradation of deoxyribose and/or DNA. For instance, Lown *et al.* [24] demonstrated DNA breaks in a cell-free system after chemical reduction with 100 μM doxorubicin. Feinstein *et al.* [39] also used a doxorubicin concentration of 100 μM to detect hydroxyl radicals that could degrade DNA in a cytochrome P450 reductase-based enzyme system, while Gutteridge and Quinlan [33] demonstrated iron-dependent formation of hydrogen peroxide (using ferrodoxin reductase) and DNA damage in a cell-free model. Bates and Winterbourn [28] reported Adriamycin-mediated deoxyribose breakdown that could be blocked by SOD and catalase in a xanthine oxidase-dependent reaction at Adriamycin concentrations as low as 10 μM. In cell-free and enzyme-free experimental systems, Eliot *et al.* [29] and Muindi *et al.* [30] demonstrated iron-mediated DNA damage as well as hydroxyl radical generation and DNA damage by Adriamycin in the range of 10–30 μM drug.

Due in part to the limited sensitivity of assays for the detection of free radical generation, the above studies were performed in cell-free systems using supraclinical drug concentrations. Since the anthracyclines are known to be concentrated in the cell [116, 117], these elevated drug concentrations may be appropriate for studies using isolated organelles or enzyme preparations. However, studies of free radical generation in the intact cell are particularly worthy of attention. Benchekroun *et al.* [38] demonstrated free-radical formation in rat glioblastoma cells at 50–100 μM doxorubicin; however, these are concentrations of drug that are at least a log order higher than that which is routinely achieved in the clinic. Bustamante *et al.* [37] described the generation of hydrogen peroxide in MCF-7 cells after exposure to 0.1 μM Adriamycin. However, since these reactive oxygen species were detected after 9 days, hydrogen peroxide generation is unlikely to represent a primary response and instead may reflect a delayed metabolic response to other unidentified perturbations in cell function. Ubezio and Civoli [118] also reported hydrogen peroxide generation in human colon adenocarcinoma cells after an overnight incubation with doxorubicin; however, the lowest drug concentration for detection of hydrogen peroxide after 4 hr was 4 μM.

One consequence of the intracellular generation of reactive free radical species could be the induction of DNA damage, which, unlike that associated with inhibition of topoisomerase II (see below), would not be protein-associated. Studies by Potmesil *et al.* in L1210 leukemic cells demonstrated the production of non-protein-associated strand breaks by elevated concentrations of the anthracyclines [41]; furthermore, this DNA damage could be blocked by SOD, catalase, and DMSO. The protection from DNA damage by free-radical scavengers in these as well as other studies in the intact cell is not fully explained, as both SOD and catalase are relatively large proteins that should be incapable of crossing the membrane barrier and entering the cell. Protection by such agents may be indicative of free-radical generation at the cell surface, an issue that is addressed further in the sections on lipid peroxidation and on protection against free radical toxicity.

Studies in our own laboratory are consistent with the idea that free radicals may be generated in response to doxorubicin, but only at elevated drug concentrations. We have observed non-protein-associated (and presumably free-radical-mediated) DNA strand cleavage in MCF-7 breast tumor cells at 5 μM doxorubicin, while only protein-associated strand breaks (presumably resulting from the inhibition of topoisomerase II) were evident at lower drug concentrations [56].

LIPID PEROXIDATION. The generation of free radical species could lead to lipid peroxidation (primarily of the cell membrane); however, such lipid peroxidation would not indicate whether free radicals were being generated intracellularly or extracellularly. Many studies relating to lipid peroxidation have been performed using enzyme preparations, non-physiological conditions (high oxygen tension), or supraclinical concentrations of the anthracyclines. For instance, in studies by Kharasch and Novak [119], hepatic microsomes, cardiac sarcolemma, and cardiac mitochondria were utilized to assess lipid peroxidation under high oxygen tension and with a minimum doxorubicin concentration of 25 μM. Similarly, studies of lipid peroxidation by Griffin-Green *et al.* [42] in mitochondria and microsomes utilized 50 μM Adriamycin, those by Fukuda *et al.* [45] using liver microsomes involved 100 μM drug, while Banfi *et al.* [43] reported lipid peroxidation with millimolar concentrations of the anthracyclines in human platelets. Studies in hepatocytes by Babson *et al.* [120] demonstrated that depletion of GSH and glutathione reductase by 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) resulted in increased lipid peroxidation by doxorubicin; however, these studies were performed with 100 μM doxorubicin and in the presence of pure oxygen—a non-physiological environment that would promote superoxide generation.

While approaches using cell-free systems and/or elevated drug concentrations may provide evidence for the genera-

* *Abbreviations*: BSO, buthionine sulfoximine; and SOD, superoxide dismutase.

tion of free radicals and lipid peroxidation, these studies do not serve to indicate whether free radicals and lipid peroxidation play a role in anthracycline action in the intact cell. Benchekroun and Robert utilized both clinical and supraclinical concentrations of doxorubicin (in the range of 0.1 through 20 μM) to demonstrate lipid peroxidation in C6 rat glioblastoma cells and in MCF-7 breast tumor cells [44]; however, the extent of lipid peroxidation did not appear to change significantly over this range of concentrations. In a careful study in an animal model system, Cummings *et al.* [52] failed to detect enhanced lipid peroxidation after injection of doxorubicin into a subcutaneously growing rat mammary carcinoma.

In reviewing the data generated by these many investigators, one must conclude that there is insufficient evidence to implicate lipid peroxidation (through free-radical production at the level of the cell membrane) in the antitumor effects of the anthracyclines.

INFLUENCE OF THE GSH REDOX PATHWAY ON CELL SENSITIVITY TO DOXORUBICIN. One argument in support of the involvement of free radicals in doxorubicin toxicity is the influence of alterations in the level of GSH or enzymes of the GSH redox pathway on cell sensitivity to doxorubicin. However, virtually all of the studies showing sensitization to doxorubicin through depletion of cellular GSH do so at elevated concentrations of drug. Hamilton *et al.* [121] reported that human ovarian cancer cell lines were sensitized to doxorubicin by depletion of cellular glutathione using buthionine sulfoximine, which inhibits γ-glutamylcysteine synthetase; unfortunately, these studies did not indicate the concentrations of doxorubicin utilized. Dusre *et al.* [122] demonstrated that depletion of cellular GSH sensitized multidrug-resistant MCF-7 breast tumor cells to doxorubicin over a concentration range of 5 through 25 μM and that hydroxy radical formation was increased at doxorubicin concentrations of 200 μM. However, it is not surprising that free radicals would be generated at these elevated drug concentrations. Raghu *et al.* [123] reported that resistance to doxorubicin in a subline of HL-60 leukemic cells was reversed by BSO; however, the change in sensitivity occurred over the concentration range of 17.6 through 60 μM, elevated drug concentrations consistent with the generation of free radicals. Lai *et al.* [124] demonstrated the reversal of resistance to doxorubicin by BSO in some colon cancer lines using doxorubicin concentrations in the range of 40–90 μM, while Nair *et al.* [125] reported sensitization of a resistant subline of P388 murine leukemic cells, with a change in the IC_{50} from 9.3 to 2.4 μM. Again, these findings may reflect the protective effect of GSH-associated enzymes at the higher range of drug concentrations where free radical generation is feasible.

Other studies have failed to demonstrate protective effects of GSH redox systems. Work by Ford *et al.* [126] stands in direct opposition to the studies of Dusre *et al.* [122] in that these investigators failed to detect sensitization of MCF-7-ADR cells to doxorubicin by BSO over the entire doxorubicin concentration range of 0.1 through 100 μM. Depletion of cell GSH by BSO also failed to influence human myeloma cell sensitivity to doxorubicin over the concentration range of 10^{-9} through 10^{-5} M [127] or to sensitize C6 human glioblastoma cells to doxorubicin over an extraordinarily broad range of concentrations (nanomolar through millimolar) [128].

In a comparison of two Chinese hamster ovary cell lines with an equivalent capacity to generate oxygen free radicals [129], there was no difference in sensitivity to doxorubicin (in the concentration range of 5–20 μM) despite the fact that one cell line was characterized by elevated levels of GSH and glutathione peroxidase, as well as SOD and catalase. Liebmann *et al.* [130] demonstrated that transfection of MCF-7 wild-type cells with glutathione peroxidase, an enzyme that decreases free radical formation by metabolism of hydrogen peroxide, failed to alter cell sensitivity to doxorubicin, although there was clear evidence for protection from radiation—which is known to be cytotoxic through free radical generation. Transfection with glutathione transferase also failed to promote resistance to doxorubicin in MCF-7 cells upon continuous exposure to drug concentrations in the nanomolar through submicromolar range [131]. Suppression of glutathione peroxidase activity and GSH redox cycling also failed to influence sensitivity of HL-60 human leukemic cells to daunorubicin [132].

PROTECTION AGAINST FREE RADICAL-MEDIATED TOXICITY. In the studies by Potmesil *et al.* in L1210 leukemic cells [41], the DNA strand breaks observed in the tumor cell exposed to elevated concentrations of doxorubicin could be prevented using catalase and SOD. However, as indicated above, these data raise some intriguing questions. These relatively large proteins would have difficulty in crossing the cell membrane and gaining access to the cell interior. It might be proposed that free radicals that have been generated intracellularly somehow avoid being quenched by intracellular proteins and reducing biomolecules and manage to diffuse to the cell surface where they can be inactivated by such agents as SOD and catalase. An alternative and perhaps more reasonable hypothesis is that free radicals are generated at the cell surface, presumably by membrane-mediated metabolism.

Only a few studies implicating the generation of free radicals in anthracycline antibiotic toxicity have utilized clinically relevant concentrations of doxorubicin. Doroshow [133, 134] demonstrated that catalase and SOD protected both MCF-7 breast tumor cells and L1210 leukemic cells from the cytotoxicity of doxorubicin at concentrations in the submicromolar range. However, the question of access of these biomolecules to the cell interior has not been resolved. More recently, Doroshow *et al.* [135] reported that scrape loading of MCF-7 cells with glutathione peroxidase or SOD protected MCF-7 cells at a doxorubicin concentration of 1.75 μM. Cervantes *et al.* [136] reported that *N*-acetylcysteine and DMSO, but not SOD or catalase protected A2780 cells from doxorubicin concentrations of

0.2 and 0.5 μM, supporting a role for hydroxyl radicals but not superoxide anions or hydrogen peroxide in the antitumor activity of doxorubicin. While Sinha *et al.* [137] demonstrated protection from doxorubicin toxicity by catalase or SOD in MCF-7 cells continuously exposed to a doxorubicin concentration of 10 nM, these chronic drug exposure studies may involve an alternative mode of action associated with differentiation-induction [37, 138].

One possible criticism of even those studies where proteins such as SOD or catalase are protective is that these effects cannot be proven to be specific for free radicals because of the high concentration of the protective species utilized [139]. Furthermore, in contrast to the reports by Doroshow's group, other investigators have failed to demonstrate protection against doxorubicin toxicity using SOD, catalase, and glutathione peroxidase [129–132, 140]. In a recent review, Keizer *et al.* [139] concluded that free radicals do not contribute significantly to anthracycline toxicity in the tumor cell. This conclusion is supported by two additional studies. Lenehan *et al.* [132], *using daunorubicin concentrations that fall well within the clinically relevant range*, reported that inhibition of catalase in HL-60 leukemic cells sensitized the cells to both hydrogen peroxide and *tert*-butyl hydroperoxide, but not to daunorubicin. Furthermore, in a study where Chinese hamster V79 cells were protected from the cytotoxic actions of the semiquinone of streptonigrin by the *cell permeable antioxidant* 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, there was no protection from either strand break induction or the cytotoxicity of doxorubicin [140]. In addition to the fact that DNA-binding anthracyclines such as 5-iminodaunorubicin, which cannot undergo redox reactions to generate free radicals, have been shown to be cytotoxic [73, 141], perhaps the most compelling evidence dissociating anthracyclines and free radical mediated toxicity in the tumor cell is the capacity of the iron-chelating agent (+)-1,2-bis (3–5-dioxopiperazinyl-1-yl) propane (ICRF-187) to provide *selective* protection to cardiac tissue (as described below).

FREE RADICALS AND CARDIOTOXICITY. It appears to be fairly well accepted that cardiotoxicity of the anthracyclines [142, 143] is associated with the generation of free radicals, and that cardiotoxicity involves interference with mitochondrial function and/or lipid peroxidation. An extensive series of reports from the laboratory of Doroshow [144–146] demonstrated the production of superoxide, hydrogen peroxide, and hydroxyl radicals mediated by cardiac mitochondrial NADH dehydrogenase and the generation of doxorubicin and daunorubicin semiquinone free radicals. Demant [147] reported that doxorubicin interferes with cytochrome *c* oxidase activity in pig heart submitochondrial particles, while Sokolove [148] has suggested that it is the Adriamycin aglycone metabolites that are responsible for disruption of mitochondrial function and cardiotoxicity.

A role for free radicals in the cardiotoxicity of the anthracyclines is further supported by work in intact cells and tissue. Myers *et al.* [149] reported that the free radical scavenger α-tocopherol (vitamin E) could protect mice against doxorubicin-induced cardiomyopathy without affecting the capacity of doxorubicin to interfere with DNA synthesis in the tumor cell. Rajagopalan *et al.* [150] demonstrated that hydroxy free radicals are generated in perfused rat heart at concentrations below and up to 1 μM. However, it must be emphasized that the fact that formation of these radicals was blocked by SOD, catalase, and ICRF-187 was taken to indicate that free-radical formation is occurring *outside of the cell.* Paracchini *et al.* [151] demonstrated that a spin-trapping agent could protect against the myelotoxicity and cardiotoxicity of doxorubicin without interfering with antitumor toxicity. Quite recently, Yen *et al.* [152] performed a series of studies in transgenic mouse models expressing increased levels of functional human manganese SOD in cardiac mitochondria. These animals demonstrated an unequivocal protection from doxorubicin cardiotoxicity after treatment with a clinically relevant dose of drug for 5 days. Finally, one of the strongest arguments in support of a role for the generation of free radicals in Adriamycin cardiotoxicity (and for dissociating cardiotoxicity from its antitumor effects) are the animal and clinical studies showing protection from cardiotoxicity without a reduction in antitumor effectiveness by the iron-chelating agent ICRF-187 [153–155] and potentiation of antitumor toxicity by ICRF-187 [156].

Additional studies in heart tissue that might appear to support a role for doxorubicin-mediated free radical generation in cardiotoxicity are undermined by the problems of supraclinical concentrations of drug or incompleteness of the findings. Lee *et al.* [157] demonstrated increased lipid peroxidation, loss of capacity to contract, and ultrastructural changes in papillary muscles by Adriamycin that could be prevented, at least in part, by catalase and mannitol; however, the Adriamycin concentration utilized in these studies was 100 μM. Mimnaugh *et al.* [158–160] reported the enhancement of lipid peroxidation in mouse heart and in mouse and rat livers, which could be diminished using free-radical scavengers; however, these studies were performed using concentrations of Adriamycin in excess of 300 μM, a concentration of drug that exceeds the clinically relevant dose by two orders of magnitude. Thayer [161] reported an increase in serum lipids in rats after chronic treatment with relevant doses of Adriamycin, but the source of these lipids was not identified.

It must be mentioned that certain studies appear to undermine the role of free radicals in Adriamycin cardiotoxicity. For instance, Julicher *et al.* [162] failed to detect lipid peroxidation even in vitamin E-deficient rat hearts at the extraordinarily high doxorubicin concentration of 100 μM in the perfusion medium. Demant and Wasserman [163] failed to detect alterations in lipid metabolism of cultured myocytes at clinically relevant doses of doxorubicin. Jackson *et al.* [164] concluded that free-radical induced damage was unlikely to be the primary mechanism of cell injury in Adriamycin cardiotoxicity in a rabbit model,

based on such observations as the lack of production of malondialdehyde or ethane gas both *in vitro* and *in vivo*.

A thoughtful analysis of this issue by Olson and Mushlin [165] notes a number of limitations to the free-radical hypothesis of cardiac injury, among which are the failure to demonstrate protection from physiological or biochemical effects of doxorubicin in the heart by vitamin E or *N*-acetylcysteine [155, 166]. However, these arguments appear somewhat tenuous in view of other reports, which clearly indicate that such protection does occur [149, 167].

Consequently, despite the reservations noted above, the overall weight of the current evidence appears to be consistent with a fundamental role for free radicals in the cardiotoxic actions of the anthracyclines.

III. DNA Adduct Formation and DNA Cross-Linking

Studies from the laboratory of Phillips have reported the induction of DNA adducts in cell-free systems, both enzyme mediated and in the absence of metabolic activation, generally using a drug concentration of 10 μM [49, 50, 53]. Cullinane and Phillips [50] observed the formation of Adriamycin adducts with both single-stranded and double-stranded DNA and hypothesized that DNA binding occurred via a quinone methide intermediate. Cummings *et al.* have also reported the covalent binding of doxorubicin to DNA in the cell at concentrations of 50 μM [51] and 1 mM [52]. Studies from the Sinha laboratory [46, 47] and by Wallace and Johnson [48] also support the enzymatic or chemically activated binding of anthracyclines to DNA; again, these studies generally involved drug concentrations in the millimolar range.

The possibility that DNA binding could lead to DNA cross-linking has also been explored, but again this work has generally taken place at elevated drug concentrations. Cullinane *et al.* [53] reported the non-enzymatic formation of DNA interstrand cross-links in a cell-free system over a period of 1–2 days using an Adriamycin concentration of 10 μM, and suggested that the reaction intermediate is a quinone methide. Skladanowski and Konopa [54] demonstrated a correlation between the EC_{50} values of various anthracycline derivatives and their capacity to produce DNA cross-links in HeLa cells; while these studies indicate the potential for DNA cross-linking to mediate drug action, the cross-links were generally detected only at concentrations that far exceed the EC_{90} values for these drugs and, with the notable exception of idarubicin, at concentrations that are not achieved in the clinic.

Additional, albeit indirect, evidence for the potential importance of DNA cross-links is provided by the observation that fewer cross-links are evident in drug-resistant cells as compared with drug-sensitive cells, even at equivalent intracellular concentrations of drug [55]. Further indirect evidence for the formation of reactive DNA-binding intermediates of the anthracyclines is provided by the observation that BSO sensitizes these cells to Adriamycin and daunorubicin with a concomitant increase in sensitivity to cross-link induction [55]; however, the fact that these changes occur in parallel is not unequivocal evidence for a cause-and-effect relationship.

While these findings are consistent with an alternative mode of drug action, their significance awaits verification by studies demonstrating a correspondence between DNA cross-linking and cytotoxic effects in the intact cell at clinically relevant concentrations of the anthracyclines.

IV. Interference with DNA Strand Separation and DNA Helicase

Studies in our own laboratory have demonstrated that low concentrations of doxorubicin interfere with DNA unwinding in MCF-7 breast tumor cells [56]. This finding could potentially be related to the induction of DNA interstrand cross-links described above, although the concentration of drug utilized in our own studies is two orders of magnitude lower than that reported for cross-link production [54, 55]. Alternatively, interference with DNA unwinding (or DNA strand separation) could be related to drug effects at the level of helicases, as reported by a number of laboratories.

Perhaps the earliest reports relating to drug interaction with helicase activity involved inhibition of bacteriophage T4 DNA ligase by various anthracyclines [57, 59]. More recent work by Bachur's group substantiates these observations [58, 60, 62] and speculates that the increase in overall duplex DNA stability at the G–C sites may prevent the helicases from separating the DNA strands, thereby interfering with helicase action [58, 60]. Although these studies involve the isolated enzyme system, it is interesting that the concentrations utilized to block helicase activity fall well within the clinically relevant range. These findings are further supported by a recent report from Tuteja *et al.* [61], demonstrating inhibition of both the unwinding and ATPase activities of purified human helicase II.

These reports are intriguing and raise the possibility of an alternative target for the anthracyclines. However, additional verification in intact cell systems at clinically relevant drug concentrations are necessary before the helicases can be assumed to be primary targets of drug action.

V. Membrane-Mediated Effects

The oft-quoted hypothesis for Adriamycin action at the membrane surface is based on a number of reports of Adriamycin interaction with real and artificial membranes [63–70]. Tritton and Yee [65] reported that Adriamycin coupled to an insoluble support that could not traverse the membrane was more toxic than free drug. In a subsequent report, Lane *et al.* [68] demonstrated that drug cytotoxicity was not evident below an incubation temperature of approximately 22° and concluded that there is a dissociation between drug uptake and cytotoxicity. However, drug accumulation by the cells at 0° appeared minimal; further-

more, a careful examination of the data presented in the first figure of this paper [68] leads to an alternative interpretation (if one assumes a reasonable confidence interval around each data point) supporting a direct correspondence between the extent of drug uptake and loss of cell survival.

The relative importance of intracellular and extracellular drug in the cytotoxicity of doxorubicin to L1210 leukemic cells was addressed further in a study by Vichi and Tritton [70]. In this work, shifting cells from 0° to 37° was shown to promote drug toxicity, whereas a shift to 0° blocked the toxic effects of the drug. The authors' argument that drug must be present at the cell exterior to cause cytotoxicity was based on two sets of experiments. In one series of studies, cells were exposed to drug at 0° and then suspended in medium at 37° either with or without a prior washing step. The lack of toxicity with a washing step was interpreted to indicate a requirement for drug at the cell surface; however, drug at the cell surface could easily be taken up into the cell and into the nucleus upon resuspension at 37° permitting interaction with intracellular targets. Unfortunately, no measurements were reported relating to *intracellular* drug concentrations after resuspension at 37° (i.e. at a time when drug interaction with an intracellular target could have been initiated).

In the second series of experiments [70], high levels of DNA in the medium were shown to prevent drug toxicity, presumably through binding of cell-surface associated drug to the exogenous DNA. However, these findings could also be explained by the dissociation of Adriamycin from intracellular binding sites and efflux into the medium driven by the transmembrane concentration gradient. Again, since no measurements were presented relating to intracellular Adriamycin concentrations after efflux, the contribution of intracellular targets to drug toxicity cannot be discounted. The fact that lowering the temperature of incubation interfered with drug toxicity is consistent with alternative mechanisms of drug action, since, for instance, the induction of topoisomerase II-mediated strand breaks (discussed below) and signal transduction pathways leading to cell death are likely to be interrupted at the reduced temperature. In support of this argument, studies in the same experimental system [69] demonstrated quite unequivocally that induction of DNA strand breaks was temperature-dependent in that breaks failed to occur in the cell at the reduced temperature.

While these studies as well as those of other investigators [66, 67] support the concept that polymer-associated drug may have potential utility in the treatment of cancer, these approaches are unlikely to provide insights into the mechanism of drug action under conventional clinical conditions. Furthermore, the fact that resistance to the anthracyclines is frequently mediated by the multidrug resistance pump [168, 169] supports the concept that drug must enter the cell to express its toxicity.

VI. Induction of DNA Damage through Interference with Topoisomerase II

A series of studies in the late 1970s and early 1980s by Ross and coworkers [170–173] described the induction of strand breaks in the DNA of L1210 leukemic cells by Adriamycin at concentrations ranging between 0.4 and 5 μM. The strand breaks in both single- and double-stranded DNA were found to be protein-associated and were slowly and incompletely repaired after removal of cells from the presence of the drug. In 1984, work by Tewey *et al.* [71, 72] using both cells and cell-free extracts identified topoisomerase II as the target enzyme for Adriamycin and demonstrated that the subunits of the homodimeric enzyme remain locked onto the 5′ end of the DNA molecule (staggered by 4 bases) after completing the cleavage reaction. Since these original observations, multiple studies have been published exploring the relationship between the induction of DNA strand breaks by inhibition of topoisomerase II and the cytotoxicity of the anthracycline antibiotics.

Topoisomerase II is likely to be one of the primary target sites for the activity of the anthracycline antibiotics. The strongest argument in support of this presumption may be the data indicating that tumor cells that are resistant to the anthracyclines have reduced levels or altered activity of the enzyme with a concomitant reduction in the level of drug-associated strand breaks in DNA (or a reduction in DNA-protein cross-link formation) [75, 77, 83, 86, 87]. In this context, Bellamy *et al.* [76] demonstrated that the calcium channel antagonist verapamil produced a concomitant increase in drug accumulation, double-strand breaks, and toxicity in lung tumor cells. Lawrence [81] demonstrated that ouabain (an inhibitor of the $Na^+ + K^+$-activated ATPases) produced a corresponding decrease in doxorubicin toxicity and strand break induction in hamster fibroblasts; however, no attempt was made to discriminate between protein-associated and direct DNA strand breaks in these studies. Surprisingly, both Capranico *et al.* [74] and Maniar *et al.* [82] reported that calcium channel antagonists could reverse resistance to doxorubicin in P388 leukemic cells without a corresponding enhancement of DNA damage. However, as these authors used supraclinical concentrations of drugs against the resistant cells, it is possible that alternative mechanisms of drug action such as free radical formation may have been initiated.

Studies assessing the relationship between the cytotoxicity of the anthracyclines and the induction of DNA strand breaks have, with one notable exception, provided generally equivocal results. Although studies by Goldenberg *et al.* [174] demonstrate a clear relationship between the induction of both single-stranded and double-stranded breaks in DNA and the cytotoxicity of Adriamycin in P388 leukemic cells, other investigators have failed to demonstrate a consistent relationship between strand breaks and toxicity of the anthracyclines. For instance, Zwelling *et al.* [73] reported a correlation between both single- and dou-

ble-strand break frequency and the cytotoxicity of Adriamycin in L1210 leukemic cells. However, a reanalysis of the data showing such a correlation (with exclusion of the origin) could easily lead to the alternative interpretation that cytotoxicity of Adriamycin is *independent* of strand breaks, since a steep increase in loss of survival is evident with minimally detectable alterations in DNA strand cleavage. Similar arguments can be made relating to the analysis of data correlating strand breaks and toxicity of doxorubicin in human small cell lung cancer cells [85] and in P388 cells [175]. In studies by Belvedere *et al.* [176], different degrees of DNA strand breakage are evident in a human colon carcinoma line at equivalent cytotoxicities for a group of anthracycline analogs; this again makes it difficult to argue that a constant amount of DNA damage can be associated with a predetermined level of toxicity. Furthermore, a relationship between breaks and growth inhibition is evident only within a very narrow range of strand break frequencies (i.e. below 50 rad equivalents), a range where it is virtually impossible to discriminate between different degrees of strand breakage. Studies in our own laboratory using both MCF-7 breast tumor cells and the H-35 rat hepatoma cell line [21, 23] failed to detect strand breaks at drug concentrations below 0.5 μM, and yet the antiproliferative activity of the anthracyclines was evident at concentrations as low as 0.1 μM. Although it could be argued that breaks produced by the anthracyclines are sustained or even increase with time [85], this argument does not explain cytotoxicity in the absence of strand break induction. Finally, there are anthracycline derivatives that are cytotoxic without the capacity to induce DNA strand breaks through inhibition of topoisomerase II [177].

One of the difficulties in attempting to relate the induction of DNA strand breaks to the toxicity of the anthracyclines is that Adriamycin generally produces a low level of DNA damage at a given level of toxicity when compared with other topoisomerase II inhibitors such as 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) [78, 79] or the epipodophyllotoxins [79, 178]. That is, the relationship between drug cytotoxicity and strand break induction is quite steep, with low levels of DNA damage associated with a high degree of cell killing [85, 179]. For instance, at its IC_{50}, doxorubicin produces fewer than 100 rad equivalents of DNA strand breaks in five different cell lines [89]. In a study by Zwelling *et al.* [179], the lowest concentration of doxorubicin utilized to detect (single-strand) breaks in HL-60 human leukemic cells produced a log killing of greater than 3. In a previous as well as a recent report by Binaschi *et al.* [85, 180], the same steep concentration–response curve is evident for a series of anthracycline derivatives in HL-60 leukemic cells. Another potential problem with the anthracyclines is that the concentration response for strand break induction—at least in a cell-free system—is biphasic in that the extent of strand break induction begins to decline as the drug concentration is increased [90]. Although it is not clear how this extrapolates to the intact cell [181], this unique relationship between drug concentration and DNA cleavage could be one reason why it is difficult to consistently demonstrate a relationship between DNA damage and cytotoxicity for the anthracyclines.

In an intriguing report by Glisson *et al.* [88] utilizing mutant CHO cells where drug resistance was a function of altered topoisomerase II activity (i.e. reduced drug-stimulated DNA cleavage), fusion of the resistant cells with normal lymphocytes partially reconstituted sensitivity to doxorubicin without evidence for increased expression of normal topoisomerase II or increased DNA cleavage. Although these findings appear to argue against topoisomerase II as a drug target, it is possible that alternative mechanisms of drug action may be in effect at the higher end of the concentration range utilized (1–10 μM)

Based on the conflicting data in the literature, one cannot be totally sanguine in accepting the role of protein-associated strand breaks through the inhibition of topoisomerase II as the mechanism for anthracycline toxicity. One approach for reconciling the high degree of toxicity of the anthracycline antibiotics with the low extent of DNA damage is that the site of the breaks may be a critical factor in drug action. Capranico *et al.* [84] have reported that the anthracyclines have a site specificity for cleavage different from that of other topoisomerase II inhibitors. Based on differences in sequence-specific cleavage by different anthracyclines, Binaschi *et al.* [180] recently suggested that "double-strand breaks generated by DNA topoisomerase II at some genomic loci. . . may be more prone to be converted into irreversible lesions." This conclusion is consistent with the hypothesis that gene-specific damage may play a more important role in anthracycline action than bulk damage to DNA [182].

VII. Induction of Apoptosis

There is clear evidence that one consequence of treatment with Adriamycin is the induction of apoptosis [91–95]. Skladanowski and Konopa [92] reported on the induction of DNA fragmentation and cell shrinkage associated with apoptosis at concentrations ranging between 0.7 and 10 μM in HeLa cells. Zaleskis *et al.* [93] demonstrated that doxorubicin induces apoptosis in murine thymocytes at concentrations of 0.05 and 0.5 μM but that cell death is not due to apoptosis at elevated (supraclinical) drug concentrations. Ling *et al.* [91] also observed apoptosis at 1 μM doxorubicin in P388 leukemic cells, but not at 10 μM drug. Jaffrezou *et al.* [95] recently demonstrated that treatment of either HL-60 or U-937 human leukemic cells with daunorubicin triggered apoptosis at concentrations of 0.5 and 1 μM (although these studies were performed using serum-free medium, which is likely to be highly permissive for apoptotic cell death). As in the studies by Zaleskis *et al.* [93], no apoptosis was evident at a supraclinical concentration of 5 μM. Both of these studies support the hypothesis presented throughout this review that elevated concentra-

tions of the anthracyclines may kill the tumor cell through non-physiological mechanisms.

It is of interest that studies in our own laboratory have determined that doxorubicin fails to induce apoptosis in MCF-7 breast tumor cells either with acute exposure to a concentration of 1 μM or with prolonged exposure to a concentration of 50 nM [23, 56]. These observations are likely to be related to the intrinsic refractoriness of these cells to apoptotic cell death in response to DNA damage [183–185]. Furthermore, a recent report raises some intriguing questions relating to the importance of apoptosis in doxorubicin-induced cell death [186]. In a rat fibroblast cell line that constitutively expresses c-*myc*, both Myc and p53 are shown to accelerate the onset of apoptosis after doxorubicin; however, the induction of apoptosis does not appear to influence the ultimate extent of cell killing. These studies should be taken in the context of a report by Lock and Stribinskiene [187], which suggests that the extent of apoptosis may not be a critical factor in cell sensitivity to drug action.

VIII. Growth Arrest in Response to the Anthracyclines

The signalling pathway, which may be representative of the alternative to apoptotic cell death, is growth arrest with an increase in the cell population accumulating in the G_2 phase of the cell cycle [23, 188, 189]. Recent studies by Ling *et al.* [190] have demonstrated that G_2 arrest by doxorubicin is related to the disruption of $p34^{cdc2}$/cyclin B activity. It is worthy of attention that Shapiro *et al.* [191] recently reported that Adriamycin can also produce growth arrest in G_1 after restoration of the activity of the cyclin-dependent kinase inhibitory protein $p16^{INK4A}$. However, a discussion of the signal transduction pathway(s) leading to growth arrest is beyond the scope of this commentary.

IX. Alternative Mechanisms of Drug Action

There are a number of alternative mechanisms of drug action that are worthy of mention. One involves the continuous exposure of cells to nanomolar concentrations of drug that are sustained during continuous infusion or during the days after bolus administration of drug. It appears that at these extremely low concentrations, doxorubicin can induce differentiation in leukemic cells and in breast tumor cells [37, 138, 192, 193], an observation that may have implications for combination drug therapies. There is also evidence, both from studies in a cell-free system [194] and in a breast tumor cell line [195], that doxorubicin can interfere with microtubular polymerization and with the cellular cytoskeleton. Finally, studies from our laboratory in breast tumor cells [23] and by others in the heart [196] have shown that doxorubicin can suppress the expression of specific growth-regulatory genes.

SUMMARY

In summary, it appears that the multiple mechanisms of action that have been ascribed to the anthracyclines may be related to the utilization of different drug concentrations under varied experimental conditions. When cells are exposed to drug concentrations in the submicromolar range, induction of cell differentiation (with prolonged exposure) and interference with DNA unwinding/DNA strand separation and DNA helicase may be evident. At drug concentrations that reflect the peak plasma concentration after bolus administration, the primary mechanism of drug action is likely to be through interaction with topoisomerase II, a conclusion that is echoed in a review by Cummings *et al.* [197]; it is possible, however, that the genomic site of injury plays a critical role in drug effectiveness. The interaction with the DNA–topoisomerase II complex is likely to be a primary triggering event for growth arrest and/or cell killing through a signalling pathway leading to apoptosis, at least in leukemic cells and thymocytes. At drug concentrations exceeding approximately 2–4 μM, free radical mediated toxicity and DNA cross-linking may become evident. The difference in mechanisms of action at the level of the tumor cell and the heart at a clinically relevant dose provides an avenue for development of more effective approaches for limiting cardiotoxicity while maintaining antitumor effectiveness. The reader is encouraged to examine a recent review by Doroshow [198] that also addresses many of the questions raised in this commentary.

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