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Anthracyclines: Molecular Advances and Pharmacologic Developments in Antitumor Activity and Cardiotoxicity

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Abstract——The clinical use of anthracyclines like doxorubicin and daunorubicin can be viewed as a sort of double-edged sword. On the one hand, anthracyclines play an undisputed key role in the treatment of many neoplastic diseases; on the other hand, chronic administration of anthracyclines induces cardiomyopathy and congestive heart failure usually refractory to common medications. Second-generation analogs like epirubicin or idarubicin exhibit improvements in their therapeutic index, but the risk of inducing cardiomyopathy is not abated. It is because of their janus behavior (activity in tumors vis-à-vis toxicity in cardiomyocytes) that anthracyclines continue to attract the interest of preclinical and clinical investigations despite their longer-than-40-year record of longevity. Here we review recent progresses that may serve as a framework for reapprais- ing the activity and toxicity of anthracyclines on basic and clinical pharmacology grounds. We review 1) new aspects of anthracycline-induced DNA damage in cancer cells; 2) the role of iron and free radicals as causative factors of apoptosis or other forms of cardiac damage; 3) molecular mechanisms of cardiotoxic synergism be- tween anthracyclines and other anticancer agents; 4) the pharmacologic rationale and clinical recommenda- tions for using cardioprotectants while not interfering with tumor response; 5) the development of tumor-tar- geted anthracycline formulations; and 6) the designing of third-generation analogs and their assessment in pre- clinical or clinical settings. An overview of these issues confirms that anthracyclines remain "evergreen" drugs with broad clinical indications but have still an improv- able therapeutic index.

I. Introduction

[1]Anthracyclines rank among the most effective anti-cancer drugs ever developed (Weiss, 1992). The first anthracyclines were isolated from the pigment-produc-ing *Streptomyces peucetius* early in the 1960s and were named doxorubicin (DOX[1]) and daunorubicin (DNR). As

shown in Fig. 1, DOX and DNR share aglyconic and sugar moieties. The aglycone consists of a tetracyclic ring with adjacent quinone-hydroquinone groups in rings C-B, a methoxy substituent at C-4 in ring D, and a short side chain at C-9 with a carbonyl at C-13. The sugar, called daunosamine, is attached by a glycosidic bond to the C-7 of ring A and consists of a 3-amino-2,3,6-trideoxy-L-fucosyl moiety. The only difference between DOX and DNR is that the side chain of DOX terminates with a primary alcohol, whereas that of DNR terminates with a methyl. This minor difference has important con-sequences on the spectrum of activity of DOX and DNR. Whereas DOX is an essential component of treatment of breast cancer, childhood solid tumors, soft tissue sarco-mas, and aggressive lymphomas, DNR shows activity in acute lymphoblastic or myeloblastic leukemias. As with any other anticancer agent, however, the clinical use of
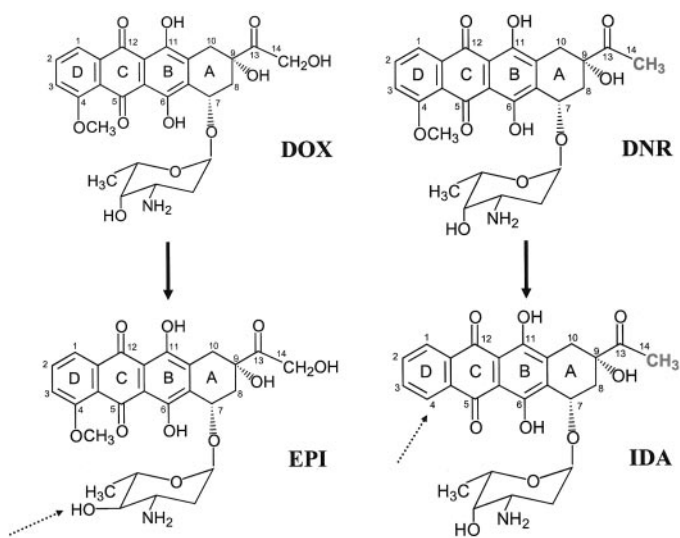
FIG. 1. Structures of DOX, DNR, EPI, and IDA. Grey-marked residues indicate that the side chain of DNR or IDA terminates with a methyl in place of a primary alcohol compared with DOX or EPI. Dotted arrows indicate structural modifications in EPI compared with DOX (axial-to-equatorial epimerization of the hydroxyl group at C-4′ in daunosamine), or in IDA compared with DNR (lack of the methoxy group at C-4 in ring D).

both DOX and DNR soon proved to be hampered by such serious problems as the development of resistance in tumor cells or toxicity in healthy tissues, most notably in the form of chronic cardiomyopathy and congestive heart failure (CHF). To avoid the latter, the maximum recommended cumulative doses of DNR and DOX were tentatively set at 500 or 450 to 600 mg/m^2, respectively.

The last 2 decades have witnessed numerous attempts to identifying novel anthracyclines that proved superior to DOX or DNR in terms of activity and/or cardiac tolerability (Weiss, 1992). The search for a "better anthracycline" has resulted in some 2000 analogs, a figure that should not sound like a surprise if one considers the number of chemical modifications or substitutions and/or conjugations that can be introduced in the tetracyclic ring, the side chain, or the aminosugar. Yet only few analogs have reached the stage of clinical development and approval; among them, epirubicin (EPI) and idarubicin (IDA) enjoy popularity as useful alternatives to DOX or DNR, respectively.

EPI is a semisynthetic derivative of DOX obtained by an axial-to-equatorial epimerization of the hydroxyl group at C-4′ in daunosamine (see also Fig. 1). This positional change has little effect on the mode of action and spectrum of activity of EPI compared with DOX, but it introduces pharmacokinetic and metabolic changes like increased volume of distribution (V_d), 4-O-glucuronidation, and consequent enhanced total body clearance (CL) or shorter terminal half-life (Robert and Gianni, 1993; Danesi et al., 2002). It is because of these kinetic and metabolic changes that EPI was soon used at cumulative doses almost double those of DOX, resulting in equal activity but not in increased cardiotoxicity (Rob-

ert, 1993). In practice, early studies of patients with advanced breast cancer demonstrated that the median doses to the development of laboratory indices of cardiotoxicity were 935 mg/m^2 EPI compared with 468 mg/m^2 DOX, and the median cumulative dose to the development of symptomatic CHF was 1134 mg/m^2 EPI compared with 492 mg/m^2 DOX (Jain et al., 1985). These figures were refined in subsequent studies, since a significantly increasing risk of CHF was documented in patients who received cumulative doses greater than 950 mg/m^2, and the recommended maximum cumulative dose of EPI was cautiously adjusted to 900 mg/m^2 (Ryberg et al., 1998). Thus, replacing DOX with EPI does not eliminate the risk of developing chronic cardiotoxicity. It should also be noted that the mechanisms underlying the reduced cardiotoxicity of EPI versus DOX might not be confined to glucuronidation and increased elimination (see *Section III.B.3.a.*). The actual mechanisms and dose dependence of the improved cardiac tolerability of EPI may therefore require further assessment in both preclinical and clinical settings.

IDA, an analog obtained from DNR after removal of the 4-methoxy group in ring D, is active in acute myelogenous leukemia, multiple myeloma, non-Hodgkin's lymphoma, and breast cancer (Borchmann et al., 1997). The broader spectrum of activity of IDA compared with DNR may be attributed to increased lipophilicity and cellular uptake and improved stabilization of a ternary drug-topoisomerase II-DNA complex [a major mechanism of anthracycline activity (see *Section II.A.1.*) (Binaschi et al. (2001)]. In addition, IDA may be administered orally [with ~10 to 30% bioavailability (Toffoli et al., 2000)], and in vitro studies have indicated that it might be more effective than DNR in tumor cell lines displaying the multidrug resistance (MDR) phenotype (Toffoli et al., 1994; Jonsson-Videsater et al., 2003). There is some controversy about whether IDA offers advantages over DOX or DNR also in regard to cardiac toxicity. Some authors conclude that oral IDA does not induce cardiotoxicity (Borchmann et al., 1997; Lipp and Bokemeyer, 1999; Toffoli et al., 2000), not even in patients previously exposed to DOX or EPI (Toffoli et al., 1997); in contrast, others have shown that IDA decreases left ventricular ejection fraction (LVEF) in anthracycline-naïve patients, and causes CHF in patients with pre-existing cardiovascular disease or previous anthracycline treatment (Anderlini et al., 1995). Thus, the cardiac safety of IDA awaits further assessment in patients properly randomized in terms of cumulative dose and individual risk factors.

Only a few more anthracyclines have attained clinical approval; these include pirarubicin, aclacinomycin A (aclarubicin), and mitoxantrone (a substituted aglyconic anthraquinone) (Fig. 2). Both pirarubicin and aclarubicin demonstrate only modest improvements over DOX and DNR in terms of drug resistance (Lothstein et al., 2001). Pirarubicin, a 4-tetrahydropyranyl doxorubicin,
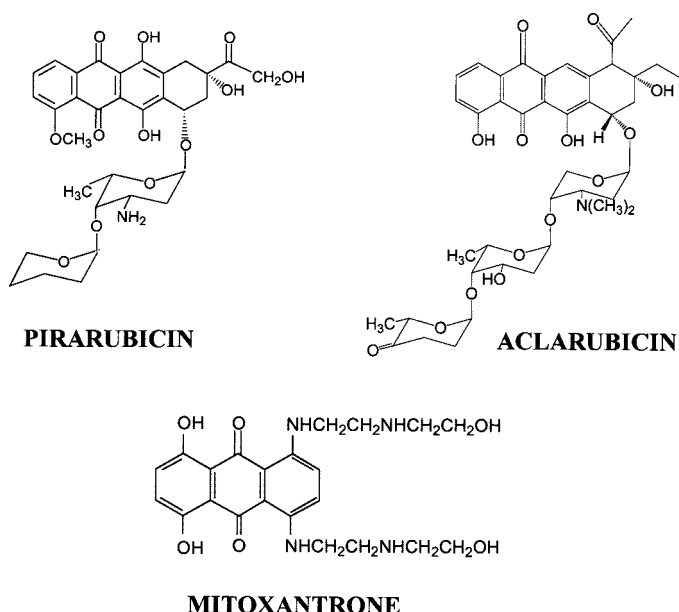
Fig. 2. Structures of pirarubicin, aclarubicin, and mitoxantrone.

PIRARUBICIN

ACLARUBICIN

MITOXANTRONE

has been reported to induce much less cardiotoxicity than DOX in animal models (Koh et al., 2002), but studies in women with metastatic breast cancer have indicated that it may cause significant decrease in LVEF or full-blown CHF at cumulative doses of 460 mg/m^2 or >500 mg/m^2, respectively (Dhingra et al., 1995). In elderly patients with non-Hodgkin's lymphoma, pirarubicin may cause severe cardiac dysfunction at cumulative doses as low as 360 mg/m^2 (Niitsu et al., 1998). Aclarubicin, a trisaccharide anthracycline, was shown to be active and cardiac-tolerable in adult patients with acute myeloblastic leukemia (Case et al., 1987; Wojnar et al., 1989). However, aclarubicin induced late cardiac events in a phase II study of adult patients with refractory acute myelogenous or lymphoblastic leukemia (Dabich et al., 1986) and proved to be inactive in women with metastatic breast cancer (Natale et al., 1993).

Mitoxantrone is active in breast cancer, acute promyelocytic or myelogenous leukemias, and androgen-independent prostate cancer. Early reports indicated that mitoxantrone was less cardiotoxic than other anthracyclines (Estorch et al., 1993), but this conclusion has been challenged in more recent studies (Thomas et al., 2002). Moreover, mitoxantrone causes chronic cardiotoxicity in patients with worsening relapsing-remitting or secondary progressive multiple sclerosis, a disease in which it showed activity worth of approval by the Food and Drug Administration (Gonsette, 2003).

These introductory remarks on the activity and toxicity of most commonly used anthracyclines are meant to indicate that a better anthracycline has yet to come. It is therefore not surprising that relatively old drugs like DOX and DNR remain the focus of clinical and preclinical research aimed at improving our appraisal of their mechanisms of activity or toxicity and at identifying new

strategies for better use in cancer patients. Likewise, the search for new analogs or formulations continues unabated. In this review we will describe and discuss some recent advances in the fields of anthracycline activity and cardiotoxicity as well as recent developments in the pharmaceutical designing and pharmacological or clinical assessment of new analogs or formulations. In preparing for this, we considered that several seminal reviews have appeared over the last 2 or 3 years and focused on the same or closely related subjects. Authoritative analyses of the mechanisms of action of anthracyclines have been provided by Binaschi et al. (2001), Laurent and Jaffrezou (2001), Perego et al. (2001), and Kim et al. (2002b), among others. Molecular mechanisms and pharmacological or clinical correlates of anthracycline-induced cardiotoxicity have been reviewed by Myers (1998), Singal et al. (2000), Kalyanaraman et al. (2002), and Zucchi and Danesi (2003), among others. The pharmacokinetic-pharmacodynamic relationships of anthracycline activity or toxicity have been reviewed by Danesi et al. (2002), and progress in the pharmaceutical designing of new anthracyclines has been reviewed by Monneret (2001), among others. Finally, mechanisms of tumor resistance and possible methods for overcoming them have been reviewed by Benjamin et al. (2000), Tan et al. (2000), Lothstein et al. (2001), and Ejendal and Hrycyna (2002), among others. In apologizing to colleagues whose reviews are not cited because of the size restrictions of our present review or because of our personal ignorance, we will focus on selected advances that, to the best of our knowledge, were not addressed in previously published commentaries or have only recently surfaced to the literature. Moreover, we will concentrate on issues that have remained a matter of unsettled controversy and that we believe are important to accommodate in a unifying picture [e.g., the role of iron and oxidative damage in antitumor activity or cardiotoxicity, or the role of apoptosis in the settings of transient/benign versus chronic/life-threatening cardiotoxicity (*Sections II.* and *III.*)]. Wherever possible, advances in analogs or new formulations (*Section IV.*) will be discussed within the framework of accepted or controversial mechanisms described for antitumor activity or cardiotoxicity.

II. Antitumor Activity of Anthracyclines

A. General Considerations

Despite extensive clinical utilization, the mechanisms of action of anthracyclines in cancer cells remain a matter of controversy. In a seminal commentary the following mechanisms were considered: 1) intercalation into DNA, leading to inhibited synthesis of macromolecules; 2) generation of free radicals, leading to DNA damage or lipid peroxidation; 3) DNA binding and alkylation; 4) DNA cross-linking; 5) interference with DNA unwinding or DNA strand separation and helicase activity; 6) direct membrane effects; 7) initiation of DNA damage via in-

hibition of topoisomerase II; and 8) induction of apoptosis in response to topoisomerase II inhibition (Gewirtz, 1999). An important issue that was raised in that commentary pertained to the concentrations at which DOX and other anthracyclines exhibited a mode of action or another. In particular, it was pointed out that several in vitro experiments reported in the literature had been performed at concentrations of DOX which were considered too high compared with peak or steady-state plasma concentrations (C_{max}, C_{ss}) observed in patients after standard bolus infusions (~5 μM and 25–250 nM, respectively). It was therefore concluded that any study involving intact cells exposed to >1 to 2 μM DOX needed a cautionary re-evaluation. The same cautionary issue was considered in examining studies with subcellular fractions, often performed with submillimolar concentrations of anthracyclines (Gewirtz, 1999). When examined in this context, topoisomerase II remains an attractive and persuasive mechanism to explain the antitumor activity of DOX and other approved anthracyclines at clinically relevant concentrations.

1. Anthracyclines as Topoisomerase II Poisons. Topoisomerases modify the topology of DNA without altering deoxynucleotide structure and sequence. They can cause transient single-strand (topoisomerase I) or double-strand (topoisomerase II) DNA breaks that are resealed after changing the twisting status of the double helix. This activity confers an important role on topoisomerases as the supercoiling of the DNA double helix is modulated according to the cell cycle phase and transcriptional activity (Binaschi et al., 2001).

Anthracyclines act by stabilizing a reaction intermediate in which DNA strands are cut and covalently linked to tyrosine residues of topoisomerase II, eventually impeding DNA resealing. The formation and stability of an anthracycline-DNA-topoisomerase II ternary complex rely on defined structural determinants. The planar ring system is important for intercalation into DNA, as rings B and C overlap with adjacent base pairs and ring D passes through the intercalation site. The external (nonintercalating) moieties of the anthracycline molecule (i.e., the sugar residue and the cyclohexane ring A) seem to play an important role in the formation and stabilization of the ternary complex. In particular, the sugar moiety, located in the minor groove, is a critical determinant of the activity of anthracyclines as topoisomerase II poisons. Topoisomerase II inhibition increases after removal of aminosubstituents at C-3' in the sugar or of the methoxy group at C-4 in ring D (as already mentioned for IDA); moreover, the nature of 3'-substituents greatly influences the sequence selectivity of anthracycline-stimulated DNA cleavage (Binaschi et al., 2000, 2001). Doxorubicin has been reported to also inhibit topoisomerase I, an effect shared by IDA and investigational IDA analogs bearing a disaccharide moiety in which the second sugar retains an axial orientation relative to the first one (Guano et

al., 1999). The cell-killing activity of anthracyclines is weakly but significantly dependent on cellular topoisomerase I content, suggesting that inhibition of topoisomerase I may represent an ancillary mode of action of anthracyclines (Guano et al., 1999). Topoisomerase II-mediated DNA damage is followed by growth arrest in G_1 and G_2 and programmed cell death (Perego et al., 2001; Zunino et al., 2001). It follows that tumor cells may become resistant to anthracyclines because of altered topoisomerase II gene expression or activity (Lage et al., 2000). In clinical settings, the degree of apoptosis induction correlates with tumor response and patient's outcome (Buchholz et al., 2003).

2. Anthracyclines and Apoptosis: Role of DNA Damage and p53. Doxorubicin, as many other genotoxic agents, activates p53-DNA binding. On the basis of the crucial role of p53 in the execution of some forms of apoptosis, it has been proposed that p53 could play an important function in anthracycline cytotoxicity. Preclinical and clinical studies support this concept (Penault-Llorca et al., 2003; Ruiz-Ruiz et al., 2003; Stearns et al., 2003), but negative reports have also appeared (Inoue et al., 2000; Perego et al., 2001; Bertheau et al., 2002; Gariboldi et al., 2003). Uncertainties about the role of p53 in anthracycline-induced apoptosis may be attributed to such various factors as heterogeneity of the tumors examined or of the methods used for assessing p53 status and tumor response (Bertheau et al., 2002).

An additional factor of consideration pertains to the role of p53 in regulating cell cycle transition in DOX-treated cells. In fact, DOX-dependent p53 activation contributes to the induction of the WAF1/CIP1 p21 gene product, a strong inhibitor of cyclin-dependent kinases involved in G_1 to S transition. Whereas this mechanism has been proposed to contribute to G_1 arrest of p53-proficient cells, it has also been suggested that WAF1 expression might protect cells from DOX because the G_1 block facilitates DNA repair before the cells undergo DNA replication. It is in keeping with this concept that constitutively high levels of WAF1/CIP1 protein were shown to associate with chemoresistance in acute myelogenous leukemia (Zhang et al., 1995). On the other hand, the ability of p53-deficient cells to progress through the S phase may be a favorable event, since the expression of the α-isoform of topoisomerase II is increased during DNA synthesis (Perego et al., 2001). Further complexity is introduced by recent data showing that p53 might be important not only in connecting DNA damage to downstream execution of apoptosis but also in determining the net levels of DNA strand breaks induced by DOX (Dunkern et al., 2003). How precisely this occurs cannot be said at this time. Studies of p53-proficient versus -deficient cells showed comparable levels of expression and activity of topoisomerase II in the two cell types, yet p53-proficient cells exhibited more DNA damage (Dunkern et al., 2003). One possibility is that p53 interacts with topoisomerase II and inhibits its li-

gase function, eventually amplifying the net levels of formation of irreversible strand breaks (Cowell et al., 2000; Dunkern et al., 2003). All such issues clearly require refinements, since many human tumors show p53 mutations that bear important implications for chemotherapy.

Uncertainties about the complex interplay between p53 and anthracycline-induced apoptosis are also due to the presence of alternative networks that are not bound to an inhibition of topoisomerase II nor do they always require functional p53, and therefore extend beyond the tentative list of mechanisms provided by Gewirtz (1999) in his thoughtful analysis. For example, present knowledge suggests that clinically relevant concentrations of anthracyclines trigger a cyclical cascade of sphingomyelin hydrolysis and formation of ceramide, which in turn activates downstream cell death effector-mediated pathways not always involving the p53 checkpoint [e.g., c-Jun N-terminal kinase (JNK) stimulation and activation of c-Jun/AP-1 (Laurent and Jaffrezou, 2001); serine-threonine Akt degradation and down-regulation of the Akt/protein kinase B survival pathway (Martin et al., 2002)]. Moreover, it is becoming increasingly evident that anthracyclines can directly release cytochrome *c* from mitochondria, thereby inducing apoptosis regardless of DNA damage or signaling pathways or p53 status (see also *Section III.B.1.*) (Green and Leeuwenburgh, 2002; Clementi et al., 2003). Needless to say, these are just a few of the plethora of mechanisms that have been characterized in recent years in relation to the mode of action of anthracyclines. Because authoritative commentaries of these mechanisms have already appeared (Laurent and Jaffrezou, 2001; Perego et al., 2001; Kim et al., 2002b), we will focus on the most recent advances in DNA damage by anthracyclines, with particular reference to the discovery of novel mechanisms for the nuclear import of anthracyclines; the role of oxidative damage to DNA; and the identification of telomeric DNA as a potential new target of anthracyclines.

B. Advances in DNA Damage by Anthracyclines

1. Role of the Proteasome. Proteasomes are cytoplasmic and nuclear proteinase complexes involved in nonlysosomal mechanisms of protein degradation. The 26S proteasome (composed of a 20S core particle and two 19S cap structures) plays a crucial role in the normal turnover of cytosolic and nuclear proteins and also plays a role in the processing and degradation of regulatory proteins that control cell growth and metabolism (Adams, 2003; Cusack, 2003). The last few years have witnessed an emerging role of the proteasome in modulating anthracycline activity. Proteasomes are present both in the nucleus and in the cytoplasm, but transformed cells and proliferating tissues usually exhibit a preferential accumulation of the proteasome in the nucleus (Kiyomiya et al., 2001b). Conditions typical of solid tumors (like glucose starvation or hypoxia) may accentu-

ate nuclear localization of the proteasome, probably through an increased expression of nuclear localization signals in the α-type subunits of the 20S proteasome (Ogiso et al., 2002). In xenografted human tumors, this is accompanied by development of the resistance phenotype, mediated by proteasome degradation of topoisomerase IIα and reverted by administration of proteasome inhibitors (Ogiso et al., 2000).

An important recent advance pertains to the definition of a multistep mechanism by which the proteasome transports DOX into the nucleus. In step 1, DOX enters cancer cells by simple diffusion and binds with high affinity to the proteasome in cytoplasm. In step 2, DOX binds to the 20S proteasomal subunit, forming a DOX-proteasome complex that translocates into the nucleus via nuclear pores (an ATP-dependent process facilitated by nuclear localization signals). Finally, in step 3, DOX dissociates from the proteasome and binds to DNA due to its higher affinity for DNA than for proteasome (Kiyomiya et al., 2001b). Elucidation of these mechanisms offers one more clue to explaining the reduced activity of anthracyclines in cells with increased nuclear sequestration of the proteasome, since accumulation of the proteasome within the nucleus would diminish the net levels of proteasome available for complexation of DOX in cytosol and its transport toward DNA.

Of particular note is the fact that anthracyclines bind to an allosteric site of the chymotrypsin-like protease activity of 20S proteasome, acting as reversible noncompetitive inhibitors of the protease (Figueiredo-Pereira et al., 1996). The biochemical consequences and potential therapeutic advantages of DOX-proteasome interactions may therefore be 2-fold: increased targeting of the anthracycline at the nucleus and accumulation of undegraded proteins that signal apoptosis. The occurrence of both mechanisms was confirmed by studies in which 1) the nuclear uptake and activity of structurally different anthracyclines correlated with their binding affinity to the proteasome (Kiyomiya et al., 2002b); and 2) DOX-treated cells accumulated proteasome-committed ubiquinated proteins and underwent apoptosis to an extent similar to that induced by inhibitors targeted at the catalytic site of the proteasome (Kiyomiya et al., 2002a). Mechanisms and consequences of DOX-proteasome interactions are sketched in Fig. 3.

Proteasome inhibitors are used as novel therapeutic agents for inducing apoptosis through reduced degradation of the inhibitory subunit (IκBα) and consequent reduced activation of an important tumor survival factor like Rel/nuclear factor κB (NF-κB), for example (Adams, 2003; Cusack, 2003). The fact that inhibitors and anthracyclines bind to distinct catalytic or allosteric sites of the proteasome offered a rationale to design schedules in which the two drugs were given in combination and showed additive or synergic effects compared with single agent treatments. The potential value of such strategy was confirmed by studies in which subtoxic levels of the
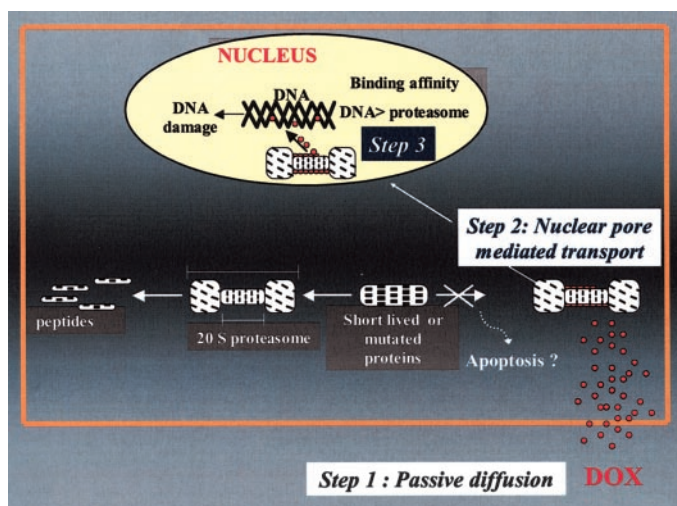
FIG. 3. Three-step sequence explaining proteasome-mediated transport of DOX into the nucleus and accumulation of undegraded proteins. Adapted, with modifications, from Kiyomiya et al. (2001a).

FIG. 4. One-electron redox cycling of anthracyclines.

proteasome inhibitor PS-341 sensitized multiple myeloma cell lines and patient cells to DOX, including cells resistant to either drug or cells isolated from a patient who had relapsed after proteasome inhibitor monotherapy (Mitsiades et al., 2003).

2. Role of Free Radicals. One-electron addition to the quinone moiety in ring C of DOX and other anthracyclines has long been known to result in formation of a semiquinone that quickly regenerates its parent quinone by reducing oxygen to reactive oxygen species (ROS) like superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). This futile cycle is supported by a number of NAD(P)H-oxidoreductases [cytochrome P450 or -b_5 reductases, mitochondrial NADH dehydrogenase, xanthine dehydrogenase, endothelial nitric oxide synthase (reductase domain)] (Vasquez-Vivar et al., 1997; Minotti et al., 1999). During this cycle the semiquinone can also oxidize with the bond between ring A and daunosamine, resulting in reductive deglycosidation and formation of 7-deoxyaglycone (Fig. 4). Due to their increased lipid solubility, aglycones intercalate into biologic membranes and form ROS in the closest proximity to sensitive targets (Gille and Nohl, 1997; Licata et al., 2000). One-electron redox cycling of DOX is also accompanied by a release of iron from intracellular stores (see *Sections III.B.1.a.* and *III.B.1.b.*); ligand binding interactions of DOX with released iron then result in formation of 3:1 drug-iron complexes that convert $O_2^{\cdot-}$ and H_2O_2 into more potent hydroxyl radicals ($^{\cdot}OH$) (Myers, 1998; Minotti et al., 1999). Oxidative damage has therefore been considered an important mechanism of anthracycline activity in tumor cells.

Although no doubt exists about whether DOX and other anthracyclines possess the chemical requisites to generate free radicals in cancer cells, too often this is seen at supraclinical drug concentrations. In those cases when cancer cells were exposed to clinically relevant

concentrations of DOX, there was a long lag phase between drug administration and detection, e.g., of H_2O_2. This raised the possibility that free radicals were formed in response to delayed perturbation of cell metabolism and function rather than in response to the activation of the primary drug (Gewirtz, 1999). An alternative explanation may be that available methods lack sufficient sensitivity to probe discrete amounts of free radicals in cells exposed to clinically relevant concentrations of anthracyclines. Another important concept to be kept in mind when considering the role of free radicals in anthracycline activity pertains to the function of ROS as signaling molecules rather than as mediators of oxidative post-translational modifications of cell constituents. Thus, ceramide formation occurs after ROS activation of neutral sphingomyelinase, and ROS can also modulate the activity of several kinases or transcription factors that control cell cycle and pro- or anti-apoptotic networks (Laurent and Jaffrezou, 2001; Bezombes et al., 2002; Kim et al., 2002b; Martin et al., 2002). In scrutinizing the importance of ROS, one should therefore distinguish their role in signaling events (probably mediated by minute amounts of ROS that escape detection by available techniques) and the role of ROS as direct oxidizing agents (probably requiring higher levels of ROS formation by supraclinical concentrations of anthracyclines).

The patterns of DNA damage in anthracycline-treated cancer cells seem to support the notion that direct oxidative lesions only occurred if cancer cells were exposed to supraclinical concentrations of anthracyclines. Concentrations of anthracyclines below 5 μM, and hence of potential clinical significance, caused formation of protein-associated DNA single- and double-strand breaks, which reflected anthracycline inhibition of topoisomerase II; in contrast, the formation of nonprotein-associ-

ated strand breaks, i.e., DNA lesions caused by free radical formation and reactivity on the DNA backbone, only occurred when the cells were treated with supraclinical concentrations of DOX (reviewed by Gewirtz, 1999).

Similar concerns hold true when considering lipid peroxidation as a possible mechanism of antitumor activity induced by DOX. With one noticeable exception, regarding a selective induction of lipid peroxidation by 1 μM DOX in mouse lymphocytic leukemia cell line L1210 but not in pig kidney proximal tubular epithelial cell line LLC-PK1 (Kiyomiya et al., 2001a), there is apparent evidence to conclude that anthracyclines do not induce lipid peroxidation in cancer cells at clinically relevant concentrations. Under defined conditions, a dissociation actually exists between anthracycline cytotoxicity and lipid peroxidation. This was the case when noncytotoxic amounts of docosahexaenoic acid (22:6 N-3) synergized the cytotoxicity of DOX in glioblastoma cell lines A-172 and U-87 MG and bronchial carcinoma cell lines A-427 and SK-LU-1, whereas lipid peroxidation showed no or very small increases over background levels (Rudra and Krokan, 2001). Supportive or disproving evidence for the formation of free radicals in cancer cells and a role for oxidative damage in anthracycline activity are reported in Table 1.

Recent advances in the field of lipid peroxidation introduce some cautionary issues about how lipid peroxidation was measured and evaluated in relation to the action of DOX and ROS in cancer cells. In most studies, lipid peroxidation was assayed as the formation of thiobarbituric acid (TBA)-reactive materials; although of practical value for experiments with isolated subcellular fractions, this popular assay lacks sufficient sensitivity and specificity for in vivo experiments or studies with intact cells (Minotti, 1993). Moreover, the TBA assay is popularly believed to measure malondialdehyde (MDA), but it is now clear that it actually detects a broad array

of aldehydes and alkenals or peroxides. The possibility therefore exists that anthracyclines did induce lipid peroxidation in cancer cells, but the low sensitivity (and specificity) of the TBA assay may have failed to produce unambiguous evidence that such process had indeed occurred. Perhaps more importantly, the lack of specificity of the TBA assay tells us nothing about the most important pathologic consequence of MDA formation, which is that of linking lipid peroxidation to DNA damage.

3. Lipid Peroxidation and DNA Damage: Malondialdehyde-DNA Adducts. Mass spectroscopy techniques now show that MDA, like other enals, can react at the exocyclic amino groups of deoxyguanosine (dG), deoxyadenosine (dA), and deoxycytidine (dC) to form alkylated products such as etheno adducts from dA, dG, and deoxycytidine; 8-hydroxypropanodeoxyguanosine adducts from dG; a pyrimidopurinone (M_1dG) adduct from dG (Fig. 5) (Marnett et al., 2003). MDA is therefore mutagenic in human cells, with the majority of MDA-induced mutations occurring at GC base pairs and consisting of large insertions and deletions (Niedernhofer et al., 2003). These mutations probably are preceded by premutagenic lesions like DNA interstrand cross-links recognized by the nucleotide excision repair system (Niedernhofer et al., 2003).

In proliferating cells the formation of M_1dG is accompanied by cell cycle arrest and inhibition of cyclin E- and cyclin B-associated kinase activities in both wild-type p53 and p53-null cell lines (Ji et al., 1998). MDA-DNA adducts therefore seem to be closely connected to cell cycle checkpoints possibly relevant to the cytostatic properties of anthracyclines. Importantly, anthracyclines can form M_1dG not only by increasing the levels of formation of MDA but also by favoring oxopropenyl transfer from preformed MDA to DNA; in fact, very low concentrations of DOX and DNR increase MDA-dependent DNA oxopropenylation severalfold, an effect due to

TABLE 1
Free radical formation, DNA damage and lipid peroxidation in tumors: supporting and disproving evidence

	Supportive Evidence	Disproving Evidence
Free radical formation	Free radical formation in rat glioblastoma cells exposed to DOX	Supraclinical concentrations of DOX
	H_2O_2 formation in human colon adenocarcinoma cells	16-h lag prior to H_2O_2 detection (delayed rather than primary metabolic perturbation?)
	Generation of H_2O_2 in MCF-7 cells exposed to 0.1 μM DOX	H_2O_2 detected 9 days after DOX (delayed rather than primary metabolic perturbation?)
DNA damage	Production of non-protein-associated strand breaks in L1210 leukemic cells exposed to DOX	Supraclinical concentrations of anthracyclines
	Nonprotein associated DNA strand cleavage in MCF-7 breast tumor cells at > 5 μM DOX	Protein-associated strand breaks at <5 μM DOX
Lipid peroxidation	Lipid peroxidation in mouse lymphocytic leukemia cells, but not in kidney tubular epithelial cells, exposed to 1 μM DOX[a]	
		No enhanced lipid peroxidation after injection of DOX into a subcutaneously growing rat mammary carcinoma.
	Lipid peroxidation in rat glioblastoma and MCF-7 breast tumor cells treated with low to supraclinical concentrations of DOX	Lack of dose dependence
		[b]Enhanced cytotoxicity but not lipid peroxidation in glioblastoma and bronchial carcinoma cells exposed to DOX and docosahexaenoic acid

Based on Gewirtz (1999), except [a](Kiyomiya et al., 2001a) and [b](Rudra and Krokan 2001).
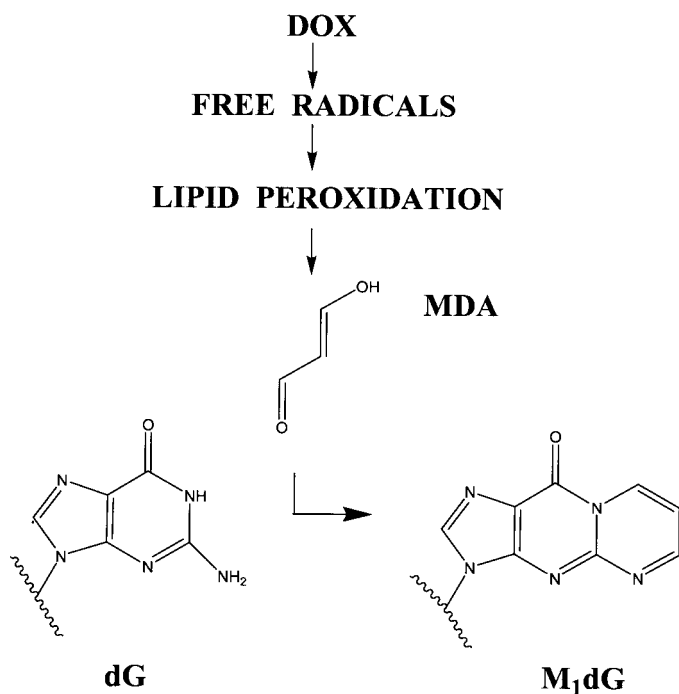
FIG. 5. DOX, lipid peroxidation, and formation of MDA-DNA adducts.

the DNA-intercalating and minor groove-binding properties of the anthraquinone and daunosamine moieties (Plastaras et al., 2002). Thus, both oxidative stress-MDA formation and DNA intercalation-oxopropenylation may enable anthracyclines to increase the cellular levels of M_1dG (Plastaras et al., 2002). These reactions establish potential new links between anthracycline-dependent generation of ROS, induction of lipid peroxidation, and DNA intercalation and damage; they also highlight the importance of replacing the TBA assay with appropriate mass spectral analyses in detecting cellular levels of MDA and related DNA adducts (Otteneder et al., 2003).

4. Oxidative Base Lesions as in Vivo Markers of Free Radical Formation and DNA Damage by Anthracyclines. Studies by Doroshow et al. introduce novel information on DNA oxidative damage induced by DOX under pharmacokinetic conditions. Using gas chromatography/mass spectrometry with selected ion monitoring these investigators examined oxidative modifications of DNA in peripheral blood mononuclear cells (PBMC) from breast cancer patients receiving DOX as slow intravenous infusion. Under these conditions, the steady-state plasma level of DOX was as low as 0.1 μM when the drug was infused for 96 h at a total dose of 165 mg/m². Before DOX infusion, all PBMC contained 13 different DNA oxidized bases; after DOX infusion, at least nine of these bases increased ~4-fold over baseline, with the most remarkable increases regarding thymine glycol (ThyGly), 5-hydroxyhydantoin (5-OH-Hyd), 5-(hydroxymethyl)uracil (5-OH-MeUra), 4,6-diamino-5-formamido-pyrimidine (FapyAde) and, to a lesser extent, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) (Doroshow et al., 2001b).

The spectrum of oxidative DNA base damage induced by DOX reproduced that caused by ionizing radiation (Gajewski et al., 1990), suggesting that bases were damaged by ROS (presumably ·OH radicals) generated after redox cycling of DOX. Other typical fingerprints of ·OH-dependent DNA oxidation like 8-oxo-dG and 8-oxo-dA were not detected in PBMC after DOX infusion; however, the observed increases of FapyAde and FapyGua clearly demonstrated that both 8-oxo-dG and 8-oxo-dA had been formed to some significant extent after DOX infusion, as FapyAde and FapyGua derive from imidazole ring opening of 8-oxo-dA and 8-oxo-dG (Douki et al., 1997).

In addition to providing unequivocal evidence for an oxidative stress induced by DOX under clinical conditions, the studies by Doroshow and associates offer important insights into the mechanisms of DOX activity and mutagenicity. Among the bases that were shown to increase in PBMC, ThyGly and FapyGua and 5-OH-Ura or 5-OH-MeUra have an established mutagenic potential (Jaruga and Dizdaroglu, 1996), mediated by GC→CG transversions (FapyGua) or GC→AT transitions and GC→CG transversions (5-OH-Ura), for example. Moreover, ring-fragmented FapyGua and ThyGly block DNA replication or increase reading error frequency by DNA polymerase, resulting in cytotoxic or mutagenic DNA lesions (Doroshow et al., 2001b; Marnett et al., 2003). DNA polymerase dysfunction may also occur as a result of conformational changes in DNA induced by the presence of oxidized DNA.

These results are important in many respects. As mentioned earlier, popularly accepted mechanisms of anthracycline activity have been characterized in model systems requiring supraclinical concentrations of anthracyclines. Inhibition of DNA and RNA synthesis or of specific DNA polymerases does not escape this bias, which may explain the lack of correlation between DOX-induced inhibition of DNA or RNA synthesis and tumor cell killing in experimental settings (Gewirtz, 1999). Likewise, the formation and disappearance of topoisomerase II-mediated DNA breaks do not always correlate with tumor cell killing or seem to be of too modest an extent to explain the antitumor potency of anthracyclines, unless one assumes that double-strand breaks occur at genomic loci unusually prone to be converted into irreversible lesions (Binaschi et al., 1997; Gewirtz, 1999). The observation that DOX infusions induce detectable levels of potentially cytotoxic, oxidized DNA bases therefore unravels an alternative mechanism to explain the action of DOX under clinically relevant conditions. In addition, it has long been known that combining DOX with cyclophosphamide causes a dramatic increase of the risk of secondary malignancies, most often acute myelomonocytic leukemia (Hoffmann et al., 1995). Thus, if DOX-induced DNA base oxidation occurs in hematopoietic precursors the same way it occurs in PBMC, this may represent an important mechanism to

explain the development of secondary hematologic malignancies.

There are other aspects in Doroshow's work that call for consideration. One of particular note is that the spectrum and net levels of oxidized DNA bases detected in PBMC after 96-h DOX infusions were appreciably broader and higher than those characterized in lymphocytes from patients treated with a short intravenous bolus of 70 mg of EPI/m^2 (Olinski et al., 1997). This has been attributed to a greater systemic exposure to DOX, leading to a depletion of intracellular antioxidants and/or an overruling of repair systems [e.g., glycosylases for Fapy adducts (Hazra et al., 2001)]. In considering that the slow infusion schedule was adopted for reducing the risk of cardiotoxicity while also maintaining good antitumor activity (Synold and Doroshow, 1996; Doroshow et al., 2001b), one cannot escape the conclusion that the free radical-generating activity of DOX correlates with not only C_{max} but also with the total AUC. This issue will be re-examined when addressing the correlates between cardiotoxicity and slow infusion versus bolus anthracyclines (see *Section III.D.1.*).

5. Anthracycline-Formaldehyde Conjugates and DNA Virtual Cross-Linking. Anthracyclines have long been known to form unstable covalent bonds to DNA when redox-activated in chemical systems with NAD(P)H oxidoreductases and transition metals. Two types of covalent bonding have been described: more stable drug-DNA cross-links and less stable drug-DNA adducts. Again, the concentrations required to promote the formation and/or to allow the detection of either cross-links or adducts often exceeded those achievable in patients, making the pathophysiologic relevance of such findings uncertain (Gewirtz, 1999). Seminal work by Taatjes, Koch, and associates has led to an-in-depth reappraisal of this picture. They have shown that iron-mediated free radical reactions enable anthracyclines to produce formaldehyde (HCHO, FORM) from carbon cellular sources like spermine and lipids (Taatjes et al., 1997, 1998, 1999; Taatjes and Koch, 2001). Elevated levels of HCHO have been detected in DOX-sensitive cancer cells but not in DOX-resistant cancer cells equipped with higher levels of ROS-detoxifying enzymes (Kato et al., 2001). Doxorubicin and HCHO then react to give a conjugate (DOXFORM) in which two anthracycline molecules bind together with three methylene groups, two forming oxazolidine rings and one binding the oxazolidines together at their 3'-amino nitrogens. DOXFORM eventually hydrolyzes to give an active monomeric metabolite in which the carbon of HCHO is recovered in the form of a Schiff's base at the aminogroup of daunosamine. Similar reactions occur with EPI and DNR but not with anthracyclines lacking a 3'-amino group (Cutts et al., 2003).

Anthracycline-FORM conjugates have attracted interest because of their unique ability to intercalate into DNA by covalent bonding of the Schiff's base with the 2-amino group of a G-base in the minor groove of DNA. If the interaction with DNA occurs at the trinucleotide 5'-NGC-3', then the drug intercalates between N and G and covalently bonds to the G-base on one strand using HCHO, and to the G-base on the opposing strand using hydrogen bonds. Such an unusual combination of intercalation, covalent bonding, and hydrogen bonding is referred to as the *virtual cross-linking* of DNA by anthracyclines (Taatjes and Koch, 2001) (Fig. 6). In the case of DOXFORM (and, presumably, EPIFORM and DNRFORM) the virtual cross-link slows DNA strand exchange by 640-fold relative to anthracycline-free DNA, and by 160-fold relative to DNA bearing intercalated unchanged anthracycline. Such a 160-fold difference in strand exchange rate clearly denotes the importance of the covalent linkage in the drug-DNA interaction (Zeman et al., 1998; Taatjes and Koch, 2001).

The discovery of the virtual cross-linking mechanism provided a rationale for assessing anthracycline-FORM conjugates as novel drugs with improved activity in both sensitive cells and cells that had developed resistance to anthracyclines due to overexpression of P glycoprotein (Pgp) (Gottesmann and Pastan, 1993) or reduced expression of the enzyme-mediating redox activation of anthracyclines (e.g., NADPH cytochrome P450 reductase), or increased expression of ROS detoxifying enzymes [e.g., superoxide dismutase (SOD), catalase, GSH peroxidase (Mimnaugh et al., 1991; Gariboldi et al., 2003)]. Activity in Pgp-overexpressing tumors was anticipated based on two factors: HCHO-dependent reduction of the pK_a of the protonated amino residue of anthracyclines, making this residue unprotonated at physiological pH and decreasing anthracycline affinity for Pgp (Lampidis et al., 1997); and rapid binding of anthracycline-FORM conjugates to DNA in competition with Pgp (Taatjes et al., 1998). Activity in cells with reduced levels of redox-activating enzymes or increased levels of ROS scavengers was anticipated based on the fact that preconjugation of anthracyclines with HCHO would obviate the need for a redox cycling of the anthracycline and consequent generation of HCHO from cellular carbon sources (Taatjes and Koch, 2001). In agreement with such expectations of improved activity, both DOXFORM and EPIFORM or DNRFORM were shown to exhibit enhanced toxicity to anthracycline-sensitive and -resistant tumor cells. This correlated with increased nuclear targeting of the conjugates, accumulation in DNA, prolonged cellular retention, and reduced cellular release of anthracyclines (Taatjes et al., 1999). DNA lesions attributed to the action anthracycline-FORM conjugates were shown to be unstable and to hydrolyze at rates that were reflected in a biexponential pattern of drug efflux. The faster rate of drug release was assigned to hydrolysis of more labile lesions at isolated G-bases, and the slower rate was assigned to hydrolysis of relatively fewer labile lesions at NGC sites, which is the site more directly
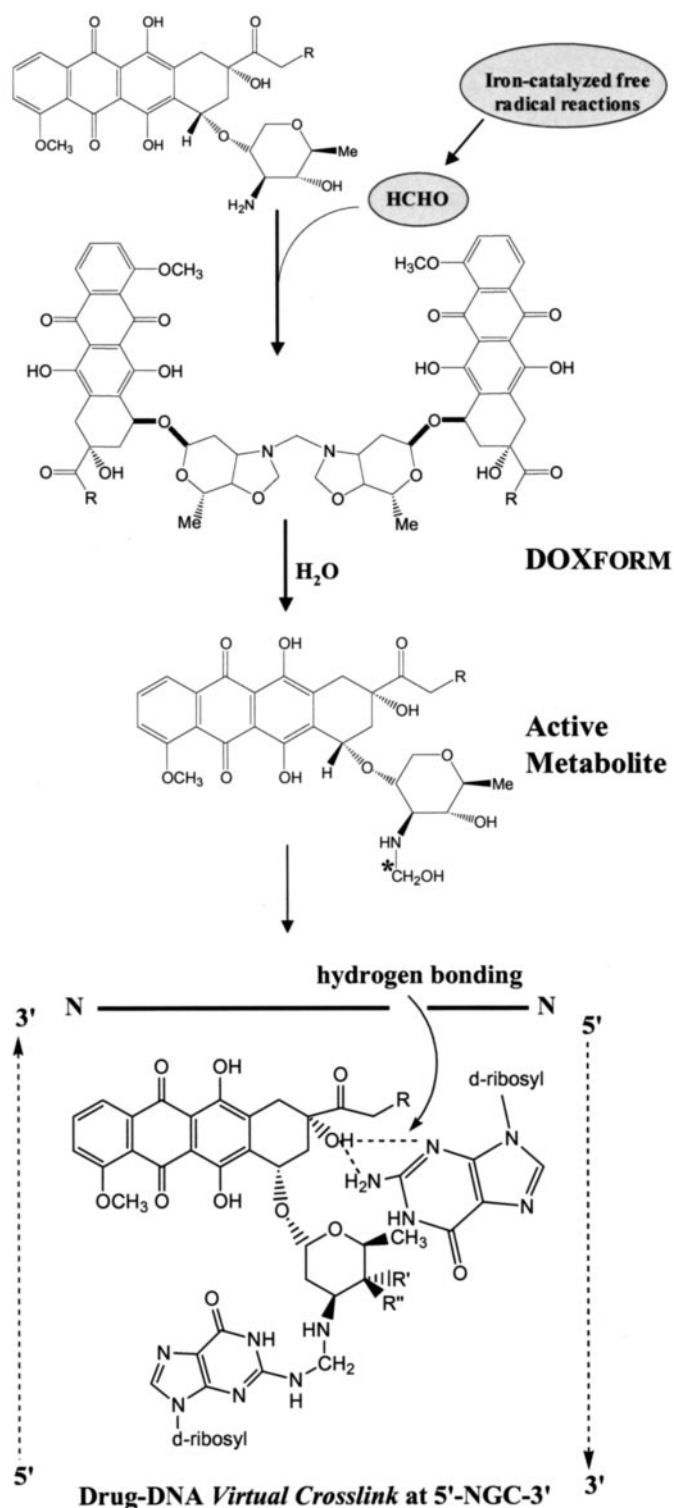
FIG. 6. Formation of DOXFORM, its hydrolysis to active metabolite, and mechanisms of drug-DNA virtual cross-link. DOX reacts with HCHO released from carbon sources through iron-catalyzed free radical reactions. Two molecules of DOX bind via three methylene groups, two forming oxazolidine rings and one binding the oxazolidines together at their 3'-amino nitrogens. Similar reactions occur with EPI or DNR. DOXFORM then hydrolyzes to give a monomeric metabolite in which the carbon of HCHO (asterisk-marked) is recovered bound to the aminogroup at C-'3 in daunosamine. In this mechanism two DOX are each covalently bonded to a 2-amino group of a G-base on one strand of DNA via a methylene group and hydrogen bonded to a G-base on the opposing strand, *virtually cross-linking* the DNA. R = —OH (DOX, EPI) or —CH3 (DNR); Me, methyl residue in daunosamine. Based on Taatjes et al. (1997, 1998, 1999), Taatjes and Koch (2001).

linked to the formation of a virtual cross-linking (Taatjes and Koch, 2001).

EPIFORM, the lead compound in a program of development of anthracycline-FORM conjugates, has been evaluated in the National Cancer Institute human tumor cell screen and shown to be more active than EPI in all but one cell line. Of note, EPIFORM significantly exceeded the toxicity of DOX or EPI to the most resistant breast cancer cell line, MCF-7/Adr, and to the most resistant prostate cancer cell line, DU-145 (Taatjes and Koch, 2001). EPIFORM also proved more active than EPI in efficacy trials conducted in a mouse mammary carcinoma model (Dernell et al., 2002). In regard to comparisons between DOX and DOXFORM, studies in HeLa S3 cells showed that both drugs induced apoptosis, but DOXFORM was effective at concentrations 1 order of magnitude lower than DOX and well in the range of clinically achievable concentrations (86 nM anthracycline equivalents) (Burke and Koch, 2001).

Further evidence for the improved activity of anthracycline-FORM conjugates comes from experiments in which DOX was administered in combination with drugs that released HCHO in the cell, like AN-9 or HMTA. Pivaloyloxymethyl butyrate (AN-9) was developed as a butyric acid-releasing prodrug. Butyric acid is known to induce cell differentiation via inhibition of histone deacetylase, but its clinical use would be limited by rapid clearance. The advantage of AN-9 is that it undergoes hydrolysis within the cell, releasing butyric acid, pivalic acid, and HCHO. Doxorubicin and AN-9 proved to be synergistic when administered simultaneously to neuroblastoma or breast adenocarcinoma cells or when the administration of DOX preceded that of AN-9; however, the reverse sequence (AN-9 → DOX) resulted in antagonism (Cutts et al., 2001). These studies demonstrated that the levels of DOX-DNA adducts increased when the drugs were administered in the synergistic sequence but decreased when the antagonistic schedule was used.

Hexamethylenetetramine (HMTA) hydrolyzes intracellularly to release six molecules of HCHO. In neuroblastoma cells, HMTA increased the levels of formation of cytotoxic DOX-DNA adducts, and the IC_{50} of DOX + HMTA proved to be 3-fold lower compared with DOX single agent. Of note, DOX-DNA adducts were formed in a pH-dependent manner, with 4-fold more detected at pH 6.5 compared with pH 7.4 (Swift et al., 2002). While in agreement with the known acid lability of HMTA, the pH dependence of anthracycline-FORM-DNA interactions offers further advantages in light of the low pH of solid tumors.

Possible mechanisms of resistance to the formation of anthracycline-FORM conjugates have to be considered nonetheless. Once formed inside the cell upon DOX-induced oxidative stress or hydrolysis of AN-9 or HMTA, HCHO would be trapped by GSH as hydroxymethyl-GSH, which in turn would become a substrate for GSH-

dependent HCHO-dehydrogenase, resulting in generation of formate and recycling of GSH (Deltour et al., 1999). Overexpression of HCHO-dehydrogenase has therefore been considered a potential mechanism of resistance to the formation of anthracycline-FORM conjugates. Careful investigation by Brazzolotto et al. (2003) seems to refute such possibility, as the expression levels of HCHO-dehydrogenase in DOX-resistant human small-cell lung carcinoma cells were actually lower than in sensitive parental cells. Moreover, DOX treatment was shown to decrease the expression of HCHO-dehydrogenase in both sensitive and resistant cells (Brazzolotto et al., 2003). The apparent ineffectiveness of HCHO-dehydrogenase in mediating resistance to DOX may relate to the fact that mammalian isoenzymes, in contrast to microbial homologs, are not induced by substrate (Edenberg, 2000). An alternative explanation is that the reaction rate between HCHO and the aminoresidue of DOX, or endogenous cellular moieties, is fast enough to allow HCHO to escape metabolization by HCHO-dehydrogenase. Not surprisingly, recent studies have led to the proposal that GSH-dependent HCHO-dehydrogenase may be involved in controlling the levels of nitroso-thiols rather than HCHO (Liu et al., 2001).

6. Anthracyclines and Telomeric DNA. Telomeres are the ends of linear chromosomes of eukaryotic cells. In most eukaryotes, telomeres consist of as many as 500 to 3000 5′-TTAGGG-3′ repeats and serve to protect the end of chromosomes from degradation and ligation. The length of telomeres is primarily controlled by telomerase, a ribonucleoprotein composed of a catalytic protein subunit (telomerase reverse transcriptase, TERT), a telomerase-associated protein, and a stably associated RNA moiety, which serves the function of an intrinsic template for the elongation of telomeres. It has been well established that telomerase is not active in most somatic tissues; therefore, telomeres shorten gradually with age, both in vitro and in vivo, and such erosion is sensed by the cells as a clock to switch toward a p53-mediated senescence program (Chin et al., 1999). Conversely, telomerase is activated in the majority of cancer-derived cell lines and malignant tumors, a finding suggesting that telomerase is pivotal to cell immortalization and tumorigenesis or tumor aggressiveness (Hahn, 2003). Supportive evidence is offered by, among several other reports, increased development of breast cancer in transgenic mice overexpressing mTERT (Artandi et al., 2002); induction of massive apoptosis in human acute leukemia cells transfected with a dominant-negative human TERT (Nakajima et al., 2003); inverse correlation between telomerase activity and overall or disease-free survival in non-small-cell lung carcinoma patients (Marchetti et al., 2002); positive correlation between attenuation of telomerase activity and inhibition of cellular growth or induction of apoptosis in immortal breast can-

cer cell lines transiently transfected with hammerhead ribozyme cleaving human TERT mRNA (Ludwig et al., 2001).

Interestingly, telomeres do not always shorten after telomerase inhibition and consequent induction of apoptosis. This suggests the existence of telomerase-independent mechanisms of telomere elongation (Kim et al., 2002a); it also suggests that telomerase may extend the lifespan of the cell by alternative mechanisms such as capping of free G-rich single-stranded telomeric DNA, which otherwise would become exposed to the nucleoplasm and could trigger cell cycle arrest or apoptosis, depending on the cellular context (Ludwig et al., 2001).

In a significant number of experimental systems, there was a long delay between inhibition of telomerase and cessation of cell growth or induction of apoptosis, and this lag was especially evident in cells exhibiting long telomeres (Herbert et al., 1999). Because telomeres accumulate single-strand breaks and shorten more rapidly after exposure to agents inducing oxidative stress and DNA damage (von Zglinicki et al., 2000), combining telomerase inhibition with anthracycline treatment has been considered a new option for improved cancer treatment. The therapeutic benefit of combining an anthracycline regimen with telomerase inhibition was also anticipated by experimental evidence for multiple crosstalks between DOX activity and changes in telomerase activity or regulation of telomerase by pro- or anti-apoptotic factors (e.g., p53 and ceramide or Bcl-2, respectively). Breast cancer cells acutely exposed to DOX exhibited an increase in p53 activity, a decline in telomerase activity, and replicative senescence characterized by G_0G_1 arrest (Elmore et al., 2002). Similarly, DOX-sensitive gastric carcinoma cells responded to anthracycline administration with a decline of both telomerase activity/hTERT mRNA and Bcl-2 protein levels, whereas DOX-resistant cells exhibited no such change (Yoon et al., 2003) or exhibited telomere-elongating mechanisms that were not mediated by telomerase (Kim et al., 2002a). Finally, elevation of endogenous ceramide inhibited telomerase and contributed to G_0G_1 arrest of human lung adenocarcinoma cells exposed to nontoxic concentrations of DNR (Ogretmen et al., 2001). It was therefore expected that concomitant administration of DOX and telomerase inhibitor(s) resulted in additive or synergic effects in telomerase-positive/anthracycline-sensitive cells. In agreement with such expectations, DOX induced more apoptosis in breast cancer cells in which telomerase had been muted with the ribozyme technology (Ludwig et al., 2001) and formed more DNA double-strand breaks in neoplastic cells derived from telomerase RNA-null mice (Lee et al., 2001). These results clearly illustrate telomerase inhibition as a novel therapeutic approach in combination with DOX and other anthracyclines. It is hoped that clinically impractical strategies like ribozyme cleavage of telomerase mRNA or vector transfection of dominant-negative te-

lomerase subunits will soon be replaced by more doable measures like administration of natural telomerase inhibitors, which are proving promising in preclinical screens [e.g., telomestatin (a natural product isolated from *Streptomyces annulatus*) or epigallocatechin gallate (a major tea polyphenol)] (Kim et al., 2003a; Naasani et al., 2003).

There are several important factors to be taken in account when considering the biological aspects and clinical perspectives of pharmacological interventions targeted at telomeres. One factor pertains to p53, whose mutations or absence attenuate or abrogate the therapeutic benefit of combining an anthracycline with antitelomerase measures [as one would expect if p53 served to relay the senescence program signaled by telomeres shortening (Lee et al., 2001; Elmore et al., 2002)]. Thus, the presence or absence of a functional p53 will dictate the appropriateness of combining DOX or other anthracyclines with antitelomerase treatments. Another important factor pertains to the role of telomere length versus telomere dysfunction. In some studies the overexpression of hTERT in tumor cells was able to compensate for DOX-induced down-regulation of telomerase and prevented telomere shortening; however, all such changes did not preclude DOX from inducing proliferative senescence (Elmore et al., 2002). Such an apparent dissociation between telomere length and cellular senescence is reconciled based upon the appearance of telomerase-independent cytogenetic changes, which are induced by the anthracycline and are referred to as telomere dysfunction (chromosomal ends with no detectable telomere signals or signal-free ends, aneuploidy, and end-to-end chromosome fusions). In cellular systems there are cases when anthracycline sensitivity and formation of double-strand breaks correlate with telomere dysfunction rather than telomerase activity (Lee et al., 2001; Elmore et al., 2002). Thus, cytogenetic assessment of telomere dysfunction will soon become as important as evaluation of telomerase activity in predicting tumor chemosensitivity. One last factor of consideration pertains to the impact of combined antitelomerase chemotherapy regimens on normal cells. Telomere shortening decreases the capacity to cope with stresses such as wound healing and blood cell depletion, especially in aged animals; thus, a potential elevation of the hematotoxic side effects of telomerase inhibitors should be a prominent consideration as clinical trials move forward, especially in view of reports demonstrating that anthracycline-based chemotherapy is per se capable of reducing telomerase activity and telomere length in leukocytes of patients (Schroder et al., 2001). The risk of developing secondary malignancies in response to telomere shortening and genetic instability should also be considered and weighed against the actual benefit of combining anthracyclines with antitelomerase therapy.

III. Cardiotoxicity of Anthracyclines

A. Morphology, Dose Dependence, Risk Factors

Dilative cardiomyopathy and CHF develop after completion of cumulative anthracycline regimens, usually within a year, but very late forms of cardiac dysfunction have been described (Steinherz et al., 1991).[2] The ultrastructural features of anthracycline-induced cardiomyopathy, characterized in patients' endomyocardial biopsies, include the loss of myofibrils, dilation of the sarcoplasmic reticulum, cytoplasmic vacuolization, swelling of mitochondria, and increased number of lysosomes. This morphologic pattern is seen also in mice and rats or rabbits treated with adequate doses of anthracycline, indicating the existence of a species-independent final pathway of morphologic damage (Singal et al., 2000). The severity of morphologic damage is inversely correlated to the levels of Pgp in the endothelium of both arterioles and capillaries of heart samples, showing that a close link exists among the administered dose of DOX, its accumulation in the heart, and the development of cardiomyopathy (Meissner et al., 2002).

In a seminal retrospective study of 399 patient records, DOX-induced cardiomyopathy and CHF proved to be dose-dependent, and their incidence rose to unacceptably high levels when the cumulative dose of the anthracycline exceeded 500 mg/m^2 (Lefrak et al., 1973). Thus, CHF developed in >4, >18, or ~36% of patients who had received cumulative doses of 500 to 550, 551 to 600, or ≥601 mg/m^2, respectively (Lefrak et al., 1973). In another retrospective review of several thousand patients receiving DOX-containing chemotherapy, the risk of CHF correlated with patient age, total anthracycline dose, and dose schedule (Von Hoff et al., 1979). Valvular, coronary, or myocardial heart disease and a long-standing history of hypertension were recognized as independent risk factors of developing cardiomyopathy at cumulative doses of DOX below 500 to 550 mg/m^2. Previous mediastinal irradiation or concurrent administration of other chemotherapeutics (e.g., cyclophosphamide) was also considered to increase the risk of developing cardiomyopathy, but neither factor turned out to influence the incidence of CHF once the effects of age, schedule, and cumulative dose were taken into account (Von Hoff et al., 1979).

Compared with adults, children were shown to have a reduced risk of cardiomyopathy at any given cumulative dose of anthracycline (Von Hoff et al., 1979), but other reports suggested that the risk of cardiomyopathy may actually be increased in children, particularly in those

[2] Anthracyclines can also cause acute cardiotoxicity. This occurs shortly after initiation of an anthracycline regimen (usually within a week) and consists of arrhythmias, hypotension, and mild depression of contractile function. With current treatment protocols, acute toxicity is infrequent, occurring in no more than ~1% of patients, and it is usually reversible. An even rarer acute complication may consist of myocarditis and pericardial effusion, usually occurring a few weeks after anthracycline administration (Zucchi and Danesi, 2003).

who received mediastinal irradiation or irradiation modalities that included the lower part of the heart (Pinkel et al., 1982). Moreover, the incidence of cardiac toxicity was shown to increase in female patients (Lipshultz et al., 1995) or in those who had longer follow-up, consistent with the fact that children may develop cardiotoxicity at longer intervals after treatment completion (sometime as late as 15 years after anthracycline regimens). The current thinking is that children present a pattern of development of cardiotoxicity distinct from that observed in adults (Lipshultz et al., 2002b). This is also indicated by the fact that anthracycline-treated children sometime develop restrictive rather than dilative cardiomyopathy (Zucchi and Danesi, 2003).

The sharp increase in the incidence of cardiomyopathy at cumulative doses above 550 to 600 mg of DOX/m^2 has formed the basis to set an empirical dose limit of 500 mg of DOX/m^2 as a strategy to minimize the risk of cardiomyopathy. However, ultrastructural changes in endomyocardial biopsies and/or impaired contractility after provocative tests (e.g., exercise echocardiography) have been documented in patients treated with reportedly safe cumulative doses. Concerns about whether nonsymptomatic cardiac dysfunction eventually surfaces in the form of late cardiac events, diminishing or even abrogating the benefit of cumulative doses below 500 mg/m^2, have never been satisfied. This issue is of particular importance when DOX is used as adjuvant therapy for women with early breast cancer. The problem of late cardiac morbidity caused by subthreshold dose levels of DOX in this defined patients population has been reappraised in a retrospective analysis of women who had received DOX (median total dose of 300 mg/m^2) or cyclophosphamide/methotrexate/fluorouracil as adjuvant therapy. Results of the analyses showed that nonsymptomatic cardiac dysfunction (defined as pathologic or borderline decrease of LVEF) was higher in women treated with DOX (8%) than in women receiving cyclophosphamide/methotrexate/fluorouracil (2%), but there was no compelling evidence of an excessive risk of late symptomatic cardiac events in patients treated with DOX (Zambetti et al., 2001). Given the uncertain clinical meaning and future course of asymptomatic dysfunction, the balance between risks and benefits was still considered largely in favor of using an appropriate subthreshold dose of DOX in adult patients who may be cured of their neoplastic disease (Zambetti et al., 2001).

B. Mechanisms

Anthracyclines have long been said to induce cardiotoxicity by mechanisms other than those mediating their antitumor effectiveness, a concept which has raised hopes to design strategies for protecting the heart while not diminishing tumor response. Here we describe the most recent advances that may help to shed light into the mechanisms by which anthracyclines induce cardiotoxicity in experimental or clinical settings.

1. Advances in Apoptosis: in Vitro Studies. Until few years ago, apoptosis was disregarded as a possible mechanism underlying dilative cardiomyopathy and CHF induced by anthracyclines. This picture has now changed dramatically. In regard to the receptor-mediated (extrinsic) pathway, DOX was shown to increase apoptosis induced by recombinant Fas ligand (rFasL) in neonatal rat cardiomyocytes (Yamaoka et al., 2000). In regard to the mitochondrial (intrinsic) pathway, several studies have shown that DOX induces apoptosis by favoring cytochrome *c* release and consequent formation of the apoptosome complex [apoptosis activating factor (Apaf-1)/cytochrome *c*/pro-caspase-9] through up-regulation of Bax (which induces cytochrome *c* release by facilitating mitochondrial pore opening) (Wang et al., 1998a,b) or down-regulation of Bcl-X$_L$ (member of the Bcl-2 protein family that blocks cytochrome *c* release) (Kim et al., 2003b; Kitta et al., 2003), for example.

Apoptotic responses may reflect multiple links between redox cycling of DOX, p53 induction by $O_2^{\bullet-}$ and H_2O_2, and transcriptional activation of Bax gene by p53 (Miyashita and Reed, 1998); they may also reflect the direct opening of the mitochondrial permeability transition pore by $O_2^{\bullet-}$ and H_2O_2 formed during redox cycling of DOX (Green and Leeuwenburgh, 2002; Clementi et al., 2003). Accumulation of the more lipophilic 7-deoxyaglycone of DOX in the inner mitochondrial membrane greatly enhances electron deviation from the regular pathway of respiratory chain to oxygen, leading to increased formation of $O_2^{\bullet-}$ and H_2O_2 and consequent amplification of mitochondrial dysfunction (Gille and Nohl, 1997). Accordingly, aglycones are considerably more potent than DOX at increasing the permeability of the inner mitochondrial membrane, coupled with calcium release, swelling, collapse of membrane potential, oxidation of [NAD(P)H], and transition of mitochondria from the condensed to the orthodox conformation (Sokolove, 1994; Clementi et al., 2003). Redox cycling and deglycosidation therefore enable DOX to induce the first two steps of mitochondrial death pathway triggered by oxidative stress in cardiac myocytes, which are *priming* (induced by H_2O_2 and consisting of progressive changes of the inner mitochondrial membrane in face of conserved membrane potential) and *sudden depolarization* (Akao et al., 2003). Priming and depolarization then would be followed by fragmentation, which is massive mitochondrial swelling and cytochrome *c* release coupled with plasma membrane alterations like exposure of phosphatidylserine, and eventual loss of membrane integrity and cellular fragmentation (Akao et al., 2003).

One of the reasons why cardiomyocytes would be more susceptible than other tissues to apoptosis induced by DOX is that cardiomyocytes exhibit low levels of catalase and readily undergo inactivation of selenium-dependent GSH-peroxidase-1 (GSH-Px1) after exposure to DOX (Doroshow et al., 1980; Siveski-Iliskovic et al., 1995). Since catalase acts via the less efficient peroxi-

datic pathway at low H_2O_2 concentrations, GSH-Px1 detoxifies most H_2O_2 due to its low K_m for H_2O_2 (Kalyanaraman et al., 2002). Inactivation of GSH-Px1 would therefore eliminate an important defense against the redox cycling of DOX, favoring the priming of apoptosis by H_2O_2. Doxorubicin decreases also the protein levels and activity of cytosolic CuZn SOD, suggesting that reductions of diverse antioxidant enzymes represent a common response to anthracycline treatment (Li et al., 2002).

The number of cellular mediators linking DOX to the intrinsic pathway of apoptosis is growing steadily (see Table 2 for a few representative examples). In adult rat cardiomyocytes, DOX activates acidic sphingomyelinase(s), favoring the accumulation of ceramide. Consecutive waves of ceramide then couple the activity of voltage-independent B-type Ca^{2+} channels with the opening of the mitochondrial transition pore, but the overall pathway is truncated if cardiomyocytes are supplemented with carnitine, known to inhibit acid sphingomyelinase(s) (Andrieu-Abadie et al., 1999; Henaff et al., 2002). Doxorubicin induces apoptosis also by activating p38 mitogen-activated protein kinases (MAPK), and inhibitors of p38 MAPK α and β isoforms prevent apoptosis (Kang et al., 2000). This apoptotic pathway is attenuated also by cardiac-specific overexpression of cysteine-rich metallothioneins known to exhibit antioxidant properties, a finding linking the activation of p38

MAPKs to the formation of ROS by DOX (Kang et al., 2000). Finally, very recent studies show that DOX and DNR can induce apoptosis of cultured cardiomyocytes through down-regulation of the zinc finger transcription factor GATA-4 (Kim et al., 2003b), an effect presumably coupled with down-regulation of Bcl-X_L (Kitta et al., 2003).

Doxorubicin-induced apoptosis of cardiac cells is prevented by a number of survival factors signaled by phosphoinositide kinase (PI-3K)-serine/threonine kinase (Akt) (see also Table 2). The insulin growth factor-1 attenuates apoptosis by inducing activation of PI-3K/Akt coupled with phosphorylation/inhibition of caspase 9 (Wu et al., 2000). The leukemia inhibitory factor, an endogenous ligand of the cytokine receptor glycoprotein 130, attenuates apoptosis through PI-3K/Akt-dependent phosphorylation and mitochondrion-to-cytoplasm translocation of Bad (another member of the protein family promoting cytochrome c release) (Negoro et al., 2001). The role of JNK is more controversial, equally good evidence existing for apoptotic or antiapoptotic responses following activation of this kinase in cardiomyocytes. In embryonic stem cell-derived cardiac myocytes (resembling fetal or neonatal cardiac myocytes), JNK attenuates apoptosis, an effect that is opposite to that seen when JNK conspires with AP-1 in mediating apoptosis of tumor cells induced by ceramide (see *Section II.A.2.*). This may reflect different effects of JNK on

TABLE 2
Doxorubicin induces apoptosis of cardiomyocytes: in vitro studies

Cell Line	Treatment	Pathway Mechanism	Inhibitors/Survival Factors	Reference
H9c2	DOX	Intrinsic (early mitochondrial dysfunction, cytochrome c release)		Green and Leeuwenburgh, 2002
	DOX	Intrinsic (Bax upregulation, cytochrome c release)		Wang et al., 1998a
				Wang et al., 1998b
Neonatal rat cardiomyocytes	rFasL + DOX	Extrinsic		Yamaoka et al., 2000
	DOX	Intrinsic	LIF-inhibits apoptosis via Bax phosphorylation/translocation	Negoro et al., 2001
	DOX	Intrinsic	IGF-1 inhibits apoptosis via PI-3K/Akt activation and caspase 9 phosphorylation/inhibition	Wu et al., 2000
	DOX	Intrinsic	JNK-c-JUN activation, ATF 3 expression, p53 downregulation	Nobori et al., 2002
Adult mouse cardiomyocytes	DOX	P38 MAPKs activation	Metallothioneins inhibit apoptosis	Kang et al., 2000
	DOX	Intrinsic (activation of acid sphingomyelinases, ceramide formation, coupling of voltage-independent B-type Ca^{2+} channels with the opening of transition pore)	L-Carnitine protects by inhibiting acid acid sphingomyelinase(s)	Andrieu-Abadie et al., 1999
				Henaff et al., 2002
HL-1 or adult rat cardiomyocytes	DOX, DNR	Intrinsic (down-regulation of Bcl-X_L)		Kim et al., 2003b
				Kitta et al., 2003

IGF-1, insulin growth factor-1; LIF, leukemia inhibiting factor; MAPK, p38 mitogen-activated protein kinases; PI-3K/Akt, phosphoinositide kinase-serine/threonine kinase; rFasL, recombinant Fas ligand.

transcriptional control. Treatment of neonatal rat cardiomyocytes with DOX causes activation of JNK and c-JUN coupled with expression of activating transcription factor (ATF) 3, a member of the ATF/cyclic adenosine monophosphate (cAMP)-responsive element binding protein family of transcription factors (Kang et al., 2002). Under these defined conditions, ATF 3 expression contributed to protecting cardiomyocytes from DOX-induced apoptosis, an effect at least in part attributable to down-regulation of p53 (Nobori et al., 2002).

Of note, DOX-induced apoptosis of isolated cardiomyocytes proceeds through activation of NF-κB (Wang et al., 2002). This is opposite to what was observed in cancer cells, in which NF-κB activation usually inhibits apoptosis induced by DOX. How precisely DOX activates NF-κB remains unknown, but enhanced phosphorylation and degradation of the inhibitory domain seem not to be involved (Wang et al., 2002). The dual role of NF-κB (antiapoptotic in cancer cells/proapoptotic in cardiomyocytes) is shown by studies in which SN50, a cell-permeable peptide blocking nuclear translocation of NF-κB, increased apoptosis induced by DOX in human retinoblastoma cells (Poulaki et al., 2002) but inhibited apoptosis induced by DOX in rat adult cardiomyocytes (Wang et al., 2002). In tumor cells SN50-dependent inhibition of NF-κB and consequent enhanced apoptosis were accompanied by up-regulation of Bax and down-regulation of Bcl-2 (Poulaki et al., 2002), but how cardiomyocytes would switch such responses in opposite directions remains to be established.

a. Doxorubicin, Iron, and Apoptosis: Role of Ferritin.
In cardiomyocytes exposed to DOX, activation of NF-κB and apoptosis were shown to occur after redox cycling of the anthracycline and consequent formation of $O_2^{\bullet-}$ and H_2O_2. Thus, overexpression of GSH-Px1 attenuated NF-κB activation and apoptosis, and the same effect was obtained by treating cardiomyocytes with cell-permeable scavengers of $O_2^{\bullet-}$ and H_2O_2 (Wang et al., 2002). The proapoptotic effects of $O_2^{\bullet-}$ and H_2O_2 were mediated by a cellular pool of low-molecular-weight iron, since apoptosis was also prevented by antitransferrin receptor (TfR) antibodies or cell-permeable iron chelators (Kotamraju et al., 2002).

Although the requirement for iron in ROS-induced apoptosis is explained based on its ability to convert $O_2^{\bullet-}$ and H_2O_2 into $^{\bullet}OH$ or equally reactive iron-peroxo complexes (Minotti, 1993), less clear is how such a pool would be formed inside cardiomyocytes. Cells have very little or no free iron available for catalyzing free radical reactions (Cairo et al., 2002); therefore, DOX-induced apoptosis must be preceded by severe dysregulation of iron homeostasis, possibly mediated by a release of iron from intracellular stores. In vitro studies suggest that one-electron redox cycling of DOX and consequent formation of DOX semiquinone and $O_2^{\bullet-}$ are accompanied by release of iron from ferritin, the most important iron storage protein. The superoxide anion is small enough to penetrate the transprotein channels of ferritin and has a reduction potential lower than that of polynuclear ferric oxohydroxide stored in the ferritin core; a combination of steric and thermodynamic factors thus enables $O_2^{\bullet-}$ to reach and reduce the iron core of ferritin, promoting the release of iron in its Fe(II) form (Minotti, 1993; Minotti et al., 1999) (Fig. 7A). The semiquinone of DOX has a reduction potential even lower than that of $O_2^{\bullet-}$ but is too large to penetrate the transprotein channels of ferritin. The current thinking is that the semiquinone of DOX releases iron through indirect mechanisms mediated by electron tunneling, for example (Fig. 7B).

This picture changes when ferritin iron movements are studied in isolated cardiomyocytes exposed to transferrin (Tf)-bound iron in the presence or absence of DOX. According to the most recent studies (Kwok and Richardson, 2003), the vast majority of Tf-bound iron is incorporated in ferritin following internalization of Tf-Tf receptor complexes, but in a few hours ferritin-bound iron is released back into cytosol or other compartments to fulfill the metabolic requirements of the cell. Under such defined conditions, iron release probably occurs through the action of physiologic reductants (ascorbate, cysteine, reduced flavins) that either penetrate transprotein channels of ferritin or donate electrons to ferritin iron by tunneling-type mechanisms (see also Fig. 7B). Doxorubicin does not interfere with the process of iron uptake and deposition in ferritin but diminishes a subsequent release of iron induced by cellular reductants, presumably because $O_2^{\bullet-}$ induces post-translational modifications of ferritin that decrease its ability to accommodate reductants or to release Fe(II) after Fe(III) reduction (Fig. 7C) (Kwok and Richardson, 2003). Another possibility is that anthracyclines diminish ferritin iron release by interfering with lysosome- and/or proteasome-mediated degradation of the ferritin protein (Kwok
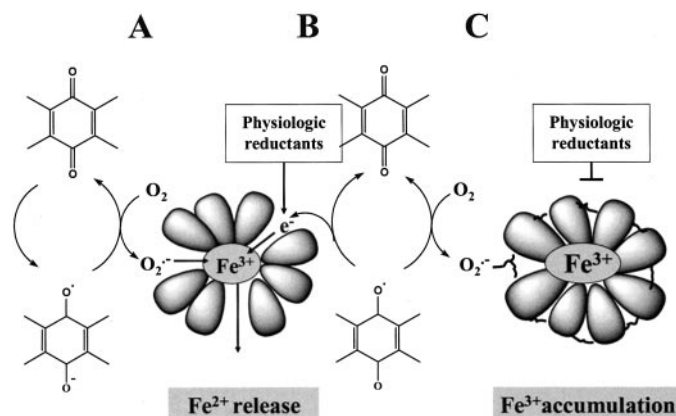
FIG. 7. Doxorubicin and ferritin: iron release versus iron accumulation. Doxorubicin releases Fe(II) through direct interactions of $O_2^{\bullet-}$ with the ferritin core (A) or through electron (e^-) tunneling from its semiquinone to the iron core, a mechanism likely shared by physiologic cellular reductants (B). Alternatively, $O_2^{\bullet-}$ induces post-translational modifications of ferritin that impede iron release induced by physiologic reductants, leading to an accumulation of Fe(III) in ferritin (C). Based on Minotti (1993), Minotti et al. (1999), Kwok and Richardson (2003).

and Richardson, 2004). In either case it is unclear whether an accumulation of iron in ferritin would be cardiotoxic or cardioprotective. The release of ferritin-stored Fe is critical to important processes such as DNA or cytochrome synthesis; hence, the ability of anthracyclines to prevent ferritin iron release might be viewed as a novel mechanism of toxicity that interrupts iron-dependent metabolic processes. Alternatively, one could argue that an increased storage of Fe inside ferritin might serve as a mechanism to diminish iron-catalyzed free radical reactions and consequent apoptotic events.

b. Doxorubicin, Iron, and Apoptosis: Role of Cytoplasmic Aconitase/Iron Regulatory Protein-1. An important issue to be kept in mind is that cell-permeable iron chelators have very little effect in mobilizing iron from ferritin (Kwok and Richardson, 2003), yet they protected cardiomyocytes from apoptosis induced by DOX (Kotamraju et al., 2002; Hasinoff et al., 2003a) or DNR (Sawyer et al., 1999). This suggests that DOX-induced accumulation of iron in ferritin has no effect in inducing apoptosis but actually represents a protective mechanism to prevent free radical reactions that otherwise would promote cell death. The beneficial effects of iron chelators against DOX-induced apoptosis must therefore reflect their ability to intercept iron ions that are released from cellular stores other than ferritin. One such site of iron delocalization has been identified in cytoplasmic aconitase, counterpart of the mitochondrial enzyme that reversibly isomerizes citrate to isocitrate by virtue of its catalytic [4Fe-4S]cluster.

The fourth iron atom of the cluster (needed for aconitase activity and referred to as Fe_a) is easily removable by products of the redox cycling of DOX, such as $O_2^{\cdot-}$ (Brazzolotto et al., 1999) or H_2O_2 (Gardner et al., 1995). This results in formation of a [3Fe-4S] protein devoid of aconitase activity. Different reactions pathways are seen when DOX, or other anthracyclines, undergo two-electron reduction of the side chain C-13 carbonyl moiety, a reaction mediated by NADPH-dependent cytoplasmic aldo/keto- or carbonyl-reductases and resulting in formation of secondary alcohol metabolites like doxorubicinol (DOXol), daunorubicinol (DNRol), or epirubicinol (EPIol) (Minotti et al., 1995; Licata et al., 2000; Mordente et al., 2003) (Fig. 8).

Anthracycline secondary alcohol metabolites are more reactive than $O_2^{\cdot-}$ or H_2O_2 toward the [4Fe-4S] cluster of cytoplasmic aconitase and release both Fe_a and the remaining three iron centers (referred to as $Fe_{\beta1-3}$) (Minotti et al., 1998, 2001b). Thus, cytoplasmic aconitase releases one or four iron atoms depending on which

particular anthracycline metabolite or byproduct reacts with its [4Fe-4S] cluster. Stoichiometric calculations indicate that the [4Fe-4S] cluster of cytoplasmic aconitase is a prevailing source of releasable iron in cardiomyocytes exposed to DOX (Minotti et al., 1995; Kalyanaraman et al., 2002).

The consequences of cluster iron release are more complex if one appreciates that the physiologic role of cytoplasmic aconitase is not confined to catalyzing isomerization of citrate to isocitrate but extends to modulating the expression level of TfR and ferritin. In fact, cluster iron release converts cytoplasmic aconitase into an iron regulatory protein (IRP)-1 which binds with high affinity to conserved iron-responsive elements (IRE) in the untranslated regions of TfR and ferritin mRNAs, increasing stability of the former while also decreasing translation of the latter (Cairo and Pietrangelo, 2000; Cairo et al., 2002, 2003). Conversion of the cluster-containing aconitase to the clusterless IRP-1 therefore facilitates iron uptake over iron sequestration, eventually expanding the cellular pool of free iron. This process occurs spontaneously in iron-depleted cells and serves as an adaptive mechanism to restore metabolic functions requiring adequate iron levels (Cairo and Pietrangelo, 2000; Cairo et al., 2002); the same process is highly toxic if cells have sufficient iron for metabolic purposes but the aconitase→IRP-1 switch is induced by DOX (Minotti et al., 1998, 1999, 2001b, 2004) (Fig. 9).

Understanding the different functions of the cluster-containing aconitase versus the clusterless IRP-1 changes our appraisal of DOX-induced apoptosis. From a structure-activity viewpoint, it becomes evident that DOXol may be considerably more toxic than and $O_2^{\cdot-}$ or
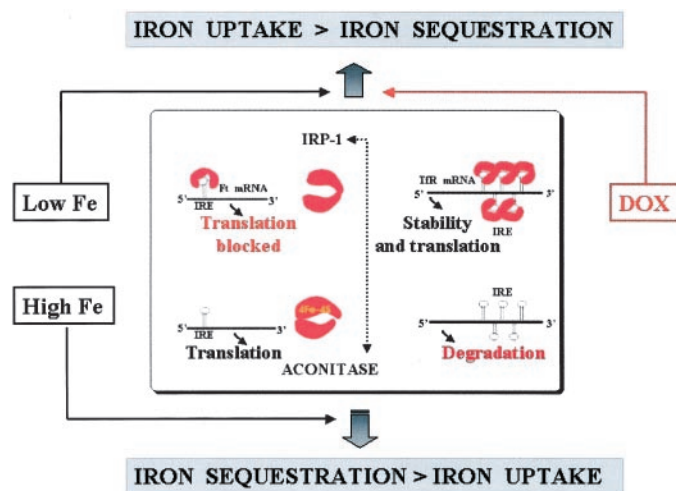
FIG. 9. The aconitase→IRP-1 switch: physiologic adaptation to iron deficiency and pathologic response to DOX. Cluster disassembly switches cytoplasmic aconitase to IRP-1, which enhances the stability of TfR mRNA but decreases the translation of ferritin (Ft) mRNA. Consequent increase of iron uptake over sequestration serves as an adaptive response to compensate for iron deficiency. The same switch, induced by DOX in iron-replete cardiomyocytes, represents a pathologic response to the action of the anthracycline. Based on Minotti et al. (1998, 1999, 2001b, 2004), Cairo and Pietrangelo (2000), Cairo et al. (2002).
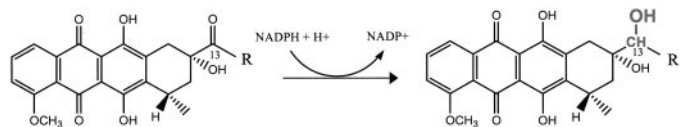
FIG. 8. Enzymatic reduction of anthracyclines to C-13 secondary alcohol metabolites.

H_2O_2, as DOXol releases more iron from the Fe-S cluster. Moreover, concomitant release of iron from the cluster into the cytoplasm and inappropriate facilitation of iron uptake over sequestration rationalize the protective efficacy of chelators and anti-TfR antibodies against DOX-induced cardiac apoptosis, obviating conceptual limitations linked to the uncertain role of ferritin as a source of releasable iron.

Activation of IRP-1, coupled with iron-mediated free radical formation and apoptosis, has been demonstrated in bovine aortic endothelial cells or H9c2 cardiomyocytes exposed to DOX (Minotti et al., 2001b; Kotamraju et al., 2002) and forms the basis to formulate a new hypothesis that accommodates the role of DOX metabolites, ROS, and iron as molecular determinants of apoptosis in cardiomyocytes, as well as the protective efficacy of antioxidants and anti-TfR antibodies or cell-permeable chelators. In this framework, iron released from Fe-S clusters or acquired from extracellular fluids through IRP-1-dependent up-regulation of TfR reacts with $O_2^{\cdot-}$ and H_2O_2 and leads to apoptosis via free radical reactions, which activate NF-κB or induce mitochondrial dysfunction (Fig. 10).

2. Advances in Apoptosis: in Vivo Studies. Despite solid evidence for the induction of apoptosis in cardiomyocytes exposed to DOX in vitro, there is controversy about whether apoptosis contributes to cardiotoxicity induced by DOX in vivo. Cardiac apoptosis has been documented in rats given multiple injections of DOX over a 2-week period (Arola et al., 2000); however, there was no additive effect of repeated dosing, and major indices of apoptosis returned to baseline values before
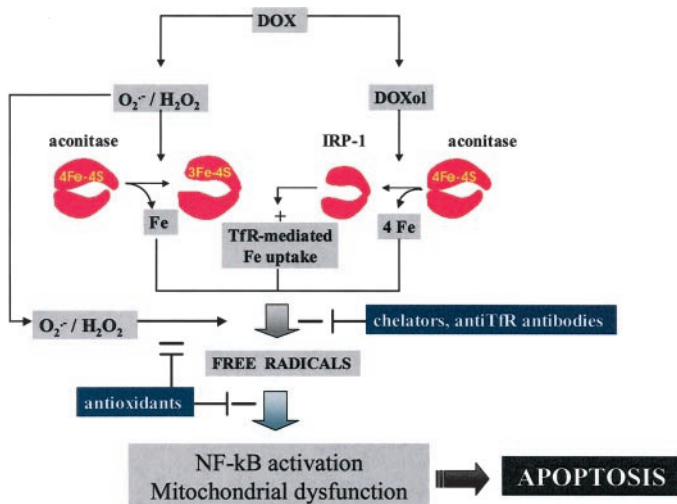
FIG. 10. Role of IRP-1, iron, and ROS in cardiomyocyte apoptosis induced by DOX. DOX-derived $O_2^{\cdot-}$ and H_2O_2 or DOXol release iron from the [Fe-S] cluster of cytoplasmic aconitase. DOXol releases more iron than $O_2^{\cdot-}$ or H_2O_2, switching aconitase to an IRP-1, which further expands the pool of free iron by up-regulating TfR-mediated iron uptake while also decreasing ferritin translation. Reactions of iron with $O_2^{\cdot-}$ and H_2O_2 then amplify free radical reactions, which mediate apoptotic responses. Apoptosis is inhibited by antioxidants, anti-TfR antibodies, or iron chelators. Based on Minotti et al. (1998, 1999, 2001b, 2004), Cairo et al. (2002), Kalyanaraman et al. (2002), Kotamraju et al. (2002).

treatment completion and development of chronic cardiomyopathy (Arola et al., 2000). Although very low levels of myocyte apoptosis may be sufficient to cause dilated cardiomyopathy in laboratory animals (Wencker et al., 2003), the short-term wave of apoptosis induced by DOX in the rat should be opposed to the fact that, in patients with heart failure, apoptosis becomes gradually more evident as the disease progresses toward its end stage (Saraste et al., 1999). An additional caveat is introduced by the fact that the dose intensity (2.5 mg/kg every 48 h) was too high to offer information of translational value, a concern that might apply to other studies showing cardiac apoptosis in DOX-treated rats (Childs et al., 2002). On the other hand, studies performed with the spontaneously hypertensive rat (one of the best animal models for inducing anthracycline-dependent chronic cardiomyopathy) showed that multiple injections of DOX caused apoptosis in occasional endothelial cells, interstitial dendritic cells, or macrophages but not in myocytes (Zhang et al., 1996).

These conflicting reports raised the possibility that apoptosis was important in the settings of acute cardiotoxicity but not of progression of cardiac damage toward chronic cardiomyopathy and heart failure (Auner et al., 2001). This issue has been addressed by treating rats with DOX at lower dose intensity and by examining their heart at regular times during and after treatment (Nakamura et al., 2000). Under such defined conditions of follow-up, the number of apoptotic myocardial cells increased over the first 2 weeks after treatment completion, concomitant with abrupt deterioration of functional indices of myocardial contractility. These findings show that there may be conditions when apoptosis occurs at increased rates while cardiac functions deteriorates, as one would expect if there were cause-and-effect relations between apoptosis and cardiomyopathy. In another study, rats were treated with cumulative doses of DOX and hearts were examined at different post-treatment times. Under such rigorous and well defined conditions, apoptosis became evident as early as 4 days after the last dose of DOX but declined at 10 and 16 days post-treatment. At 21 days the number of apoptotic cells increased again and included cells of the conducting system (Kumar et al., 2001). These results revealed that there may be a lengthy post-treatment period during which apoptosis is silenced in face of the progressive development of cardiomyopathy. Whether apoptosis is a coincidental finding or a causal component in the pathogenesis of anthracycline-induced cardiomyopathy therefore remains open to debate.

3. Multifactorial Processes in Chronic Cardiotoxicity. Uncertainties about the actual role of apoptosis as the only or prevailing mechanism of anthracycline-induced cardiomyopathy justify continued efforts toward identification of other contributing factors. The current thinking is that chronic cardiomyopathy develops after summation and mutual feedback of diverse processes

such as increased membrane lipid peroxidation; inhibition of nucleic acid and protein synthesis; release of vasoactive amines; changes in adrenergic function and adenylate cyclase; abnormalities in Ca^{2+} handling; reduced expression of specific genes possibly caused by altered expression and function of DOX-sensitive transcriptional regulatory proteins; impairment of membrane binding, assembly, and enzymatic activity of mitochondrial creatine kinase; induction of nitric oxide synthase, leading to nitric oxide and peroxynitrite and converse nitration/inactivation of myofibrillar creatine kinase or nitration/activation of metalloproteinases (Table 3).

Whether and how these processes contribute to inducing cardiotoxicity in laboratory animals or patients is controversial, and it is not clear how precisely iron and ROS intervene in these multiple settings. For example, chronic anthracycline administration inactivates and reduces expression of Ca^{2+}-ATPase of the sarcoplasmic reticulum, yet transgenic mice that overexpressed this ATPase proved to be more susceptible rather than more resistant to cardiomyopathy induced by cumulative doses of DOX (Burke et al., 2003). Anthracyclines lacking the quinone moiety (like 5-imino DNR) induce less severe alterations of calcium homeostasis and contractility, but it is now evident that this is due to an abrogation of direct interactions of the quinone moiety with labile −SH groups in calcium channels rather than a suppression of ROS formation (Shadle et al., 2000).

Uncertainties are introduced also by the differential effects of iron chelators, antioxidants, or free radical scavengers in protecting laboratory animals or patients from cardiotoxicity induced by anthracyclines. With few exceptions (Heon et al., 2003), an iron chelator like dexrazoxane prevents chronic cardiotoxicity in all laboratory animals tested (see also *Section III.D.3.*); in contrast, antioxidants afford protection in rodents but not in dogs or pigs. Perhaps more importantly, antioxidants like vitamin E or *N*-acetylcysteine neither prevent nor delay the development of cardiomyopathy in patients treated with cumulative doses of anthracyclines (reviewed by Minotti et al., 1999; Ladas et al., 2004). Thus, a dissociation exists between the role of iron and that of free radicals, as if iron contributed to inducing chronic cardiomyopathy by mechanisms not always related to its ability to generate ˙OH or equivalent reactive species.

A best known example of such dissociation is offered by modifications of lipid peroxidation levels in the heart of patients or laboratory animals exposed to DOX. In a limited clinical study, performed by collecting blood samples from the coronary sinus of cancer patients, lipid peroxidation products did not increase but actually decreased after DOX infusion (Minotti et al., 1996). Similarly, cyclooxygenase-2 inhibitors were shown to aggravate cardiac apoptosis or necrosis induced by DOX in the rat while not increasing but actually diminishing the levels of formation of 8-iso-PGF$_2\alpha$, an isoprostane product of free radical-dependent arachidonic acid peroxidation (see also *Section III.C.3.*) (Dowd et al., 2001). Most recently, DOX and other anthracyclines were shown to inhibit lipid peroxidation by reducing peroxidases or pseudoperoxidases (like H_2O_2-activated myoglobin) through the hydroquinone moiety in ring B (Reszka et al., 2001; Menna et al., 2002). This process resulted in an oxidative degradation of anthracyclines to lower mass products (Cartoni et al., 2004), a finding consistent with the concept that anthracyclines may serve as "suicide substrates" for cellular lipid oxidants.

a. Pharmacokinetics of Secondary Alcohol Metabolites. Structure-activity and metabolism studies now offer clues to explain dissociation(s) between iron and free radicals during the course of development of chronic cardiomyopathy. Analyses of post-mortem cardiac samples, obtained from patients exposed to cumulative doses

TABLE 3
Multiple mechanisms of cardiotoxicity induced by DOX

Mechanism	Reference
Lipid peroxidation	Li and Singal, 2000
	Pacher et al., 2003
Inhibition of nucleic acids and protein synthesis; release of vasoactive amines	Olson and Mushlin, 1990
Changes in adrenergic function and adenylate cyclase	Kalyanaraman et al., 2002
	Singal and Iliskovic, 1998
	Singal et al., 2000
Inhibition of spontaneous or caffeine-induced sarcoplasmic reticulum Ca^{2+} release	Olson et al., 2000
	Shadle et al., 2000.
Reduced expression of:	Arai et al., 1998
	Gambliel et al., 2002
α-Actin, myosin light chain 2 slow, troponin I	Ito et al., 1990
SR Ca^{2+}-ATPase and Ca^{2+}-gated Ca^{2+} release channel (RyR-2)	Jeyaseelan et al., 1997a
Phospholamban, calsequestrin	Jeyaseelan et al., 1997b
Rieske iron-sulfur protein	Torti et al., 1998
ADP/ATP translocase, phosphofructokinase, Mt-CK	Zhou et al., 2001b.
Irreversible decrease in mitochondrial Ca^{2+} loading and ATP content	Zhou et al., 2001a
Impairment of membrane binding, assembly, and activity of MtCK	Tokarska-Schlattner et al., 2002
Peroxynitrite-dependent nitration/inactivation of Mt-CK or nitration/activation of metalloproteinases	Mihm et al., 2002
	Pacher et al., 2003
	Weinstein et al., 2000

M-CK, myofibrillar creatine kinase; Mt-CK, mitochondrial creatine kinase; RyR-2, ryanodine receptor 2; SR, sarcoplasmic reticulum.

of DOX, demonstrate that DOXol is by far the most abundant anthracycline metabolite retained inside cardiomyocytes (Stewart et al., 1993). Moreover, studies conducted by reconstituting DOX with cytosolic fractions of ex vivo human myocardial samples show that anthracycline/protein ratios reproducing acute treatment facilitate conversion of DOX to aglycones (that is, metabolites with improved membrane diffusion and ROS-mediated toxicity), whereas anthracycline/protein ratios reproducing chronic treatment switch DOX metabolism toward formation of DOXol (Licata et al., 2000).

An additional mechanism for explaining the prevailing formation of DOXol during the course of chronic anthracycline regimens and cardiomyopathy pertains to the ability of H_2O_2 to induce aldo/keto-reductases similar to those reducing the side chain carbonyl group of DOX (Spycher et al., 1997). The up-regulation of these enzymes, induced by redox cycling of DOX and formation of H_2O_2 during the early phases of anthracycline treatment, may pave the road for increased reduction of DOX to DOXol during the chronic phase of drug administration. Such a facilitated conversion of DOX to DOXol would then be accompanied by a reduced formation of ROS, as DOXol exhibits a significantly increased K_m for one-electron quinone reductases (Gervasi et al., 1986). Preferred conversion of DOX to DOXol therefore establishes possible links to explain reduced formation of ROS and/or reduced sensitivity to antioxidants during the course of chronic cardiomyopathy (Licata et al., 2000; Minotti et al., 2004).

Pharmacokinetic and functional evidence for the importance of anthracycline secondary alcohol metabolites can be summarized as follows: 1) in laboratory animals, the development of chronic cardiomyopathy usually coincides with an accumulation of DOXol in the heart (Olson and Mushlin, 1990); 2) in senescent rats, an increased susceptibility to cardiomyopathy correlates with an enhanced conversion of anthracyclines to their secondary alcohol metabolites (Cusack et al., 2002); 3) anthracyclines lacking the side chain carbonyl group (e.g., C-13 deoxy DOX) or exhibiting a reduced affinity for C-13 reductases (e.g., the novel disaccharide anthracyclines described in *Section IV.B.1.c.*) induce less severe or progressive cardiomyopathy in the rat (Cirillo et al., 2000; Gambliel et al., 2002; Sacco et al., 2003); 4) mice with cardiac-specific overexpression of anthracycline carbonyl reductases exhibit an increased conversion of DOX to DOXol and an accelerated course of development of cardiomyopathy (Forrest et al., 2000); and 5) mice with genetic deletion of carbonyl reductases form less DOXol and show reduced cardiotoxicity (Olson et al., 2003).

Biochemical evidence supports a role for secondary alcohol metabolites also in humans. For example, studies performed with cytosol from ex vivo human myocardial biopsies indicate that EPI forms significantly less alcohol metabolite than equimolar DOX (Minotti et al.,

1995, 2000), suggesting that limited conversion to EPIol might be an important determinant of the reduced cardiotoxicity induced by EPI (in addition to the well established pharmacokinetic factors like improved glucuronidation and CL).

There are, of course, potential caveats in the "alcohol metabolite hypothesis" of chronic cardiomyopathy. One argument is offered by the fact that DNR and IDA generate higher plasma levels of their alcohol metabolites DNRol or IDAol compared with DOX (Lu et al., 1986), but their cardiotoxicity is similar to or less severe, respectively, than that of DOX (see *Section I.*). Another argument originates from a study in which rats were treated with several doses of DNR or DNRol, but only DNR proved effective at depressing cardiac contractility, despite the fact that the two regimens produced comparable levels of DNRol in cardiomyocytes (Platel et al., 2001). Daunorubicinol also proved to be less toxic than DNR when the two anthracyclines were used in rat isolated heart (Platel et al., 2001). In considering these arguments one should keep in mind that alcohol metabolites are slightly but significantly more polar than their parent anthracyclines; therefore, they exhibit both a reduced partitioning from extracellular fluids inside cardiomyocytes and an altered intracellular distribution compared with metabolites that are formed endogenously (Danesi et al., 1988). Caution should therefore be exercised when attempting to establish cause-and-effect relations between cardiotoxicity induced by a given anthracycline and the levels of its alcohol metabolite in plasma.

Other potential arguments originate from the fact that both DNR and IDA can form higher levels of alcohol metabolites in laboratory animals' cardiac tissues compared with DOX, a finding which does not correlate with cardiotoxicity induced by these anthracyclines. In this respect, recent studies conducted by perfusing rat heart with IDA have shown that a major pool of IDAol would be formed in the vascular wall rather than in cardiomyocytes, transiently increasing coronary resistance rather than inducing negative inotropism (Kang and Weiss, 2003). These findings indicate that an additional determinant of cardiac toxicity or tolerability pertains to the compartment within which alcohol metabolites are formed and suggest that anthracyclines undergoing metabolization in cellular types other than cardiomyocytes might prove to induce different patterns of cardiotoxicity compared with anthracyclines' exhibiting preferred metabolization in the vulnerable myocytes. Having taken this factor into account, one can find that the negative inotropism induced by anthracyclines in rats (Sacco et al., 2003) or in isolated rat heart (Minotti et al., 2001a) does correlate with the levels of alcohol metabolite formed in cardiomyocytes. Finally, recent studies have shown that both DOX and DNR are metabolized to alcohol metabolites in the heart of laboratory animals by carbonyl-type reductases, whereas in human heart DOX

and DNR are metabolized by aldo/keto- or carbonyl-reductases, respectively (Mordente et al., 2003). Because the regulation of expression of aldo/keto- or carbonyl-reductases by H_2O_2, for example, might prove to be quite different, these results anticipate that the net levels of formation of DOXol, DNRol, or IDAol in the heart of patients might not be the same as those observed in the heart of laboratory animals.

The pros and cons of the alcohol metabolite hypothesis of cardiotoxicity clearly need to be weighed from several standpoints. In general, studies performed by reconstituting anthracycline metabolism in human heart extracts concur in establishing good relations between the levels of formation of alcohol metabolites and the clinical cardiotoxicity of anthracyclines (Minotti et al., 1995, 2001c; Licata et al., 2000).

b. Iron-Dependent and -Independent Mechanisms of Toxicity by Secondary Alcohol Metabolites. Doxorubicinol has been reported to induce cardiotoxicity by mechanisms which may or may not involve iron. In regard to iron-independent mechanisms, early reports by Olson and Mushlin (1990) showed that DOXol was several times more potent than DOX at inhibiting the Ca^{2+}-Mg^{2+} ATPase of sarcoplasmic reticulum, the f_0-f_1 proton pump of mitochondria, and the Na^+-K^+ ATPase and Na^+-Ca^{2+} exchanger of sarcolemma. Secondary alcohol metabolites also proved superior to their parent anthracyclines at inhibiting spontaneous or caffeine-induced release of Ca^{2+} from sarcoplasmic reticulum (Olson et al., 2000). These mechanisms have not been confirmed by others (Zucchi et al., 2000), which may be explained by experimental variables such as the amount of Ca^{2+} loaded in sarcoplasmic reticulum vesicles prior to an exposure to anthracyclines or their alcohol metabolites (see Olson et al., 2000). More recently, Olson and colleagues showed that DOXol mediates gene suppression effects previously attributed to DOX. In fact, down-regulation of the Ca^{2+}-gated Ca^{2+} release channel (ryanodine receptor 2/RyR2) of sarcoplasmic reticulum and the consequent decline of closely related contractile parameters such as left ventricular fractional shortening were considerably more evident in rabbits given DOX than in rabbits given C-13 deoxy DOX (Gambliel et al., 2002). These findings bring secondary alcohol metabolites into the arena of anthracycline-induced gene suppression and anticipate one more advantage in replacing DOX with analogs forming no or fewer secondary alcohol metabolites (see also *Section IV.B.1.c.*).

In regard to iron-dependent mechanisms of toxicity, it is now evident that sustained, chronic conversion of DOX to DOXol perturbs the aconitase/IRP-1 machinery beyond the aconitase→IRP-1 switch that occurs in acute settings. Three independent studies (Minotti et al., 1998, 2001b; Brazzolotto et al., 2003) indicate that DOXol eventually converts aconitase/IRP-1 into a "null protein," i.e., a protein devoid of RNA binding activity and unable to recover aconitase activity by reassembling

its Fe-S cluster. Such a simultaneous loss of RNA binding and aconitase activities is attributed to oxidative modifications of cysteine residues that mediate interactions of the clusterless IRP-1 with IREs and serve to coordinate iron atoms in Fe-S clusters needed for aconitase activity (e.g., Cys^{437}) (Minotti et al., 1998). There is controversy about how precisely these labile cysteines would be damaged by anthracyclines. Some believe that the null protein forms after an attack of aconitase/IRP-1 by anthracycline-Fe complexes, regardless of any previous action of DOXol on Fe-S clusters (Kwok and Richardson, 2002); others have shown that the formation of anthracycline-Fe complexes must be preceded by disassembly of Fe-S clusters, induced by DOXol and releasing sufficient iron for subsequent anthracycline-Fe ligand-binding interactions (Minotti et al., 1998, 2001b; Brazzolotto et al., 2003). Whereas the latter mechanism seems to enjoy more solid evidence than the former, there is agreement on the fact that ROS scavengers would have little or no effect on the action of anthracycline-Fe complexes on aconitase/IRP-1, as if these complexes oxidized labile $-SH$ groups by ROS-independent mechanisms (Minotti et al., 1998; Kwok and Richardson, 2002). A role for ROS in the conversion of aconitase/IRP-1 into a null protein has been demonstrated in cellular systems, but this would be confined to synergizing the action of anthracycline-Fe complexes formed by previous collisions of DOXol with Fe-S clusters (Minotti et al., 1998, 2001b).

What are the pathologic consequences of converting IRP-1 into a null protein? In terms of iron homeostasis, the null protein would be unable to sense iron levels and to adapt the processes of iron uptake or sequestration to the metabolic needs of the cells. It is also important to note that in addition to modulating ferritin and TfR levels, IRP-1 can regulate mRNAs for other enzymes closely related to iron utilization (erythroid aminolevulinate synthase), uptake (DMT1/Nramp2), and release (ferroportin 1/IREG1/MTP1). Hence, the influence of IRP-1 on the iron status of the cell extends over a number of regulatory and metabolic pathways (Cairo and Pietrangelo, 2000). In addition, permanent inactivation of cytoplasmic aconitase reduces substrate supply to the cytoplasmic isoform of isocitrate dehydrogenase, decreasing formation of $[NADH + H^+]$ by this enzyme and contributing to an altered redox and/or metabolic balance of the cell (Narahari et al., 2000).

Irreversible conversion of aconitase/IRP-1 into a null protein therefore anticipates a general metabolic impairment, as well as the loss of iron homeostasis and a possible misplacement of iron ions at cellular sites that govern the contraction-relaxation cycle of the heart but lose their function after steric occupation by this metal (e.g., RyR2) (reviewed by Minotti et al., 1999). Chronic formation and cluster reactivity of DOXol, coupled with ROS-independent inactivation of aconitase/IRP-1 by anthracycline-iron complexes, rationalize the protective ef-

ficacy of chelation therapy against chronic cardiomyopathy and serve as new clues to explain the apparent lack of protection by antioxidant interventions.

Cells also contain an IRP-2 that is highly homologous to IRP-1 (79% at the amino acid level) but lacks aconitase activity due to its inability to assemble a [4Fe-4S] cluster (Cairo et al., 2002). IRP-2 responds to iron deprivation or supplementation by undergoing de novo synthesis or proteasome-mediated degradation, respectively. DOXol has no effect on IRP-2, which is instead highly sensitive to ROS-dependent oxidative modifications and consequent proteasome-mediated degradation (Cairo et al., 2002). In an acute setting, ROS-dependent IRP-2 degradation may represent a protective stratagem to counterbalance transient activation of IRP-1 by DOXol and to sequester free iron in newly formed ferritin (Minotti et al., 2001b; Cairo et al., 2002; Corna et al., 2004).[3] Whether IRP-2 inactivation serves some protective role also during chronic toxicity has not been established.

c. Unifying Mechanisms of Chronic Cardiomyopathy. Iron-dependent and -independent mechanisms of cardiotoxicity induced by alcohol metabolites seem to be linked by cause-and-effect relations. In fact, dexrazoxane has been shown to prevent down-regulation of RyR2 induced by chronic administration of DNR to rats, if suppression of gene expression were preceded by disruption of iron homeostasis (Burke et al., 2000). This concept forms the basis to recapitulate the mode of action of alcohol metabolites in a comprehensive picture of chronic cardiomyopathy. As shown in Fig. 11, DOXol may act by altering iron homeostasis (through the conversion of aconitase/IRP-1 into a null protein) or by disrupting calcium homeostasis and related energy and contractile events (through inhibition of ATPases and suppression of RyR2 expression). The two mechanisms share multiple links. The formation of a null protein extends its negative influence on the energy and redox balance of the cell, and the loss of iron homeostasis/compartmentalization aggravates redox/energy impairment and down-regulation of RyR2 expression. All such mechanisms would be sufficient to induce chronic cardiomyopathy, but their noxious consequences might very well be amplified if some viable cardiomyocytes had been lost due to apoptosis caused by iron and ROS. We propose that this framework accommodates prevailing hypotheses of cardiotoxicity and serves as avenues to further investigation in this field.

C. Enhancement by Other Agents

Cardiotoxicity induced by DOX may occur at lower cumulative doses if the anthracycline is given in combination with new approved agents like taxanes or trastuzumab. In laboratory animals, cardiotoxicity induced

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[3] ROS may further contribute to increasing ferritin levels by inducing transcription of the ferritin gene (Corna et al., 2004).
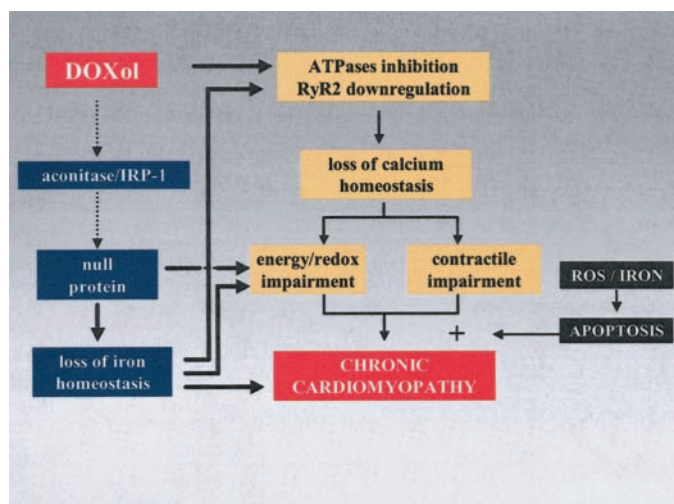
FIG. 11. Proposed mechanism of chronic cardiomyopathy, linking iron and ROS-dependent apoptosis with iron-dependent and -independent actions of DOXol. Based on Olson and Mushlin (1990), Minotti et al. (1998, 1999, 2001a,b, 2004), Burke et al. (2000), Olson et al. (2000), Cairo et al. (2002), Gambliel et al. (2002), Sacco et al. (2003).

by DOX is aggravated also by cyclooxygenase-2 inhibitors (coxibs), new therapeutic agents that might be considered for use in anthracycline-containing clinical regimens. Here we review the pharmacological mechanisms and possible clinical readouts of enhanced cardiotoxicity observed when combining DOX with these agents.

1. Taxanes. Taxanes [paclitaxel (PTX) and docetaxel (DCT)] are microtubule inhibitors that induce apoptosis in breast cancer and inhibit tumor angiogenesis (Grant et al., 2003; Valero and Hortobagyi, 2003). Combining DOX with a taxane therefore came as a natural step toward an improved treatment of metastatic breast cancer. Trials incorporating bolus DOX followed by a 3-h infusion of PTX evidenced very high complete response rates and overall response rates, but an unexpectedly high incidence of CHF at cumulative doses of 480 mg/m^2 DOX was also observed (Gianni et al., 1995). Attempts to elucidate the mechanisms of enhanced cardiotoxicity induced by DOX→PTX compared with DOX single agent showed that PTX increased plasma exposure to DOX; this should translate into a commensurate increase of DOX uptake in the heart, resulting in increased cardiotoxicity (Gianni et al., 1997). Patients receiving DOX→PTX cycles also presented with an increased plasma exposure to DOXol (Gianni et al., 1997); however, an elevation of DOXol in plasma cannot explain a greater incidence of cardiac events, as DOXol is too polar for partitioning from extracellular fluids into cardiomyocytes. The effect of PTX in increasing plasma exposure to DOX and DOXol has been attributed to a competition of PTX and its vehicle Cremophor EL (BASF Wyandotte, Wyandotte, MI) for biliary Pgp, leading to a reduced elimination of the anthracycline and its metabolite (Gianni et al., 1997).

Studies conducted by reconstituting DOX metabolism in human cardiac cytosol in the presence of clinically

relevant concentrations of PTX now show that the taxane can also act by influencing DOX metabolization to toxic species. In fact, PTX increased the conversion of DOX to DOXol by mechanisms that did not require Cremophor EL but reflected allosteric modulation of aldo/keto-reductases by the taxane (Minotti et al., 2001c). These observations recapitulate DOX-PTX interactions in a sequence of events in which the clinical formulation of PTX (vehicle + taxane) reduces elimination of DOX and increases its penetration in the heart, whereas PTX per se facilitates conversion of DOX to DOXol inside cardiomyocytes (Minotti et al., 2001c; Perotti et al., 2003). Of note, DOXol formation was not increased by vinorelbine, a tubulin-active alkaloid that does not increase cardiotoxicity induced by DOX (Bruno et al., 1998). A novel PTX analog (BMS-184476, 7-methylthiomethyl paclitaxel) similarly failed to increase DOXol formation in human cardiac cytosol, nor did it alter DOX pharmacokinetics or cause any severe decline of LVEF when assessed in combination with a cumulative dose of 350 mg of DOX/m^2 in a limited number of women with metastatic breast cancer (Sessa et al., 2004). These results highlight the importance of a taxoid structure and of critical residues of PTX in modulating anthracycline pharmacokinetics and metabolism; they also suggest that cardiotoxicity only increases when DOX is combined with agents that accelerate its conversion to DOXol in the heart (Minotti et al., 2001c; Sessa et al., 2004).

Strategies for reducing the cardiac toxicity of DOX→PTX schedules included separating the two agents by longer than 4 h to avoid pharmacokinetic (or metabolic) interactions or decreasing the cumulative dose of DOX. Retrospective analysis of women treated with PTX and DOX according to different schedules and intervals between drugs' administration shows that a upper limit of 360 mg/m^2 DOX defines a safe range for the application of the active two-drug combination (Gianni et al., 2001). The safety of this threshold has been confirmed in a recently completed phase III trial in which anthracycline-naïve metastatic breast cancer patients were randomized to receive bolus DOX followed 30 min later by a 3-h infusion PTX infusion or a standard DOX-cyclophosphamide regimen (Biganzoli et al., 2003).

An alternative strategy to minimize the enhanced cardiotoxicity induced by DOX→PTX compared with DOX single agent includes the replacement of PTX with its closely related analog docetaxel (n-debenzoil-N-tert-butoxycarbanil-10-desacetyl paclitaxel). This strategy was justified by the apparent lack of pharmacokinetic interactions between DOX and DCT (D'Incalci et al., 1998). Phase II studies showed that such a lack of pharmacokinetic interactions correlated with the apparent cardiac safety of DOX→DCT compared with DOX→PTX (Misset et al., 1999; Nabholtz et al., 2000). More recently, a phase III trial of DOX→DCT versus DOX-cyclophosphamide as first-line chemotherapy for metastatic breast cancer confirmed that DOX→DCT offered improved time to progression and objective response rates compared with DOX-cyclophosphamide while not increasing the incidence of CHF (Nabholtz et al., 2003). An important cautionary factor to be taken into account when interpreting these trials pertains to the cumulative dose of DOX administered in combination with DCT. In the phase III trial of DOX→DCT versus DOX-cyclophosphamide, the median cumulative dose of DOX did not exceed 378 mg/m^2 (Nabholtz et al., 2003). This precludes direct comparison with the DOX→PTX trials in which CHF occurred after 420 to 480 mg/m^2 DOX (Gianni et al., 1995). When evaluated in the human cardiac cytosol model in regard to its ability of increasing DOX to DOXol conversion, DCT had effects similar to PTX (Minotti et al., 2001c). While indicating that modulation of aldo/keto-reductases may be an intrinsic feature of both PTX and DCT, these findings raise caution against the potential toxicity of combining DCT with cumulative doses of DOX higher than 360 to 400 mg/m^2 that were proven safe in available clinical studies.

An additional strategy for reducing the risk of CHF associated with anthracycline-PTX regimens includes substituting EPI for DOX. In a study conducted on breast cancer patients, the risk of developing CHF was reasonably low (7.7%) at a cumulative dose of 720 mg/m^2 EPI, formally corresponding to the cumulative dose of 360 mg/m^2 DOX, which proved to be safe in the DOX→PTX trials (Gennari et al., 1999). However, the risk of CHF increased to 48.7% when the cumulative dose of EPI given in combination with PTX reached 1080 mg/m^2 (Gennari et al., 1999). This figure is significantly higher than that determined for EPI single agent or in combination with agents other than taxanes (15% cumulative risk of CHF at 1000 mg/m^2 EPI) (Ryberg et al., 1998), and it raises the possibility that PTX might increase the cardiotoxicity of EPI the same way it increases that of DOX. Interestingly, pharmacokinetic studies of patients receiving bolus EPI followed immediately by PTX infusion showed that PTX increased plasma exposure to EPIol and to the more polar EPIol or EPI glucuronides without affecting EPI elimination, a result pointing to metabolic rather than pharmacokinetic interference of PTX with EPI (Grasselli et al., 2001).

Data on the cardiac safety of combining EPI with DCT are more preliminary. In a phase I/II study, EPI-DCT proved to be an active and safe regimen in poor-prognosis patients with advanced breast cancer (Pagani et al., 2000). However, the median cumulative dose of EPI reached in the study (495 mg/m^2) was too low to draw firm conclusions in comparison with other anthracycline-taxane trials.

2. Trastuzumab. Trastuzumab is a humanized monoclonal antibody approved for the treatment of women with metastatic breast cancer whose tumors overexpress p185^{HER2}, product of the protooncogene

HER2 (also known as *c-erbB-2* or *neu*). p185^{HER2} is a transmembrane receptor tyrosine kinase belonging to the epidermal growth factor family. HER2 is amplified and the p185^{HER2} protein is overexpressed in 20 to 25% of human breast cancers. These alterations associate with poor prognosis. Single agent trastuzumab produces durable objective responses in patients whose tumors previously failed to respond to standard chemotherapies for metastatic breast cancer. When combined with PTX or DOX, trastuzumab offers improved response rate, response duration, time to progression, time to treatment failure, and median overall survival compared with chemotherapy alone.

Early during the course of pivotal trials, patients treated with trastuzumab developed cardiac dysfunction and CHF that were similar to those induced by anthracyclines; the incidence of cardiac events was 7% in women treated with trastuzumab alone but increased to 28% in women treated with trastuzumab plus an anthracycline and cyclophosphamide (Feldman et al., 2000). In another study anthracycline-naïve patients were randomized to receive DOX (or EPI)-cyclophosphamide alone or with trastuzumab, whereas patients with previous anthracycline treatment were randomized to receive PTX or PTX-trastuzumab. Under such defined conditions, severe cardiac dysfunction occurred in as many as 27% of patients who received anthracycline-cyclophosphamide-trastuzumab. The incidence was much lower (8%) in patients given anthracycline-cyclophosphamide alone and in patients receiving PTX-trastuzumab or PTX alone (13 and 1%, respectively) (Slamon et al., 2001). Similar figures emerged from a retrospective review of records for patients enrolled in seven phase II and III trials. All patients treated with trastuzumab were found to be at increased risk for cardiac dysfunction. The incidence was greater in patients receiving DOX-cyclophosphamide-trastuzumab (27%), with a sharp increase of dysfunction risk at cumulative doses of approximately 300 mg/m^2 DOX. The incidence was substantially lower in patients receiving PTX-trastuzumab (13%) or trastuzumab alone (3–7%) (Seidman et al., 2002).

Clinical presentation and treatment outcome of cardiovascular dysfunction was different in subgroups receiving trastuzumab plus chemotherapy. As many as 64% of patients treated with trastuzumab plus DOX-cyclophosphamide presented with significant functional impairment, whereas only 20% of patients in the PTX-trastuzumab group exhibited significant functional impairment. Moreover, response to conventional CHF treatment was far superior in patients receiving PTX-trastuzumab than in patients receiving DOX-cyclophosphamide-trastuzumab. Treatment response was even less impressive in heavily pretreated patients who received trastuzumab single agent, but this refractoriness might well be attributed to reduced performance and functional capacity due to more advanced disease (Seid-man et al., 2002; Perez and Rodeheffer, 2004). Finally, analysis of potential risk factors associated with trastuzumab-induced cardiac events showed that only age was of statistical significance, which was observed in patients receiving DOX-cyclophosphamide-trastuzumab but not in other subgroups. These findings show that trastuzumab "primes" breast cancer patients to develop cardiotoxicity, especially in presence of previous or concomitant anthracycline therapy (Gianni, 2001).

The cardiotoxic synergism between trastuzumab and anthracyclines cannot be attributed to pharmacokinetic interactions similar to those described for anthracycline-taxane regimens. In fact, a study conducted on women who received trastuzumab in combination with both DOX and PTX for improved treatment of HER2-positive advanced breast cancer ruled out that trastuzumab caused any major effect on the disposition of and plasma exposure to DOX, PTX, or their major metabolites (Bianchi et al., 2003). In another study on breast cancer patients treated with trastuzumab plus EPI and DCT, there was no major change in the pharmacokinetics of EPI and its major metabolites (Lunardi et al., 2003). The mechanisms through which trastuzumab enhances the cardiotoxicity of anthracyclines should therefore be searched downstream of antibody-receptor interactions. Along with neuregulin and the erbB4 (HER4) coreceptor, HER2 plays an essential role in signaling pathways that are central to cardiac development and cardiomyocyte survival. Thus, neuregulin 1β activates both HER2 and HER4 receptor tyrosine kinase activity and promotes growth, myofilament organization, and survival of isolated cardiac myocytes to apoptotic challenges; conversely, HER2-mutant mice develop a severe dilated cardiomyopathy (Ozcelik et al., 2002).

The heart expresses low levels of HER2 compared with breast cancer cells; however, cardiomyocyte HER2 seems to exhibit an unique susceptibility to trastuzumab. This may be due to localization of HER2 and HER4 in the transverse tubules of ventricular myocytes, a site favoring contacts of HER2-HER4-neuregulin complexes with proteins of the sarcomeric Z-band known to play a central role in cytoskeleton-driven signaling (Schneider et al., 2002). Because the myofilament apparatus is an exquisite target of anthracyclines (Lim et al., 2004), synergic interactions between trastuzumab and anthracyclines at this subcellular level might prove to be highly deleterious. Consistent with this possibility, studies in adult rat ventricular myocytes have shown that neuregulin 1β promotes concomitant activation of HER2, extracellular signal-activated kinase 1/2 (Erk 1/2), and Akt, proving able to minimize myofilament disarray induced by administration of DOX. In contrast, anti-HER2 antibodies cause rapid phosphorylation of HER2 but not Erk1/2 or Akt, eventually inducing down-regulation of HER2 and aggravating myofilament disarray induced by DOX (Sawyer et al., 2002). These results indicate that concerted activation of HER2, Erk 1/2, and

Akt serves as a salvage pathway against the damaging effects of DOX and unravel the theory that anti-HER2 antibodies may interrupt such pathway by dissociating HER2-dependent signals from Erk 1/2 and Akt (Sawyer et al., 2002).

Preliminary results suggest that trastuzumab enhances ROS formation in HER2 overexpressing breast cancer cells, presumably through modulation of a membrane oxidase linked to tyrosine phosphorylation (Doroshow et al., 2001a). If trastuzumab-enhanced ROS formation also occurs in the heart, this might serve as an additional mechanism to explain increased cardiotoxicity of trastuzumab-DOX compared with trastuzumab or DOX single agents.

3. Cyclooxygenase-2 Inhibitors. Cyclooxygenases, the enzymes that convert arachidonic acid to prostaglandin H_2, can be grouped in constitutive and inducible isoforms referred to as COX-1 and COX-2, respectively. COX-2 seems to play a key role in tumorigenesis by stimulating epithelial cell proliferation, inhibiting apoptosis, stimulating angiogenesis, enhancing cell invasiveness, mediating immune suppression, and increasing the production of mutagens (reviewed by Singh and Lucci, 2002). Studies of mouse models of mammary tumorigenesis and human breast cancer cell lines provide evidence that COX-2 overexpression plays an important role in the pathogenesis, progression, and metastatic diffusion of malignant breast cancer in humans. Moreover, a causal link exists between COX-2 activity and development of an MDR-1 phenotype, which would have implications for clinical resistance of tumors where COX-2 is overexpressed (Patel et al., 2002). There is therefore growing interest in using specific coxibs as novel and effective agents for the prevention and treatment of breast cancer.

The combination of DOX with coxibs is limited by the facts that DOX induces COX-2 in cardiomyocytes and that such induction serves as a salvage pathway against cardiac injury inflicted by the anthracycline (Dowd et al., 2001). The current thinking is that certain products of COX-2 activity, such as prostacyclin, have protective effect(s) against DOX toxicity (Dowd et al., 2001). Coxibs might therefore prove to exacerbate cardiotoxicity induced by DOX if the two drugs were considered for use in combination therapy of breast cancer or other cancers characterized by COX-2 overexpression and known sensitivity to anthracyclines. In agreement with these premises, the coxib SC236 aggravated acute cardiotoxicity and apoptosis induced by a single dose of DOX administered to adult rats (Dowd et al., 2001). Ongoing clinical trials will define the cardiac safety or toxicity of combining coxibs with DOX.

D. Prevention

1. Slow Infusion. Early in the 1980s, administering DOX by continuous infusion over 48 to 96 h rather than by the standard ~15-min bolus proved to be an effective mean to reduce severe morphologic changes in endomyocardial biopsies and the development of clinically evident CHF, while not diminishing tumor objective response in patients receiving cumulative doses of 600 mg/m^2 DOX (Legha et al., 1982). The safety and efficacy of replacing bolus administration with slow infusions in adult patients have been confirmed ever since, suggesting that plasma and tissue C_{max}, rather than AUC, might determine the development of chronic cardiotoxicity (Danesi et al., 2002). In agreement with this picture, studies with rabbits showed that both plasma and left ventricular peak concentrations of DOX and DOXol were significantly higher following bolus than slow infusion (Cusack et al., 1993).

The benefit of replacing bolus administration with slow infusions is less clear or controversial when this schedule is adopted in pediatric settings. A study in children treated with 60 mg/m^2 DOX over 24 h or 75 mg/m^2 over 72 h showed a reduced incidence of CHF (1 of 97 patients) compared with historical control patients treated with the bolus schedule (13% of patients with CHF) (Berrak et al., 2001). In contrast, in a trial of 240 children with acute lymphoblastic leukemia who were randomized to receive continuous (over 48 h) or bolus infusion of 30 mg/m^2 DOX every 3 weeks up to a median cumulative dose of 340 mg/m^2, there was no compelling evidence of reduced incidence of dilated cardiomyopathy or left ventricular hypertrophy 1.5 years after leukemia diagnosis (Lipshultz et al., 2002a). The current thinking is that the protective benefit obtained by lowering the peak dose might be offset by damage due to the longer exposure of cardiomyocytes to DOX, as if the entire AUC were at least as important as C_{max} in determining late cardiotoxicity in children (Lipshultz et al., 2002a). As anticipated, this concept would be consistent also with the fact that DNA damage in peripheral blood mononuclear cells was considerably more evident after slow infusion than bolus administration.

2. Antioxidants. We have discussed that the mechanisms of cardiotoxicity induced by anthracyclines may be quite different from those mediating their antitumor effectiveness (e.g., the proapoptotic effects of NF-κB activation in cardiomyocytes versus its antiapoptotic effects in cancer cells, or the proapoptotic effects of JNK-AP-1 activation in ceramide-challenged cancer cells versus the antiapoptotic effects of JNK-ATF 3 activation in certain cardiac cell lines). At present, however, there is no specific method to exploit such differences in terms of selective protection of cardiomyocytes against damage induced by anthracyclines. The vast majority of protective strategies therefore focused on administering drugs or natural compounds that improved the antioxidant defenses of cardiomyocytes against anthracycline-derived ROS. This approach was conceptually strengthened by the observations of reduced acute or chronic cardiotoxicity in mice that overexpressed mitochondrial MnSOD (Yen et al., 1996) or cysteine-rich metallothio-

neins (Sun et al., 2001). Most frequently used antioxidants included probucol (a lipid-lowering drug); piperidine nitroxides; lipophilic spin traps; melatonin; vitamins (A, E, and C); and thiol-containing reducing agents (GSH, *N*-acetylcysteine, *S*-allylcysteine, amifostine) (reviewed by Singal et al., 1997, 2000; Singal and Iliskovic, 1998; Minotti et al., 1999; Zucchi and Danesi, 2003). In general, these compounds did not interfere with anthracycline activity in tumor cells, raising hopes of designing pharmacologic interventions that improved the therapeutic index of anthracyclines. Unfortunately, as discussed earlier, the cardioprotective efficacy of antioxidants like vitamin E or *N*-acetylcysteine was quite limited or disappointing in large-sized animals such as dogs or pigs; moreover, clinical studies failed to document mitigation of cardiomyopathy in patients given robust doses of vitamin E or *N*-acetylcysteine (see *Section III.B.3.*).

In laboratory animals, promising results have also been obtained with rutoside-type flavonoids. In some studies, flavonoids were shown to act by exerting direct positive inotropism (van Acker et al., 2001), but the prevailing belief is that they act by scavenging free radicals generated by DOX and/or by chelating iron (Husken et al., 1997; van Acker at al., 1997). More recently, however, flavonoids were shown to inhibit carbonyl reductases that converted DOX to DOXol in rabbit heart extracts; conversely, flavonoids were shown to stimulate DOXol formation in human heart extracts, presumably through inhibition of carbonyl reductases and consequent preferred interaction of DOX with aldo/keto-reductases (Mordente et al., 2003). The mechanism

of action of flavonoids in laboratory animals might therefore be more complex than previously believed, as it might easily involve inhibition of alcohol metabolite formation rather than interception of ROS or chelation of iron. Moreover, flavonoids might prove to be of reduced clinical benefit if they also increased DOXol formation in the heart of patients.

3. Iron Chelators (Dexrazoxane). Back in the 1970s, dexrazoxane (former proprietary name, ICRF-187; also code-named ADR 529) was shown to prevent histologic lesions and contractile dysfunction induced by anthracyclines in laboratory animals or isolated heart models. Dexrazoxane did not interfere with DOX distribution, metabolism, or excretion, nor did it appear to reduce the antitumor potency of the anthracycline; moreover, dexrazoxane caused limited damage on mitotically active tissues (bone marrow, testes, and gastrointestinal epithelia) and diminished both acute and chronic cardiotoxicity in all animal models tested, from mice to dogs or swine (reviewed in Minotti et al., 1999). Dexrazoxane is a bisketopiperazine that undergoes stepwise hydrolysis of the two piperazine rings to form one-ring open intermediates; the latter eventually hydrolyzes to give a diacid-diamide (code-named ADR 925) that is structurally reminiscent of EDTA and chelates iron bound to low-molecular-weight cellular ligands or coordinated within 3:1 anthracycline/Fe complexes (Fig. 12). Studies with beating heart myocytes exposed to radiolabeled dexrazoxane indicate that drug uptake is extraordinarily rapid, approaching its maximum level within 1 min (Doroshow, 1995). This unique pattern of cellular diffusion anticipates rapid formation of ADR 925 inside the heart,
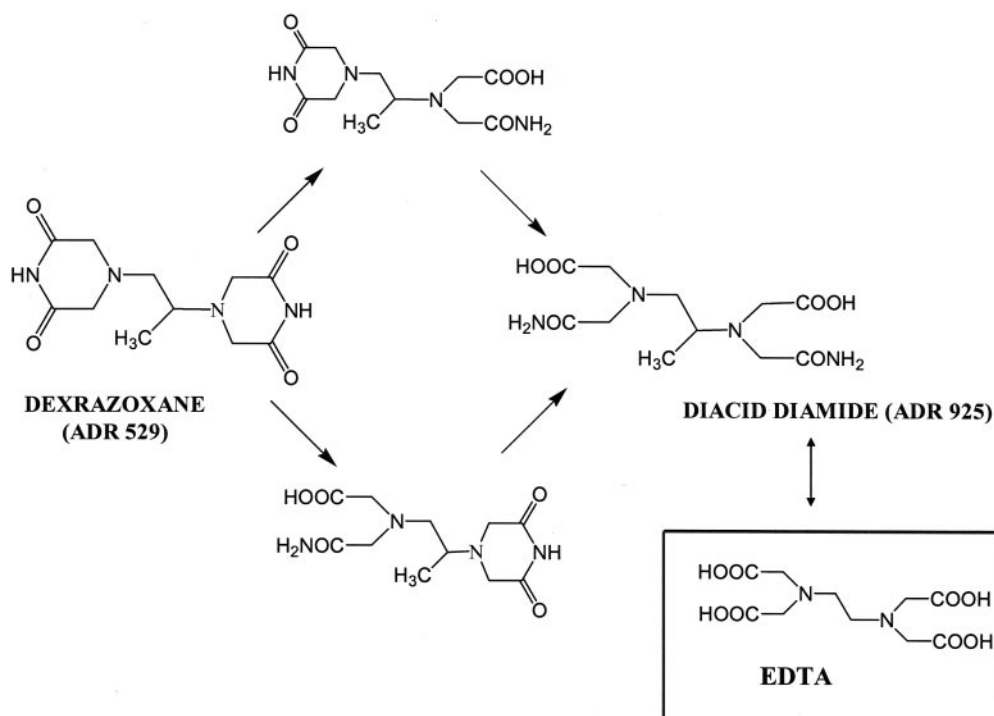
FIG. 12. Two-step hydrolysis of dexrazoxane to an EDTA-like diacid diamide.

but most recent studies show that sizable amounts of ADR 925 are rapidly formed also in plasma, likely through the action of extracellular dihydroorotases (Schroeder et al., 2002; Schroeder and Hasinoff, 2002). Extracellularly formed ADR 925 may contribute to inducing sideruria observed in patients during the course of dexrazoxane administration (Tetef et al., 2001), but recent studies show that it would not contribute to protecting the heart because of its limited access to iron pools in critical cellular compartments or impaired cellular uptake caused by complexation with calcium (Hasinoff et al., 2003b).

When administered by i.v. push or slow infusion to women with advanced breast cancer 15 to 30 min before DOX at escalating doses up to a 10:1 dose ratio to the anthracycline, dexrazoxane did not perturb DOX distribution, metabolism, or excretion but clearly reduced the incidence of cardiotoxicity (Speyer et al., 1992). In a randomized clinical trial, patients who received dexrazoxane prior to DOX could be treated with more cycles and higher cumulative doses of DOX (700–1000 mg/m^2 or more) than patients in the control group (Speyer et al., 1992). Moreover, cardiac protection was observed in patients with and without previous chest-wall radiation or other risk factors for developing cardiomyopathy (Speyer et al., 1992). An exacerbation of myelotoxicity (usually in the form of grade 3 or 4 neutropenia) was the dose-limiting toxicity observed when combining dexrazoxane with DOX at a 10:1 dose ratio (Hochster et al., 1992).

Dexrazoxane has been approved by the Food and Drug Administration for use in patients receiving anthracycline-containing chemotherapy; however, the last few years have witnessed an in-depth reappraisal of its pharmacological properties and clinical therapeutic index. Dexrazoxane was originally investigated as an anticancer agent, and several studies have confirmed that it functions as a catalytic/noncleavable complex-forming-type inhibitor of topoisomerase II (Hasinoff et al., 1997, 2001). Dexrazoxane causes an accumulation of closed-clamp conformations of topoisomerase II on DNA (Jensen et al., 2000) and primes leukemic cells to differentiation or apoptosis (Hasinoff et al., 2001). There is therefore renewed interest in dexrazoxane as an antitumor drug, and 96-h continuous infusion schedules have been devised for optimizing its use as a single active agent or cardioprotective agent in combination with DOX (Tetef et al., 2001). On the other hand, there have been reports showing that dexrazoxane may reduce response rates in women receiving DOX for treatment of advanced breast cancer (Swain et al., 1997). The mechanism(s) underlying interference(s) of dexrazoxane with the clinical activity of DOX have not been formally elucidated, such as competition with DOX for topoisomerase II; inhibition of iron-mediated reactions like oxidation of DNA bases, formation of anthracycline-FORM, or

induction of lipid peroxidation and consequent formation of MDA-DNA adducts.

The guidelines for clinical use of dexrazoxane have therefore been revised by the American Society of Clinical Oncology, Chemotherapy, and Radiotherapy Expert Panel in documents produced in 1999 and updated in 2002 (Schuchter et al., 2002). According to the Expert Panel, the use of dexrazoxane is justified and recommended in 1) patients who have received more than 300 mg/m^2 for metastatic breast cancer and who may benefit from continued DOX treatment[4]; 2) patients who have received more than 300 mg/m^2 DOX for treatment of malignancies other than breast cancer; and 3) patients who responded to previous anthracycline-based chemotherapy for advanced breast cancer and who may benefit from continued therapy with EPI.

E. Treatment

At present there is no specific treatment for DOX-related cardiomyopathy. The efficacy of digoxin on DOX-induced cardiomyopathy and CHF is temporary (Singal and Iliskovic, 1998). β-Blockers (e.g., metoprolol, bucindolol, labetalol, and practolol) have been used with some success in children with systolic dysfunction (Hjalmarson and Waagstein, 1994). Angiotensin-converting enzyme inhibitors such as enalapril and captopril may be indicated in patients with elevated afterload and asymptomatic left ventricular dysfunction. However, most recent retrospective studies of DOX-treated long-term survivors of childhood cancer demonstrate that enalapril-induced improvement in left ventricular structure and function is transient and prone to inexorable deterioration, which suggests that any short-term improvement is related to lowered diastolic blood pressure rather than to inhibited myocardium remodeling (Lipshultz et al., 2002b). After all, cardiac transplantation remains a vital option for patients with DOX-induced full-blown cardiomyopathy and CHF (Thomas et al., 2002).

IV. Toward a Better Anthracycline

Strategies for improving the efficacy and/or cardiac safety of currently approved anthracyclines have moved along two medicinal chemistry lines: 1) development of tumor-targeted formulations and 2) development of new analogs.

A. Tumor-Targeted Formulations

According to a seminal definition (Perez-Soler et al., 1995), the pharmacological strategies for achieving tumor-targeted delivery of anthracyclines may be classified into two main groups: 1) development of carriers that assist preferential distribution of anthracyclines within tumors while not exposing healthy tissues to

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[4] Delayed dose analysis of large multicenter placebo-controlled trials shows that dexrazoxane protects also when given to patients who have already received 300 mg of DOX/m^2 (Swain, 1998).

potentially toxic levels of the drug; and 2) conjugation of anthracyclines to a carrier that specifically recognizes tumor cells. Liposomal formulations of DOX or DNR offer best examples of pharmaceutical developments of the first strategy. Extracellularly tumor activated pro-drugs and various forms of polymer-bound DOX offer good examples of pharmaceutical developments of the second strategy. Immunoliposomes cut across the two strategies (Table 4).

1. Liposomal Formulations. Compared with microspheres or nanoparticles, liposomal incorporation of drugs represents the leading method to passively target anthracyclines to tumors. The scientific foundations of using liposome-encapsulated anthracyclines have been the subject of a previous seminal review by Drummond et al. (1999). Briefly, liposome-encapsulated anthracyclines are characterized by 1) reduced V_d and CL and prolonged half-life; 2) limited conversion to aglycones or secondary alcohol metabolites; 3) preferential accumulation in areas (like tumors) characterized by discontinuous "leaky" microvasculature or in organs containing the macrophages of the reticuloendothelial system (liver and spleen); 4) prolonged release of the drug within the tumor environment; 5) partial circumvention of Pgp-mediated tumor resistance; and 6) limited accumulation in healthy tissues (like the heart) with a normal endothelial barrier (Drummond et al., 1999).

Successful achievement of such numerous goals will depend on a complex interplay between the vesicle size of the liposome carrier, its rate of plasma CL, and the stability of the liposome-drug association in the bloodstream or in the tumor environment. The accumulation of liposomes in tumors is favored not only by a leaky microvasculature but also by an insufficient lymphatic drainage. The combination of these two factors is sometime referred to as the "EPR phenomenon" (enhanced permeability and retention) (Drummond et al., 1999).

The mechanisms favoring the release of DOX from within the liposome into the tumor are less clear. The tumor microenvironment contributes to destabilizing the lipid carrier through the action of the slightly acidic pH of interstitial fluids, the release of lipases from dying tumor cells, and the release of enzymes and oxidizing agents by tumor-infiltrating inflammatory cells (Martin, 1998). In addition, phagocytic cells residing in tumors could metabolize liposomes and release free DOX, killing neighboring tumor cells via the bystander effect (Storm et al., 1988). The role of tumor-associated macrophages may nonetheless be more complex than previously believed and probably extends to alternative although poorly understood processes that contribute to increasing tumor vascular permeability (Mayer et al., 1997).

The favorable pharmacokinetics of liposomal DOX, resulting in a delivery of greater quantities of the drug to tumor cells, may also account for its activity in patients refractory to previous anthracycline treatment; for example, a liposomal formulation of DOX was shown to induce partial or even complete responses in patients with AIDS-related Kaposi's sarcoma who had experienced disease progression during the course of DOX/

TABLE 4
Selected advances in tumor-targeted anthracyclines

Carrier	Conjugate	Investigational or Approved Indications
Liposomes		
Pegylated (Doxil - Caelyx)	–	Ovarian cancer, AIDS-related Kaposi's sarcoma, multiple myeloma, high grade gliomas, glioblastoma or secondary brain tumors, head and neck squamous cell cancer, breast cancer, squamous cell carcinoma of the cervix
Uncoated, citrate-containing [Evacet - Myocet (TLC D-99)]	–	Breast cancer
Uncoated (DaunoXome)	–	AIDS-related Kaposi's sarcoma, acute myeloblastic leukaemia, multiple myeloma, non-Hodgkin's lymphoma, breast cancer
Immunoliposomes		
Pegylated or uncoated	Fab' fragments of trastuzumab	Breast cancer
Pegylated	anti-MUC-1 monoclonal antibody	Breast cancer
Pegylated	anti-CD19 monoclonal antibody	Multiple myeloma
Pegylated	Anti-GD(2) ganglioside whole antibodies	Neuroblastoma
Pegylated	Anti-aminopeptidase-N NGR peptide	Neuroblastoma
Peptides or copolymers		
L-377,202	*N*-glutaryl-[4-hydroxyprolyl]-Ala-Ser-cyclohexaglycyl-Glu-Ser-Leu	Prostate cancer
CPI-0004Na	(*N*-Succinyl-Ala-Ala-Leu)	Breast cancer, prostate cancer
Uncoded	Integrin-targeted RGD-4C peptide + plasmin-targeted (Ala-Phe-Lys)	Fibrosarcoma
PK2	tetrapeptide linked to to galactosamine-containing *N*-(2-hydroxypropyl) methacrylamide copolymers	Primary or metastatic liver cancer

All formulations contain DOX, except DaunoXome, which contains DNR.
MUC-1, mucin-1 antigen.

bleomycin/vincristine or bleomycin/vincristine regimens (Northfelt et al., 1997). Partial reversal of drug resistance has been observed also when liposomal DOX or DNR were given to cell cultures (Sadava et al., 2002). Possible clues to explaining circumvention of resistance by liposome-encapsulated anthracyclines include interference(s) with Pgp activity; exposure of cells to higher levels of the anthracyclines over longer periods of time; recruitment of anthracyclines in the endocytosis pathways and consequent reduced recognition by the Pgp transport system in the plasma membrane (Drummond et al., 1999).

The three main liposome-encapsulated anthracycline formulations that have been assessed in experimental models and clinical settings and shown to be superior to free DOX in terms of efficacy and cardiac tolerability are 1) a sterically stabilized polyethyleneglycol-coated formulation containing DOX [Doxil (Caelyx in Europe)] (Schering Plough, Kenilworth, NT); 2) an uncoated formulation in which citrate is included for increasing DOX encapsulation above the levels predicted by the maintenance of a transmembrane pH gradient (Evacet-Myocet-TLC D-99; Elau Pharma International Ltd., Dublin, Ireland); and 3) liposomal DNR (DaunoXome; Gilead, Foster City, CA).

a. Polyethyleneglycol-Coated ("Pegylated") Liposomal Doxorubicin. The highly favorable pharmacokinetics of polyethyleneglycol-coated ("pegylated") liposomal DOX (hereafter referred to as PLD) compared with free DOX have been reviewed (Gabizon et al., 1998). At a dose level of 50 mg DOX/m^2, half-life (first and second exponent of elimination), V_d and CL of PLD versus DOX are 1.4 and 46 h versus 0.06 and 10.4 h, 5.9 versus 254 liters, 0.09 versus 25.3 l/h, respectively. PLD also exhibits a much higher AUC than free DOX (902 versus 3.5 mg · h/l), but this factor should be considered with caution as the AUC of liposomal DOX refers to a drug that is mainly (95%) entrapped inside the carrier and cannot elicit responses inferred from AUC calculated for free drug (Martin, 1998). Difficulties in measuring the rate at which DOX is released from liposomes similarly preclude an accurate determination of AUC for the bioavailable drug in the tumor (Drummond et al., 1999). Despite these technical and conceptual limitations, available data attest to the favorable kinetics of PLD compared with conventional DOX; these were confirmed in a recently completed dose escalation and pharmacokinetic study in children with solid tumors (Marina et al., 2002).

Many trials have been conducted to characterize the antitumor activity, the spectrum of toxicity, and the optimal schedule of administration of PLD. This liposomal anthracycline causes less nausea, vomiting, alopecia, and stomatitis than conventional DOX (Safra et al., 2000; Judson et al., 2001; Harris et al., 2002). However, a frequent adverse event with PLD is skin toxicity, consisting of a hand and foot syndrome (palmar-plantar erythrodysesthesia syndrome). The incidence of this

syndrome depends on the dose and the interval between doses, and generally occurs when the dose intensity exceeds 10 mg/m^2 per week (Johnston and Gore, 2001). For this reason, palmar-plantar erythrodysesthesia has been primarily managed by reducing the dose size and increasing cycle duration.

PLD is active in patients who failed first-line treatment of ovarian cancer with platinum and paclitaxel (Johnston and Gore, 2001), and trials comparing PLD to topotecan (topoisomerase I inhibitor) in these patients have shown that the two drugs are equally effective in terms of disease-free survival, but PLD performs better in terms of progression-free survival and overall survival in platinum-sensitive subgroups (Gordon et al., 2001). PLD has shown good activity also in AIDS-related Kaposi's sarcoma, both in the absence (Hengge et al., 2001) and presence (Nunez et al., 2001) of antiretroviral therapy. These studies confirm the findings of a phase III trial in which PLD induced higher response rates than DOX/bleomycin/vincristine (Northfelt et al., 1998). Phase II studies in patients with multiple myeloma also showed that substituting PLD for free DOX in association with vincristine and reduced dose of dexamethasone improves safety and treatment convenience without compromising efficacy (Hussein et al., 2002).

Further advantages in using PLD have been reported in patients with glioblastoma or secondary brain tumors. A study of the distribution of radiolabeled PLD during radiotherapy showed a significantly higher accumulation of the drug in brain tumor lesions than in the normal brain, suggesting that liposomal DOX overcomes the blood-brain barrier in the tumoral areas (Koukourakis et al., 2001). Single-agent PLD has shown some activity in head and neck squamous cell cancer (Harrington et al., 2001) and in high grade gliomas (Fabel et al., 2001), but very little or no activity has been observed in pretreated advanced recurrent endometrial cancer (Muggia et al., 2002; Escobar et al., 2003), advanced gastric cancer (Thomas et al., 2001), unresectable pancreatic carcinoma (Halford et al., 2001), and anthracycline-resistant breast cancer (Rivera et al., 2002).

PLD causes less cardiotoxicity than conventional DOX. In a retrospective analysis of patients who had reached or exceeded cumulative doses of 500 mg/m^2 (range 500-1500 mg/m^2), there was no evidence of CHF secondary to cardiomyopathy, and only 5 of 42 subjects experienced a drop of 10% or more in LVEF; importantly, three of these had received previous DOX (Safra et al., 2000). In another study the cumulative doses of DOX were high, up to 1500 mg/m^2, but only 1 patient of 45 experienced cardiotoxicity, likely because of previous treatment with mitoxantrone and mediastinal radiotherapy (Lyass et al., 2000). More recently, only a moderate and transient decrease of LVEF was observed in anthracycline-pretreated patients who received PLD-gemcitabine as front line therapy of metastatic breast cancer (Rivera et al., 2003). The cardiac safety of high-

dose PLD in patients is consistent with preclinical studies in which liposome-encapsulated anthracyclines accumulated in rat heart blood vessels between muscle fibers but not within the muscle itself (Working et al., 1994).

The fact that PLD releases very little free DOX in cardiomyocytes anticipates potential advantages in combining it with drugs that would otherwise stimulate metabolization of DOX to the toxic DOXol. PLD is therefore considered for use in combination with PTX, DCT, or carboplatin-PTX (Sparano et al., 2001; Gibbs et al., 2002; Mavroudis et al., 2002; Campos et al., 2003).

b. Uncoated Citrate-Containing Liposomal Doxorubicin. This formulation has pharmacokinetic parameters less pronounced than PLD but still significantly more favorable than those of free DOX (Drummond et al., 1999). The principal indication of uncoated citrate-containing liposomal DOX is treatment of metastatic breast cancer. When used as first-line therapy, this formulation gave the same objective response as conventional DOX but significantly lower cardiotoxicity; thus, median cumulative anthracycline dose at the onset of cardiotoxicity was 785 mg/m^2 for the liposomal formulation versus 570 mg/m^2 for free drug (Harris et al., 2002). Other toxicities, commonly associated with conventional DOX, were also less pronounced with the liposomal formulation, and palmar-plantar erythrodysesthesia was infrequent (Harris et al., 2002). In a randomized phase III study of metastatic breast cancer patients, liposome-encapsulated DOX plus cyclophosphamide gave the same objective response rates as conventional DOX plus cyclophosphamide, but the liposomal formulation was clearly associated with a lower incidence of cardiotoxicity (median cumulative anthracycline dose at onset was more than 2220 mg/m^2 in the liposomal DOX/cyclophosphamide arm versus 480 mg/m^2 in the free DOX/cyclophosphamide arm) (Batist et al., 2001).

c. Liposomal Daunorubicin. The pharmacokinetics of liposomal DNR (DaunoXome) have long been known to be more favorable than those of free DNR. In early phase I/II evaluation in patients with AIDS-related Kaposi's sarcoma, equivalent doses of 80 mg/m^2 DaunoXome or DNR gave half-life, AUC, V_d and CL values of 5.2 versus 0.77 h, 375 versus 10 mg · h/l, 2.9 versus >1000 liters, and 0.4 versus 13 liters, respectively (Forssen and Ross, 1994; Gill et al., 1995). DaunoXome has been approved by the Food and Drug Administration as a first-line therapy of AIDS-related Kaposi's sarcoma, and it shows activity and tolerability as a single agent or in combination with ara-C for treatment of patients with refractory or relapsed acute myeloblastic leukemia (Cortes et al., 2001; Fassas et al., 2002). It has also been assessed in combination with dexamethasone [for treatment of patients with recently diagnosed or recurrent/refractory multiple myeloma, showing activity comparable with standard regimens (Mohrbacher et al., 2002)] and in combination with cyclophosphamide/vincristine/prednisolone [as salvage therapy in poor-prognosis non-

Hodgkin's lymphoma, showing as effectively as the same regimen containing DOX (McBride et al., 2001)].

Recently, a phase I dose-escalating study showed that DaunoXome was active in metastatic breast cancer, giving asymptomatic cardiotoxicity only in a few patients exposed to cumulative doses of 800 to 960 mg/m^2. At lower cumulative doses ($\sim$600 mg/m^2) cardiotoxicity only occurred in patients who had received 300 mg/m^2 neoadjuvant DOX (O'Byrne et al., 2002). Improved cardiac tolerability and activity in both solid and hematological malignancies anticipate broader clinical applications of DaunoXome in the near future.

d. Immunoliposomes. The technique of immunoliposomes conjugates the specificity of whole monoclonal antibodies or FAB′ fragments with the favorable pharmacokinetics and drug delivery of liposomes. A pioneering and elegant approach exploited conjugation of uncoated or pegylated liposomes to Fab′ fragments of trastuzumab to create immunoliposomes targeting HER2/neu-overexpressing cancers. Anti-HER2/neu liposomal DOX selectively bound to HER2/neu-positive cells, favoring internalization of DOX in the target cells compared with less productive accumulation of liposome-encapsulated DOX in the interstitium (Kirpotin et al., 1997; Drummond et al., 1999). When assessed in animals xenografted with human breast cancer cells, anti-HER2/neu PLD showed higher antitumor efficacy than DOX, reduced systemic toxicity, and the same prolonged circulation of nontargeted PLD without any apparent drug leakage or monoclonal antibody dissociation (Park et al., 2002). The unique efficacy of anti-HER2/neu PLD was confirmed by the fact that the complete formulation exhibited higher activity than either PLD, trastuzumab alone, trastuzumab plus free DOX, or trastuzumab plus liposomal DOX.

Other immunoliposomes, combining DOX with a monoclonal antibody against the mucin-1 antigen, have been assessed in pseudometastatic and metastatic mice models of breast cancer. The formulation was effective in treating early lesions in both the pseudometastatic and metastatic models, but limitations in the access to tumor cells in the primary tumor compromised its therapeutic efficacy in treating the more advanced lesions (Moase et al., 2001). Other investigators assessed the therapeutic applicability of using liposome-encapsulated DOX targeted against the internalizing CD19 antigens present on human multiple myeloma cells. The formulation proved to be superior to either free DOX or nonimmunotargeted liposomal DOX in eliminating CD19$^+$ B cells in a heterogeneous mixture of peripheral blood mononuclear cells from multiple myeloma patients (Lopes de Menezes et al., 2000). Excellent results were most recently obtained in mice xenografted with human neuroblastoma cells and treated with PLD covalently bound to Fab′ fragments of antibodies raised against the disialoganglioside GD$_2$ specifically overexpressed by these tumor cells (Pastorino et al., 2003b). Equally good results

were obtained by coupling liposomal DOX with an NGR peptide (Asn-Gly-Arg) that targets aminopeptidase-N, a marker of tumor-associated angiogenic endothelial cells (Pastorino et al., 2003a). All such findings clearly call for further refinements in investigational settings, especially with reference to an improved cardiac safety of antiHER2/neu PLD versus conventional DOX/trastuzumab regimens, but their development in clinical studies is highly attractive.

2. Extracellularly Tumor-Activated Prodrugs An alternative to liposomal preparations involves the synthesis of prodrugs that are unable to enter normal cells but are proteolytically activated by peptidases secreted by cancer cells [extracellularly tumor-activated prodrugs (ETAP)]. A good example is offered by L-377,202, a prodrug obtained by covalently linking DOX to *N*-glutaryl-[4-hydroxyprolyl]-Ala-Ser-cyclohexaglycyl-Glu-Ser-Leu (Fig. 13). In the presence of prostate cancer cells secreting the serine protease prostate-specific antigen (PSA), the peptide moiety of L-377,202 is hydrolyzed to release DOX or Leu-DOX, the latter being freely diffusible and activated to DOX inside the target cells. Studies in vitro have shown that L-377,202 is considerably more toxic to PSA-positive tumor cells than PSA-negative normal or tumor cells (Denmeade et al., 1998; DeFeo-Jones et al., 2000). Moreover, L-377,202 is several times more active than conventional DOX in human prostate cancer xeno-

grafts while also inducing less severe systemic or cardiac-specific toxicity. The improved therapeutic index of L-377,202 in animal models correlates with preferred hydrolysis and release of DOX or Leu-DOX in PSA-positive tumor foci (Wong et al., 2001). L-377,202 has been recently assessed in a phase I study in 19 patients with prostate cancer. At the recommended dose of 225 mg/m^2, equivalent to 90 mg of DOX/m^2 on a molar basis, two patients had a greater than 75% decline in PSA. A preferential delivery of DOX to PSA-positive tumor cells was not measured, but toxicity data indicated that it was possible to safely deliver about 1.5-fold more anthracycline compared with the conventional formulation on a molar basis (DiPaola et al., 2002).

Another ETAP with good prospects of clinical development is a tetrapeptidic derivative of DOX code-named CPI-0004Na (*N*-succinyl-β-Ala-L-Leu-L-Ala-L-Leu-DOX) (Fig. 14). CPI-0004Na does not undergo hydrolysis in blood nor does it enter the cells, but it is activated by tumor peptidases to give *N*-(L-Leu-DOX), which eventually diffuses inside the cells as described for PSA-hydrolyzed L-377,202. In mice, CPI-0004Na is significantly less toxic than DOX due to its lower accumulation in the heart and other normal tissues (Trouet et al., 2001). Thanks to its improved selectivity, higher doses of CPI-0004Na than of DOX could be administered to nude mice bearing xenografts of human breast and colon tumors. In these models CPI-0004Na showed significantly higher antitumor activity than conventional DOX did. Tissue distribution and pharmacokinetic studies were also performed, and they confirmed that the improved activity and toxicity profile of the prodrug is due to the selective release of DOX at the tumor level (Dubois et al., 2002).

The newest member of the ETAP family is a DOX prodrug that incorporates both a tumor-specific recognition site and a tumor-selective enzymatic activation sequence. The first tumor-specific sequence is the bicyclic CDCRGDCFC (RGD-4C) peptide that selectively binds to $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins highly overexpressed in invading tumor endothelial cells. The second tumor-specific sequence is a D-Ala-Phe-Lys tripeptide recognized by the tumor-associated protease plasmin, an important factor of tumor invasion and metastasis. An aminocaproyl residue was incorporated as a spacer between the
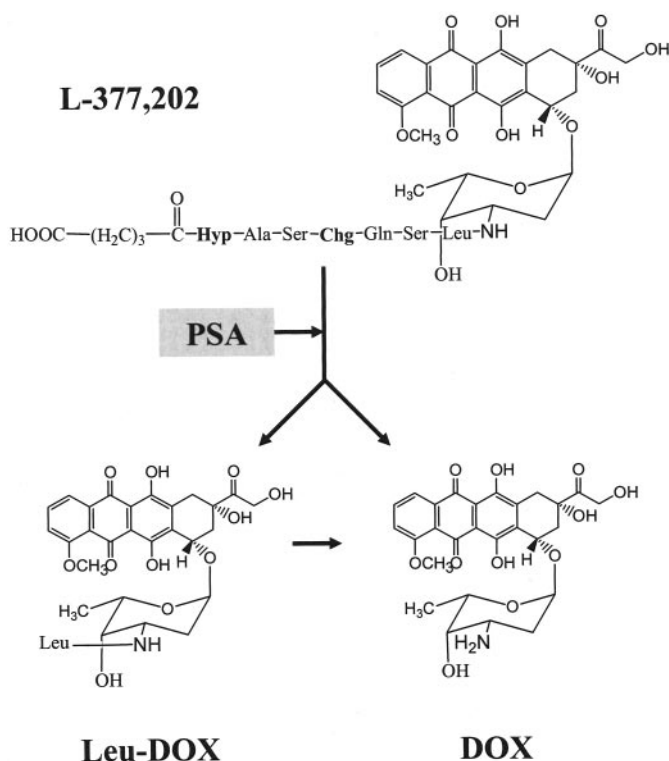
FIG. 13. PSA-dependent hydrolysis of L-377,202. L-377,202 is hydrolyzed to DOX or Leu-DOX by prostate cancer cells, which secrete the serine protease PSA. Leu-DOX is freely diffusible and is further converted to DOX inside the cells. Hyp, *trans*-4-hydroxyproline; Chg, L-cyclohexylglycine. Based on Denmeade et al. (1998), DeFeo-Jones et al. (2000), Wong et al. (2001).
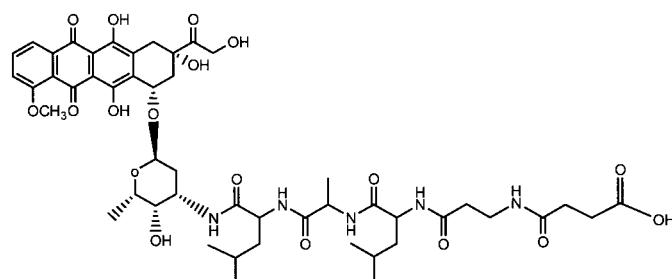
FIG. 14. Structure of CPI-0004Na (*N*-succinyl-β-Ala-L-Leu-L-Ala-L-Leu-DOX).

two peptide sequences, whereas a self-eliminating 4-aminobenzyl alcohol spacer was inserted between the plasmin substrate and DOX. In vitro, this prodrug shows plasmin-dependent cytotoxicity for endothelial cells and HT1080 fibrosarcoma cells, but improvements in water solubility and bioavailability are needed prior to its evaluation in more complex preclinical models (de Groot at al., 2002).

3. Polymer-Bound Doxorubicin Another interesting approach for improving targeted delivery of DOX to a given neoplasm may be that of linking the anthracycline to polymers recognized by receptors expressed in that particular tumor. A good example of this strategy is offered by PK2, a formulation in which DOX is linked via a lysosomally degradable tetrapeptide sequence to *N*-(2-hydroxypropyl)methacrylamide copolymers bearing a galactosamine residue that is recognized by the hepatic asialoglycoprotein receptor (Fig. 15). Results of a phase I trial in 31 patients with primary or metastatic liver cancer showed partial responses in three patients as well as significant decrease of serum α-fetoprotein levels in two additional patients. Very low levels of nonpolymer bound DOX could be detected in the bloodstream, and the study of body distribution of the radiolabeled polymer showed that PK2 selectively accumulated in the liver, allowing for the attainment of higher concentrations of DOX also in primary hepatocarcinomas (Seymour et al., 2002). In rats PK2 induced no or considerably less severe cardiotoxicity than equivalent doses of free DOX, suggesting that selective tumor targeting and improved cardiac safety might be observed in future clinical studies if PK2 were given at doses reaching or exceeding the conventional threshold of 500 mg/m^2 (Hopewel et al., 2001).

B. Analogs

The search for novel anthracyclines has been influenced by appreciation of the role of the aminosugar in stabilizing drug intercalation into DNA. New generation analogs were therefore designed during the course of studies aimed at elucidating the impact of this critical moiety on the pharmacological and toxicological properties of anthracyclines. Promising agents obtained by this strategy include anthracyclines with morpholinyl or alkyl substituents at the amino group at C-3′, and disaccharide anthracyclines in which the amino group is displaced to the second sugar. Another class of anthracyclines has been obtained by combining modifications at C-14 with modifications of the aminosugar. This latter strategy has produced congeners that no longer target DNA but potentiate apoptotic events modulated by protein kinase C (PKC).

The two strategies (nuclear-targeted versus non-nuclear-targeted analogs) will predictably influence further developments in the field of anthracycline designing. For those anthracyclines targeted at DNA, preserving the quinone-hydroquinone chromophore will remain a structural requirement. For those anthracyclines targeted at PKC or other extranuclear sites, preserving a canonical chromophore might not represent an absolute requirement, at least in principle.

Here, we will review current information on anthracyclines obtained by either strategy, particular attention being paid to those analogs that reached the stage of clinical evaluation or exhibited experimental activities that warrant validation in clinical settings.

1. Nuclear-Targeted Anthracyclines. We have discussed that anthracycline-FORM conjugates, whether preformed or generated intracellularly, offer a good example of how a given analog can gain improved nuclear targeting (see *Section II.B.5.*). A more classical approach to achieving the same kinetic advantage has been that of replacing the 3′-amino group with hydroxyl or acyl substituents. Hydroxyl substitution, like that introduced in 3′-hydroxy-DOX, does not alter the usual pattern of topoisomerase II-mediated DNA cleavage and cytotoxicity but poses problems of the drug's solubility and administration; on the other hand, acyl substitutions often reduce topoisomerase II interaction and cytotoxicity. A successful alternative involves cyclic substitutions that do not only preserve drug's cytotoxicity but also modify the mechanisms by which cytotoxicity occurs.

a. Morpholinyl Anthracyclines. Morpholinyl derivatives of DNR [MX 2 (KRN 8602)], morpholinyl, methoxymorpholinyl (MMRA), or cyanomorpholinyl derivatives
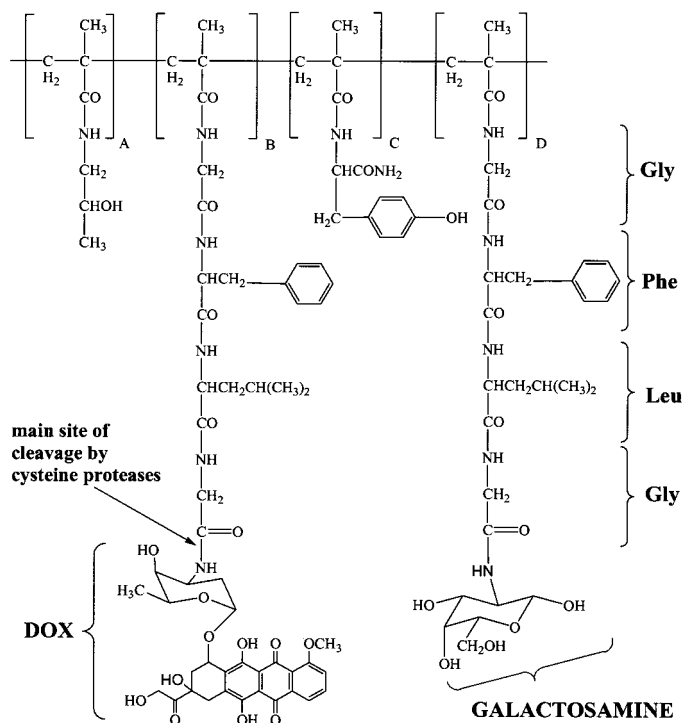
FIG. 15. Structure of PK2. PK2 is a copolymer of *N*-(2-hydroxypropyl)methacrylamide bearing DOX and galactosamine, the latter being recognized by the hepatic asialoglycoprotein receptor. DOX is released after cleavage of the bond between Gly and the aminogroup at C-′3 in daunosamine, mediated by lysosomal cathepsin-type cysteine proteases. Based on Seymour et al. (2002).

of DOX (Fig. 16) bind to DNA through a mechanism involving rearrangement/opening of the morpholino ring which then binds to DNA with a structure reminiscent of N-(2-hydroxyethyl)DOX. In the case of cyanomorpholinyl anthracyclines, the cyano group is released.

Interaction of rearranged morpholinyl anthracyclines with DNA aligns the amino group in the minor groove as a requirement for DNA alkylation. Cytotoxicity induced through this novel pathway of DNA binding and alkylation does not associate with topoisomerase II-mediated DNA cleavage but involves enhanced topoisomerase I-mediated DNA cleavage and DNA cross-linking. In vitro, this is accompanied by a ~100-fold higher potency of morpholinyl anthracyclines compared with DOX.

Morpholino-derivatives of DOX or DNR share common features like circumvention of multidrug resistance-related protein (MRP)-1-mediated resistance (Bakker et al., 1997), gain of activity after metabolization by CYP3A isoform(s) of cytochrome P 450 (Lewis et al., 1992), and reduced cardiotoxicity in animal models in face of sustained oxyradical generation (Sato et al., 1991). This latter finding either offers one more argument against the involvement of ROS in anthracycline-induced chronic cardiotoxicity or suggests that ROS are formed at cellular sites that are not critical to the development of cardiac dysfunction (Sato et al., 1991).

Studies of the relationships between different chemical modifications on morpholinyl anthracyclines and their ability to overcome resistance showed that an insertion of morpholinyl or methoxymorpholinyl groups at position 3′ of the sugar moiety confers the ability to overcome resistance in vitro and in vivo, whereas dislocation of the morpholinyl residue to C′4 was accompanied by loss of activity in vivo. On the other hand, 3′ morpholinyl or methoxymorpholinyl anthracyclines bearing substituents in the aglycone or at position 2 of the morpholino ring were as effective as their parent compounds in both sensitive and resistant models (Ripamonti et al., 1996). These observations formed the basis to conclude that position 3′ in the sugar moiety
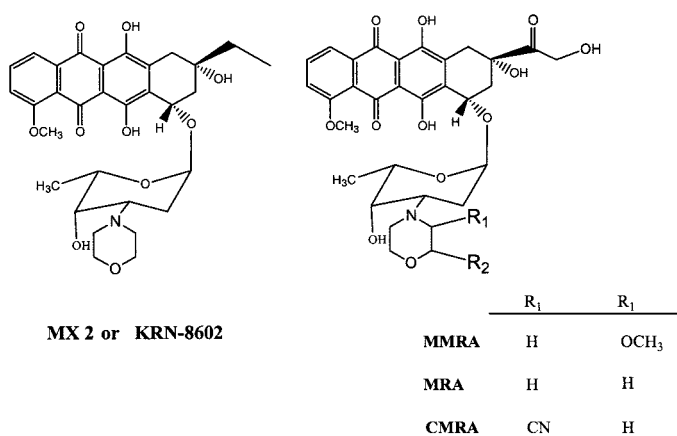
plays a crucial role in the ability of morpholinyl-anthracyclines to overcome resistance. Studies conducted by reconstituting purified MRP-1 in lipid vesicles suggest that the morpholino group enables anthracyclines to immobilize this carrier in a conformational status that does not allow ATP binding and consequently precludes ejection of the drug in extracellular fluids (Manciu et al., 2001).

Only MX 2 (KRN 8602) and MMRA (FCE 23762/PNU-152243, also referred to as nemorubicin) have been evaluated in clinical trials. In adult patients with newly diagnosed acute myelogenous leukemia, MX 2/ara-C was as effective as DNR/ara-C but induced more severe gastrointestinal and central nervous system toxicity, likely due to the ability of MX 2 to cross the blood-brain barrier. However, there was no cardiotoxicity in MX 2/ara-C patients, as opposed to a dose-dependent incidence of arrhythmias, decrease of LVEF, or overt cardiac failure induced by DNR in combination with ara-C (Takemoto et al., 1999).

The ability of MX 2 to partition in the central nervous system while not inducing cardiotoxicity was exploited to assess its safety and efficacy for treatment of patients with malignant gliomas. Two independent studies have shown promising activity in this setting (overall response rate, between 30 and 43%); noncardiac toxicity was usually mild or clinically manageable, and no evidence of cardiotoxicity was reported in either study (Clarke et al., 1999; Kuratsu et al., 2000).

MMRA (FCE 23762/PNU-152243) has been evaluated in a phase II and pharmacokinetic study of patients with chemotherapy resistant solid tumors (Bakker et al., 1998). When given by i.v. bolus, the drug exhibited extensive clearance as well as rapid and extensive distribution into tissues. The response rate was low, but hematologic or hepatic toxicities were frequent; moreover, greater than 15% reduction of LVEF occurred in 2 patients of 48. Essentially similar response rate and noncardiac toxicities were observed when PNU-152243 was given by slow infusion, but cardiotoxicity was apparently negligible (Fokkema et al., 2000). The safety and efficacy of MMRA clearly require further evaluation.

b. Alkyl Anthracyclines. Alkyl anthracyclines are obtained by introducing alkylating substituents at position C-3′ of the aminosugar of IDA. Among them, PNU-159548 is characterized by the presence of an aziridinyl moiety at C-3′ and esterification of −OH at C-4′ with a methylsulfonic group (Fig. 17). Alkyl anthracyclines do not interact with topoisomerase II but do cause DNA intercalation via the anthracycline backbone and bind covalently to guanines at the N7 position and adenines at the N3 position via the reactive alkylating group in the sugar. PNU-159548 shows good activity in murine and human cancer cells in vitro and strong antitumor efficacy in vivo, after both i.v. and p.o. administration against rapidly proliferating murine leukemias or several human tumor xenografts. In addition, PNU-159548

	R₁	R₁
MMRA	H	OCH₃
MRA	H	H
CMRA	CN	H

MX 2 or KRN-8602

FIG. 16. Structures of morpholinyl, methoxymorpholinyl, or cyanomorpholinyl derivatives of DOX or DNR.
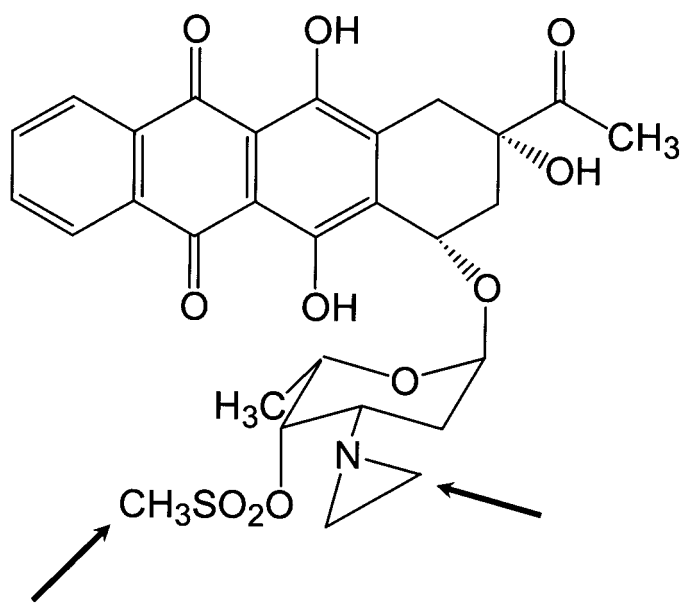
FIG. 17. Structure of PNU-159548. The arrows indicate esterification of −OH at C-4′ with methylsulfonate and substitution of an aziridinyl moiety for the aminogroup at C-3′.

is able to cross the blood-brain barrier and to delay the growth of intracranially implanted tumors (Geroni et al., 2001). As expected, based on its mechanisms of action, PNU-159548 is effective in tumors bearing alterations of topoisomerase II levels and activity; it also shows activity in cells overexpressing the MDR-1 gene and in tumors resistant to alkylating agents like cisplatin, cyclophosphamide, or melphalan (Geroni et al., 2001; Marchini et al., 2001). The latter finding suggests that PNU-159548 could preferentially bind to DNA regions different from those attacked by conventional alkylating agents, or that other intracellular targets may be involved in its mechanism of action.

Interestingly, PNU-159548 does not gain further activity in cells defective in nucleotide excision repair, a finding which should be opposed to an increased activity of cisplatin or melphalan in the same cells (Marchini et al., 2001). These results are in contrast with the strong alkylating activity of PNU-159548 toward naked DNA and suggest that, in vivo, the drug induces a more modest extent of DNA alkylation. This mode of action would be similar to that of minor-groove binders, which have been reported to produce a lower number of alkylations as compared with classical major-groove alkylating agents like cisplatin or melphalan (Marchini et al., 1998). Otherwise, it is possible that other repair enzymes are required for removing the adducts induced by PNU-159548 treatment. Of particular note is the fact that PNU-159548 shows activity in both mismatch repair-proficient and -deficient cells (Marchini et al., 2001). This may confer advantages on PNU-159548, as the loss of mismatch repair often associates with resistance to DOX and major alkylating agents or minorgroove binders (Fink et al., 1998).

In the rat, PNU-159548 induces chronic cardiotoxicity which is less than one-20th of that caused by an equimyelotoxic dose of DOX and significantly milder than that predicted based upon the equivalent dose of IDA present in the drug's backbone (Della Torre et al., 2001). These findings have been explained, at least in part, by the high plasma clearance of PNU-159548 (Marchini et al., 2001).

Because of its promising profile of activity and cardiac safety, PNU-159548 has already entered clinical evaluation. A pharmacokinetic phase I study showed adverse effects like hypersensitivity reactions and especially thrombocytopenia, the latter correlating with AUC (de Jonge et al., 2002). PNU-159548 will be evaluated in phase II studies of both heavily and minimally pretreated patients.

c. Disaccharide Anthracyclines. In an attempt to explore the effects of the aminogroup of daunosamine while also improving the pharmacological properties of anthracyclines, Animati et al. (1996) designed 4-methoxy or demethoxy analogs of DNR in which the sugar attached to the aglycone lacked the aminogroup, and daunosamine was dislocated to become the second sugar via an $\alpha(1–4)$ linkage. Structure-activity studies in experimental solid tumors showed that replacing daunosamine with a disaccharide moiety dramatically reduced the cytotoxic potency of the drugs in the 4-methoxy series. In contrast, in the 4-demethoxy series, the C-4 axial configuration (but not the equatorial configuration) conferred a cytotoxic potency and antitumor activity severalfold higher than that of DNR or IDA and virtually comparable with that of DOX (Arcamone et al., 1999). The configuration also influenced the ability of disaccharide anthracyclines to stimulate topoisomerase IIα-mediated DNA cleavage. Only glycosides with the axial orientation proved to be active, but methoxy-substituted analogs were significantly less effective than demethoxy analogs. On balance, it became apparent that the axial orientation governed optimal interactions of disaccharide anthracyclines with the DNA-topoisomerase II complex only in the absence of the methoxy group (Arcamone et al., 1999).

An extended synthesis of demethoxy anthracyclines with axial configuration of the disaccharide moiety produced an analog code-named MEN 10755 (currently given the generic name of sabarubicin). As shown in Fig. 18, MEN 10755 is a 4-demethoxydoxorubicin characterized by insertion of 2,6-dideoxy-L-fucose between the aglycone and daunosamine. Studies in human tumor xenografts revealed that MEN 10755 was more effective than DOX at poisoning topoisomerase II and stimulating DNA fragmentation. MEN 10755 did not overcome the MDR phenotype, a feature shared by other disaccharide analogs of the same family; however, MEN 10755 was remarkably active in xenografts of MX-1 breast carcinoma cells that overexpressed Bcl-2 and exhibited modest or delayed apoptotic responses to DOX (Ar-
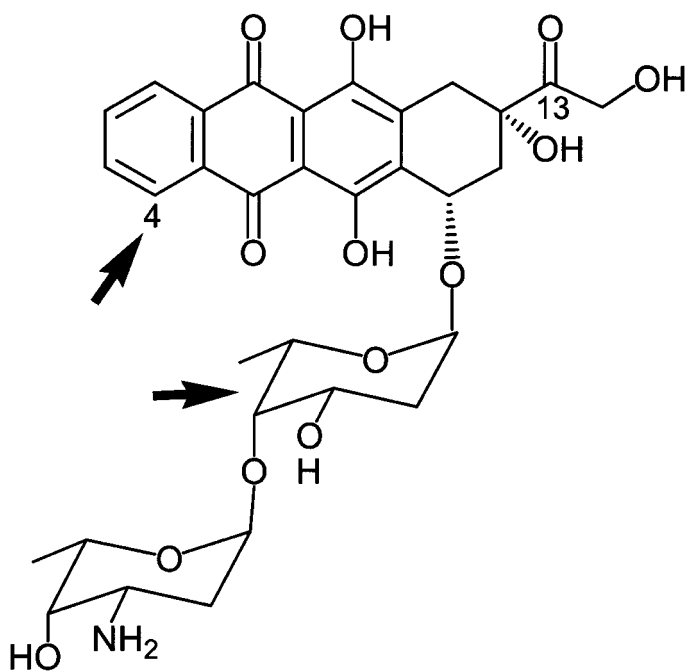
FIG. 18. Structure of MEN 10755. The arrows indicate the lack of the methoxy group at C-4 in the aglycone and the presence of 2,6-dideoxy-L-fucose between the aglycone and daunosamine.

camone et al., 1997; Pratesi et al., 1998). The different behavior of the two anthracyclines in this tumor seemed to reflect an ability of MEN 10755, but not of DOX, to phosphorylate Bcl-2 (Pratesi et al., 1998; Perego et al., 2001). Although controversy exists on the pro- or anti-apoptotic consequences of Bcl-2 phosphorylation (Kharbanda et al., 2000; Tournier et al., 2000; Deng et al., 2001), the activity of MEN 10755 in MX-1 xenografts clearly reflected phosphorylation events. In fact, Bcl-2 phosphorylation always occurred when this tumor underwent apoptosis induced by drugs endowed with different mechanisms of action (e.g., taxanes and platinum compounds) (Pratesi et al., 1998).

Early during the course of preclinical evaluation, MEN 10755 proved to exhibit high activity in the face of reduced intracellular accumulation (Arcamone et al., 1997) and preferential localization to the cytosol, giving cytoplasmic/nuclear ratios significantly higher than those of DOX (Bigioni et al., 2001). Despite its reduced cell penetration and lower nuclear concentration, MEN 10755 was as potent as DOX in eliciting DNA single- and double-strand breaks, G_2/M cell arrest, and apoptosis. This could not be attributed to an unique pattern of DNA cleavage, as sequencing of drug-induced topoisomerase II cleavage sites showed a common presence of adenine in −1 position for both DOX and MEN 10755 (Bigioni et al., 2001). Recently, the crystal structure of the complex between MEN 10755 and the DNA hexamer d(CGATCG) has been solved. The structure is similar to previously crystallized anthracycline-DNA complexes, but two different binding sites seem to arise from drug intercalation. The two halves of the self-complementary duplex

are, therefore, no longer equivalent. In one site, both sugar rings reside in the minor groove; in the other site, the second sugar protrudes out from the DNA helix and is linked, through hydrogen bonds, to guanine of a symmetry-related DNA molecule. This is the first report of an anthracycline-DNA complex in which the drug interacts with a second DNA helix, and it offers new clues to explaining the extent and persistence of DNA cleavage induced by MEN 10755 (Temperini et al., 2003).

Whereas extensive and long-lived DNA breaks contributed to the cytotoxic effects of MEN 10755, attempts to correlate them to the greater efficacy of MEN 10755 at inducing apoptosis were troublesome, as if cellular targets other than topoisomerase II were also involved. Attempts to identify such targets have been unsuccessful so far. In human ovarian cancer cells, both DOX and MEN 10755 up-regulated Fas expression, but anti-Fas antibodies did not inhibit apoptosis. Moreover, neither MEN 10755 nor DOX induced apoptosis in cytoplasts (i.e., cells deprived of the nucleus but retaining intact mitochondrial function), nor could either anthracycline induce apoptosis if the synthesis of some specific protein factor(s) was inhibited by cycloheximide. Bax and caspase-3 clearly executed apoptosis, but this was seen with both DOX or MEN 10755 (Bellarosa et al., 2001).

In a recent study of the same cell line and other carcinoma cells, MEN 10755 was shown to activate both NF-κB and p53 transcription factors, but only p53-controlled genes underwent up- or down-regulation according to the known action of p53 on their promoters. The expression of NF-κB-dependent genes remained unaltered, and neither did pharmacologic inhibition of NF-κB modify apoptosis induced by MEN 10755, as if NF-κB were nonfunctional or bound to unproductive DNA sites (Camarda et al., 2002). Whether dissociations in p53- versus NF-κB-dependent pathways are unique to MEN 10755 or to the tumor type under investigation remains to be established. MEN 10755 was also undoubtedly more active than DOX in xenografts derived from p53-mutant or p53-null small-cell lung carcinoma, or prostatic carcinoma cell lines (Perego et al., 2001). This may confer further advantages on MEN 10755 compared with DOX.

In addition to exhibiting an enlarged spectrum of activity, MEN 10755 also shows reduced toxicity in the heart. In the rat, MEN 10755 produced milder ECG alterations, smaller impairment of contractile response to β-adrenergic stimulation, and less severe myocyte lesions than those observed after treatment with equimyelotoxic doses of DOX; moreover, morphologic and functional abnormalities induced by DOX worsened with time, whereas the effects of MEN 10755 were not progressive (Cirillo et al., 2000). The cardiac safety of MEN 10755 versus DOX was attributed to pharmacokinetic factors, as studies in nude mice xenografted with human tumors showed that MEN 10755 had limited distribution not only in tumor tissues but also, more

remarkably, in the heart and other normal tissues (Gonzalez-Paz et al., 2001). Thus, it seemed that an unfavorable cellular uptake did not preclude MEN 10755 from eliciting antitumor activity, but it strongly diminished its cardiotoxicity.

Studies with human cardiac cytosol and rat isolated heart now show that the cardiotoxic potential of MEN 10755 is also limited by its impaired conversion to the secondary alcohol metabolite MEN 10755ol (Minotti et al., 2000, 2001a). Moreover, MEN 10755ol is characterized by reduced reactivity toward the Fe-S cluster of aconitase/IRP-1, a behavior due to steric interferences introduced by the presence of a disaccharide moiety (Minotti et al., 2000). The altered [Fe-S] cluster reactivity of MEN 10755ol might therefore contribute to further diminishing the cardiotoxicity of MEN 10755 if one assumed that changes in aconitase/IRP-1 activity were involved in mediating cardiac injury. Accordingly, studies of a rat chronic model of cardiotoxicity show that EPI is less cardiotoxic than DOX due its limited conversion to EPIol, whereas MEN 10755 is less cardiotoxic than both DOX and EPI due to multiple reductions in uptake, alcohol metabolite formation, and irreversible inactivation of the [Fe-S]-containing aconitase (Sacco et al., 2003) (Fig. 19).
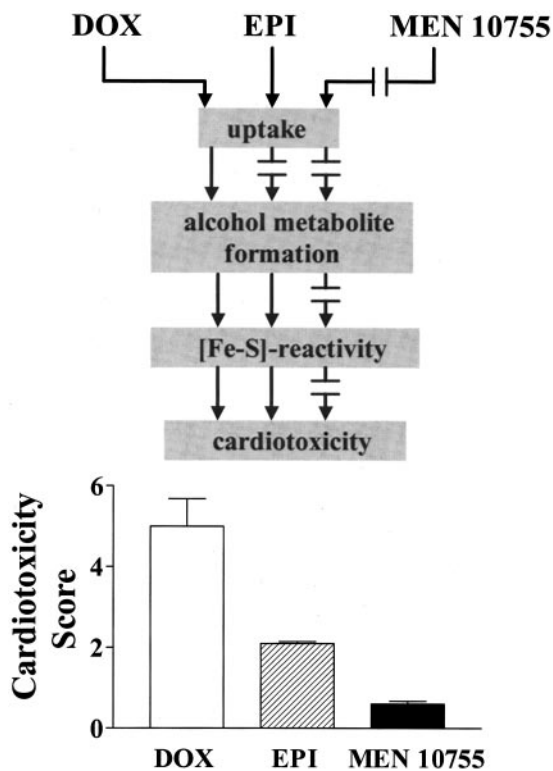
FIG. 19. Pharmacokinetic and molecular determinants of reduced chronic cardiotoxicity of MEN 10755 versus DOX and EPI in the rat. Limited conversion to alcohol metabolite reduces chronic cardiotoxicity induced by EPI compared with DOX, as evidenced by ≥50% reduction of the cardiotoxicity score (histologic lesions) at 13 weeks after treatment completion. MEN 10755 is less cardiotoxic than both DOX and EPI due to multiple reductions in uptake and alcohol metabolite formation and [Fe-S] reactivity. Based on Minotti et al. (2000, 2001a), Sacco et al. (2003).

In principle, a reduced level of formation of MEN 10755ol might contribute also to improving the antitumor activity of MEN 10755 compared with DOX. In fact, studies of cancer cells overexpressing carbonyl reductases indicate that secondary alcohol metabolites diminish sensitivity to anthracyclines (Gonzalez et al., 1995; Ax et al., 2000). The mechanism(s) by which secondary alcohol metabolites impair the activity of their parent drugs in cancer cells have not been formally elucidated.

MEN 10755 has been evaluated in clinical trials. A pharmacokinetic phase I study has been conducted in adults with solid refractory malignancies. MEN 10755 is characterized by an approximately 2-fold shorter terminal half-life, much lower total plasma CL, and much smaller V_d than DOX; moreover, very little MEN 10755ol is detected in plasma, consistent with an in vitro evidence for an intrinsic resistance of MEN 10755 to carbonyl reduction (Bos et al., 2001). In a phase I study of 24 patients with advanced solid tumors, two patients developed asymptomatic decrease of LVEF to below 50%, one after 11 cycles of MEN 10755 at the weekly dose of 30 mg/m², and one after three cycles at the dose of 45 mg/m². Six patients had stable disease, but no objective responses were seen (Schrijvers et al., 2002). In view of the fact that only one patient received more than four cycles of therapy, no conclusions on the cardiotoxic potential of the drug can be drawn at this time. The issue of cardiotoxicity will therefore be re-addressed in forthcoming phase II trials (Broker and Giaccone, 2002).

2. Non-Nuclear-Targeted Anthracyclines: 14-O-Acyl-anthracyclines. 14-*O*-acylanthracyclines are obtained by combining modifications on the aminogroup at C-3′ with addition of alkylesters, preferably 5-carbon moieties, at C-14. This approach has produced two lead compounds, *N*-benzyldoxorubicin-14-valerate (AD 198) and *N*-trifluoroacetyldoxorubicin-14-valerate (AD 32, valrubicin) (Fig. 20). The valerate moiety results in a marked increase in lipophilicity and rapid cellular penetration, an effect contributed to also by derivatization of the charged aminogroup with benzyl or trifluoroacetyl moieties. Moreover, valerate substitution at C-14 modifies the pattern of subcellular localization of the anthra-
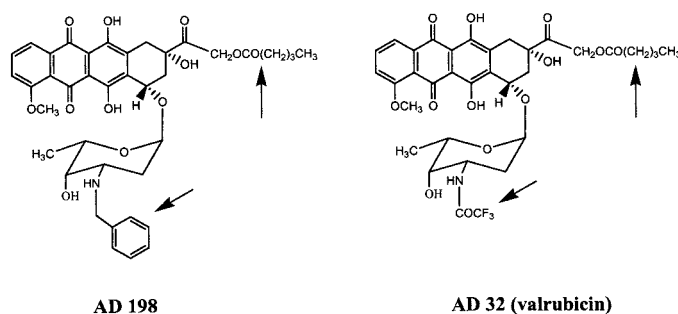
FIG. 20. Structures of AD 198 and AD 32 (valrubicin). The arrows indicate esterification of the side chain with valerate and derivatization of the C-3′ amino group with benzyl (AD 198) or trifluoroacetyl (AD 32) moieties.

cyclines, favoring localization of AD 198 and AD 32 in the perinuclear cytoplasmic region rather than in the nucleus (Lothstein et al., 1998, 2001).

Despite their poor penetration in the nucleus, both AD 198 and AD 32 were soon recognized to induce cytotoxicity which was at least equal to, if not greater than, that induced by DOX in several tumor cells. Detailed investigations also showed that AD 198 induced tumor cell apoptosis by mechanisms distinct from those of its valerate-free metabolite *N*-benzyldoxorubicin (AD 288), a DNA-intercalating agent and topoisomerase II inhibitor whose apoptotic potential was remarkably delayed by expression of Bcl-2 and Bcl-XL. In fact, AD 198 had little inhibitory activity on topoisomerase II and did not effectively intercalate into DNA; moreover, apoptosis induced by AD 198 was essentially unimpeded by Bcl-2 and Bcl-XL expression (Lothstein et al., 2001).

These observations formed the basis to conclude that AD 198 acted independently of its conversion to AD 288, presumably by targeting cell constituents located in the cytoplasm. One such target has been identified in the C1 regulatory domain of PKC isozymes, as the alkylester moiety at C-14, in combination with the A and B ring components of the anthraquinone chromophore, share spatial homologies with PKC activators like diacylglycerol and the phorbol ester 12-myristate 13-acetate (Roaten et al., 2002). The current thinking is that such spatial homologies enable AD 198 to induce rapid translocation of PKC-α and -δ in intact cells. PKC-δ has an established role as a proapoptotic protein through the association of either the holoenzyme or its catalytic fragment with mitochondria; therefore, rapid translocation and activation of PKC-δ in mitochondria, induced by AD 198, promote apoptosis via cytochrome *c* release and caspase-3 activation (Barrett et al., 2002). The fact that Bcl-2 and Bcl-XL fail to prevent apoptosis induced by AD 198 does not reflect the ability of this novel anthracycline to hyperphosphorylate or promote caspase-mediated cleavage of antiapoptotic factors. Rather, it would appear that the apoptotic signaling induced by AD 198 through translocation and/or activation of PKC-δ is potent enough to overrule the antiapoptotic effects of Bcl-2 (Barrett et al., 2002).

An additional important feature of AD 198 pertains to its ability to bypass drug resistance due to reduced topoisomerase II activity, a finding consistent with the fact that this drug is not targeted at topoisomerase II; moreover, AD 198 is lipophilic enough to escape extracellular transport by Pgp or MRP-1, and structure-activity studies have shown that this occurs only when both *N*-benzyl and 14-*O*-valerate are attached to the anthracycline. The presence of just one moiety or the other does not confer any particular advantage in circumventing Pgp- or MRP-1-mediated resistance, explaining a reduced activity of AD 288 in cells that express these transport systems (Lothstein et al., 1992).

AD 32 (valrubicin) shares some features with AD 198, such as preferred localization in the cytosol and lack of topoisomerase inhibitory activity. Studies performed in animal models of human head and neck squamous cell carcinoma suggest that valrubicin may be a catalytic inhibitor of PKC (Wani et al., 2000). This is also indicated by the fact that valrubicin synergizes with sublethal doses of ionizing radiations in head and neck squamous carcinoma cells (Wani et al., 2000), a behavior shared with established PKC inhibitors like calphostin, staurosporine, and PKC-412 (Rocha et al., 2000). On the other hand, the valerate-free metabolite of AD 32 [*N*-trifluoroacetyldoxorubicin (AD 41)] is a nuclear-targeted topoisomerase I/II poison whose activity may be hampered by resistance mechanisms typical of conventional anthracyclines. Therefore, AD 32 and AD 41 act by distinct mechanisms that resemble the different modes of action of AD 198 and AD 288, respectively. Comparisons between AD 198 or AD 32 and their valerate-free metabolites offer a good example of how many functional and kinetic advantages can be gained by changing cellular localization of a given anthracycline (Fig. 21).

After initial studies assessing the safety and efficacy of its intraperitoneal administration in patients with advanced gynecologic malignancies (Markman et al., 1996), valrubicin has been approved in the United States for the topical treatment of bladder cancer in patients who fail standard therapy with intravesical bacillus Calmette-Guerin and are not eligible for radical surgery (Kuznetsov et al., 2001). Valrubicin is also considered as first-line medical therapy for superficial bladder cancer (van der Heijden and Witjes, 2003). Intravesical instillation of valrubicin is well tolerated and can be durable.

V. Conclusions

In reviewing selected recent progresses in basic or clinically oriented research on anthracyclines, we have shown that these drugs enjoy an unexhausted repertoire of activities in cancer cells as well as in the vulnerable cardiomyocytes. The long-held assumption that anthracyclines have different modes of action in tumors or in the heart seems to be supported by the presence of distinct signal-transduction mechanisms and survival factors in these tissues (see *Section III.D.2.*). At the same time, the fact that iron represents a causative factor of both cardiotoxicity and newly discovered mechanisms of DNA damage (see *Sections II.B.2.–5.* and *III.B.1.a.* and *b.*) reveals that the beneficial and detrimental effects of anthracyclines also share common effectors. This mechanistic overlapping anticipates problems in reducing toxicity while also preserving activity, and rationalizes concerns about whether and how dexrazoxane should be used in patients receiving cumulative doses of DOX (see *Section III.D.3.*).
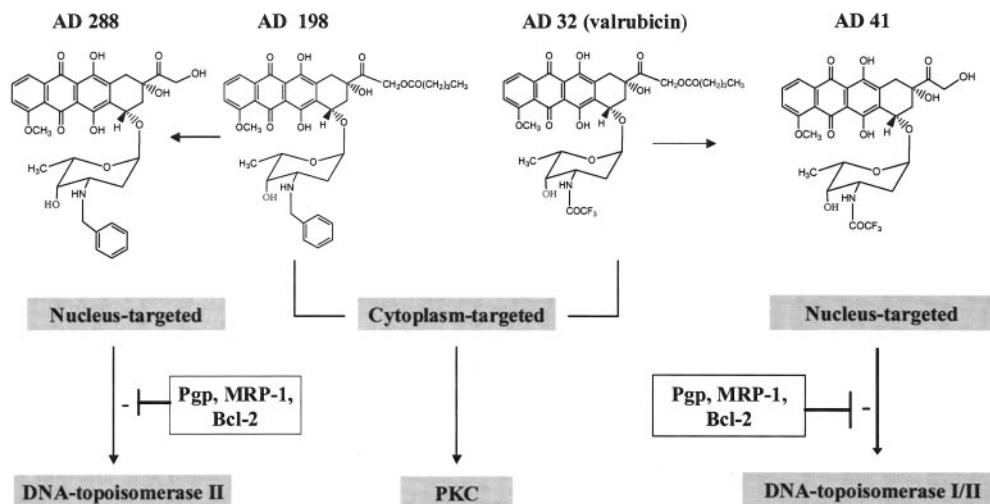
FIG. 21. Nucleus- versus cytoplasm-targeted 14-*O*-acylanthracyclines. AD 198 and AD 32 (valrubicin) localize to perinuclear cytoplasm, induce apoptosis through PKC modulation, and escape counter effects mediated by Pgp, MRP-1, and Bcl-2. Their valerate-free metabolites (AD 288 and AD 41) localize to nucleus, act through DNA-intercalation and topoisomerase II inhibition, and are counteracted by Pgp, MRP-1, and Bcl-2. Adapted, with modifications, from Lothstein et al. (2001).

Concerns might also surface if anthracyclines were considered for clinical use in combination with agents that optimized their activity in experimental models. For example, proteasome inhibitors might very well increase the nuclear uptake of anthracyclines in cancer cells, leading to an improved tumor response (see *Section II.B.1.*); yet the same inhibitors might also aggravate cardiotoxicity by interfering with a role of the 20S proteasome subunit in the cotranslational assembly of myofibrillar proteins (Foucrier et al., 2001). Antitelomerase agents might prove useful for accelerating activation of the senescence program in anthracycline-treated tumors (see *Section II.B.6.*), but they might also cause development of cardiomyopathy and CHF at subthreshold doses of DOX due to their own activity in inducing myocyte senescence/apoptosis and cardiac dilation (Leri et al., 2003; Oh et al., 2003). Thus, the unavoidable dilemma of optimizing the efficacy of anthracycline-based multiagent therapies while not exposing patients to overt cardiac damage still challenges clinicians and pharmacologists (see also *Section III.C.1–3.*).

The search for a "better anthracycline" continues. Forthcoming clinical trials will define the efficacy and safety of new formulations or analogs that performed well in preclinical models (see *Sections IV.A.* and *B.*). Appreciation of the toxicity of secondary alcohol metabolites in cardiomyocytes but not in cancer cells, at least apparently, concentrates expectations on disaccharide anthracyclines that form smaller amounts of such metabolites. Likewise, there is interest in anthracyclines that were recently designed to achieve both an increased DNA damage and an improved delivery to cancer cells. Koch and associates described the synthesis of a new DOXFORM that is targeted at estrogen receptor-positive breast cancer cells or androgen-sensitive prostate cancer cells by conjugation with 4-hydroxytamoxifen or

cyanonilutamide, respectively (Cogan and Koch, 2003; Burke and Koch, 2004). The efficacy and safety of these DOX derivatives await characterization in suitable preclinical models. And finally, a unique combination of favorable pharmacokinetics and pharmacodynamics identifies alkyl- or 14-*O*-acylanthracyclines as new agents endowed with alternative modes of actions, escaping classical mechanisms of resistance and possibly allowing for treatment modalities that spare cardiac function to some extent.

At present, however, liposomal formulations (whether pegylated or uncoated) remain the best known alternatives for improving the therapeutic index and spectrum of activity of DOX in clinical settings (Batist et al., 2002; Gabizon et al., 2003). Other tumor-targeted formulations clearly require more extended evaluation. Whereas the noncardiac toxicities of liposomal DOX should be taken in proper account, lessons from the use of this formulation in investigational or approved settings raise questions about whether increasing tumor delivery of a given anthracycline might be more productive than changing its structure and mode of action in cancer or cardiac cells. The next few years will tell us more about which strategy is better. As usual with anthracyclines, there is one more dilemma to deal with.

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