Anthracycline drug targeting: cytoplasmic versus nuclear – a fork in the road

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Abstract The anthracycline antibiotics doxorubicin (Adriamycin; DOX) and daunorubicin (DNR) continue to be essential components of first-line chemotherapy in the treatment of a variety of solid and hematopoietic tumors. The overall efficacies of DOX and DNR are, however, impeded by serious dose-limiting toxicities, including cardiotoxicity, and the selection of multiple mechanisms of cellular drug resistance. These limitations have necessitated the development of newer anthracyclines whose structural and functional modifications circumvent these impediments. In this review, we will present recent strategies in anthracycline design and assess their potential therapeutic merits. Current anthracycline design has diverged to target either cytoplasmic or nuclear sites. Nuclear targets have been broadened to include not only topoisomerase II (topo II) inhibition through ternary complex stabilization and catalytic inhibition, but also topoisomerase I (topo I) inhibition and transcriptional inhibition. In contrast, cytoplasmic targeting focuses on anthracycline binding to protein kinase C (PKC) regulatory domain with consequent modulation of activity. © 2001 Harcourt Publishers Ltd

INTRODUCTION

For three decades, the anthracycline antibiotics DOX and DNR have remained as first-line chemotherapy for a variety of solid and hematological tumors. Despite serious dose-limiting adverse effects, such as myelosuppression and cardiotoxicity, and the hindrance of drug-induced cytotoxicity by multiple mechanisms of cellular drug resistance, the persistence of DOX and DNR in the chemotherapeutic arsenal attests to the dearth of newer, substantially more efficacious anthracyclines. In assessing the vast effort applied to the design and development of more efficacious anthracyclines, it could be considered, as it has in a previous review,1 that in terms of overall therapeutic efficacy, DOX and DNR are the optimal or, at least, near-optimal anthracyclines. Yet in the last two years, novel developments in anthracycline drug action have suggested that the chemotherapeutic efficacy of DOX and DNR may yet be dramatically eclipsed by several novel anthracyclines. In this review, current strategies of anthracycline drug design will be examined in terms of improved mechanisms of cytotoxicity, decreased systemic toxicity, and circumvention of cellular drug resistance and these strategies assessed as to whether anthracycline drug development is, in fact, improving significantly beyond DOX and DNR.

THE LIMITATIONS OF DOX AND DNR

The mechanistic basis for the cytotoxicity of DOX and DNR has been attributed to one of several structural features. First, the near-planar polycyclic chromophore permits intercalation into the stacked bases of DNA and inhibition of topo II religation activity through the association of drug with the topo II/DNA ternary complex in the presence of bound ATP. This mechanism is consistent with the nuclear localization of both DOX and DNR. Second, the quinone ring, a structural feature common to anthracyclines, generates reactive oxygen species (ROS) through one-electron reduction by flavin-centered reductases4-7 or through a non-enzymatic pathway involving coordination of a ferric ion with chromophore rings B and C.8 In the presence of water molecules, this complex initiates redox cycling to produce superoxide anions.5 Stabilization of the DNA/topo II ternary complex by DOX and DNR inhibits DNA religation and promotes the accumulation of double-stranded DNA breaks. The generation of ROS leads to a wide array of cellular damage involving DNA, protein, and lipid.9 Damage induced by either mechanism can lead to increased expression of the tumor suppressor protein p53, growth arrest, and apoptosis that is dependent both on the extent of cellular damage and cell type.10-12 As expected, both the structure and cytotoxic functions of DOX and DNR provide the basis of susceptibility to multiple mechanisms of cellular drug resistance. DOX and DNR, owing to modest lipophilicity and net positive charge at physiological pH, are efficient substrates for both P-glycoprotein (Pgp) and MRP-mediated drug extrusion processes.13-15 Circumvention of topo II-mediated DNA scission by DOX and DNR can be achieved through reduced topo II activity, identified as the at-MDR phenotype.16 ROS production by DOX selects for resistant cells with either increased glutathione, metallothionein, glutathione S-transferase, Cu,Zn-superoxide dismutase or Mn-superoxide dismutase levels.17-19 Along with most, if not all, currently approved antitumor agents, DOX and DNR cytotoxicity is impeded by defects in both the cell cycle arrest and apoptotic signaling pathways. DOX-induced cytotoxicity is delayed in cells with p53 loss of function mutations20 that result in multiple effects, including the disconnection of DNA damage to apoptotic progression,21 increased Pgp expression,22 decreased bax expression,23 and increased Mn-superoxide dismutase expression.24 Within the apoptotic pathway, Bcl-2 expression abrogates mitochondrial membrane depolarization produced by either bax expression or via TNF-α family receptor-mediated caspase-8 activation. In so doing, DOX-induced DNA damage fails to trigger the irreversible stages of apoptosis.25

Of additional concern with the clinical use of DOX and DNR is the emergence of acute and chronic cardiotoxities. Cardiomyocytes are exquisitely sensitive to DOX-mediated ROS generation, given their lack of catalase activity and the elimination of glutathione peroxidase activity by DOX treatment.8 Oxidative damage to sulfhydryl groups on the calcium release channels of the sarcoplasmic reticulum26 and changes in ADP/ATP translocase content of the myocyte mitochondria27 shift cellular calcium distribution into the cytoplasm and...
induce apoptosis. ROS generation by anthracyclines is linked to acute cardiotoxicity, as manifested by ECG changes and hypertension. In contrast to acute cardiotoxicity, chronic cardiomyopathies are thought to be produced primarily by the secondary alcoholic DOX metabolite, doxorubicinol, produced by aldo-keto reductase activity. Doxorubicinol is a potent inhibitor of ion-dependent pump proteins in the mitochondria, sarcoplasmic reticulum, and sarcolemma, as well as an inhibitor of Na-K ATPase activity in the sarcolemma and the aconitase/iron regulatory protein-1. The persistence of the relatively polar DOX in cardiac tissue and its progressive conversion to the more polar and even more persistent doxorubicinol results in prolonged dose-dependent interference with calcium loading into the sarcoplasmic reticulum and consequent systolic and diastolic dysfunction.

A SMALL STEP FORWARD

Within the last ten years only a handful of anthracyclines have attained clinical approval based upon modest improvements over DOX and DNR. These include 4'-epidoxorubicin (epirubicin; EPI), 4-demethoxydaunorubicin (idarubicin; IDA), tetrahydropropyldoxorubicin (pirarubicin; PIR), and aclacinomycin A (clarubicin; ACR). All retain the characteristic nuclear targeting observed with DOX and DNR and stabilize topo II/DNA ternary complexes to extents that are comparable to or more effective than DOX. IDA, however, has also been shown to stimulate topo I-mediated DNA cleavage, while retaining a preference toward topo II/ternary complex stabilization. The trisaccharide anthracycline, ACR, is both a catalytic inhibitor of topo II, preventing the initial binding of topo II to DNA, and a topo I inhibitor by stabilizing the topo I/DNA covalent complex. This change in function has been attributed primarily to a carboxymethyl substitution at C-10 on the ring A of the chromophore.

While the potential advantage of EPI and IDA over DOX and DNR include reduced cardiotoxicity, EPI and PIR still retain the potential to induce cardiotoxicity at doses above 950 mg/m² and 360 mg/m², respectively, or lower if administration follows radiation therapy. IDA also produces fetal heart failure following its facilitated transplacental transit from maternal circulation. EPI, IDA, PIR, and ACR also demonstrate only modest improvements over DOX and DNR in terms of drug resistance. The clinical response of AML to IDA does not demonstrate a correlation with P-gp expression, as seen with DNR, although IDA, EPI, and DNR are reported to induce the expression of P-gp in both P-gp-positive and -negative AML blast cells. IDA cytotoxicity is inhibited by MRP and Bcl-2 expression in a variety of leukemic cell lines, and is likely to be affected by p53 and p21 loss of function mutations. Further, the equipotent alcoholic metabolite, idarubicinol, is a P-gp substrate. EPI and PIR are substrates for both P-gp and MRP and are susceptible to resistance through loss of p53 function. ACR-resistant cells have been reported to show decreased drug-accumulation associated with P-gp overexpression in cultured P388 murine leukemia cells. However, in human primary AML and hepatoma cells, aclacinomycin A resistance does not correlate with P-gp levels. Thus, these four clinically approved anthracyclines expand slightly upon the nuclear targeting observed with DOX and DNR by including topo I. However, their success in overcoming dose-dependent cardiotoxicity, dose-limiting myelosuppression, and cellular drug resistance appears marginal.

CONTINUED NUCLEAR TARGETING BY ANTHRACYCLINES: AMINOSUGAR ALTERATION

Anthracycline development beyond EPI, IDA, PIR, and ACR has witnessed a divergence of strategies: One design path maintains the past course of nuclear/DNA targeting, while another path of anthracycline design focuses on cytoplasmic targeting of congeners that may function as signaling modifiers rather than DNA damaging agents. The key element for each strategy is the site of anthracycline modification (Fig. 1).

Essential to the strategy of improved anthracycline efficacy was the understanding that the aminosugar is essential for anthracycline activity and that modification of the sugar could have considerable effects upon tumor cell cytotoxicity and systemic toxicity. The purported importance of the positively charged C-3’ amine in stabilizing drug intercalation into DNA was successfully challenged by replacement of the 3’-amino with small uncharged groups, such as in 3’-deamino-3’-hydroxydoxorubicin (hydroxyrubicin; WP159). Hydroxyrubicin is not only comparable to DOX in cytotoxicity through topo II/DNA ternary complex stabilization, but it also demonstrates certain potential therapeutic advantages, such as partial circumvention of Pgp-mediated drug resistance, reduced cardiotoxicity, and enhanced sensitivity to vinca alkaloids in hydroxyrubicin-resistant murine cells. However, the reduced aqueous solubility of hydroxyrubicin due to loss of charge posed a potential clinical impedance.

To improve solubility, a second aminosugar was added to the 4’-position of the first sugar to produce a novel series of 3’-deamino, 3’-hydroxy disaccharide analogs of DOX, DNR and IDA with the second aminosugar either in an axial or equatorial configuration. It has been observed that bulky substituents at the 3’-position abrogate topo II-mediated DNA cleavage in favor of other cytotoxic mechanisms without loss of cytotoxicity, as observed for the 3’-morpheolinyl or 3’-N-benzyl derivatives of DOX. However, following 4’-aminosugar addition, both axial and equatorial DNR disaccharides demonstrate a marked reduction in cytotoxicity that correlates with the reduced ability to stimulate topo II-mediated DNA cleavage. The axial disaccharide conformation of Idarubicin (MEN10746) restores the ability to stimulate topo II-mediated DNA cleavage and consequent cytotoxicity to a level comparable with DOX. While the disaccharide anthracyclines stimulate topo I-mediated DNA cleavage, this activity is approximately 100-fold less potent than the drug effects on topo II, based upon relative concentrations, and is significantly less potent than camptothecin in inhibiting topo I activity. Of particular note is the reported inability of the disaccharide anthracyclines to circumvent Pgp- or MRP-mediated drug resistance. In an attempt to address this latter therapeutic limitation, Pratesi and co-workers developed a 4’-disaccharide congenger of 4-demethoxy-3’-deamino-3’-hydroxydoxorubicin (MEN 10755). Relative to DOX, MEN 10755 demonstrates comparable or lower activity against a
Nuclear and cytoplasmic anthracyclines

Fig. 1 Structures, mechanisms of cytotoxicity, and mechanisms of cellular resistance associated with relevant nuclear-targeted and cytoplasmic-targeted anthracyclines.

variety of human solid tumor cell lines and is unable to circumvent resistance conferred by either P-gp or MRP. By contrast, in nude mice bearing human tumors xenografts, MEN 10755 demonstrates modest (1.5-fold) to significant (10-fold) improvement over DOX in the treatment of A2780 ovarian carcinoma and MX-1 breast carcinoma cells, respectively. Further, MEN 10755 cytotoxicity is reported to be less affected by Bcl-2 expression in a comparison of human lung carcinoma cell lines. MEN 10755 treatment, in fact, induces the phosphorylation of a small percentage of the total Bcl-2 content of the lung carcinoma cells tested, suggestive of hyperphosphorylative inactivation akin to Bcl-2-expressing cells treated with taxol. The significance of these results is obscured by the comparison of lung carcinoma cells of different histotypes and with multiple biochemical alterations including p53 loss of function and increased levels of either glutathione, EGF-R, or c-erbB-2 expression, all of which can alter cell response to DOX. Systemically, MEN 10755 is reported to be less acutely cardiotoxic than DOX and EPI based upon relative contractile impairment in equimolar drug-treated isolated rat hearts. The reduced cardiotoxicity correlates with a markedly reduced release of Ca^{2+} from cardiomyocyte sarcoplasmic reticulum. Taken together, these results suggest that of the family of 4'-disaccharide anthracyclines, only MEN
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10755 may hold potential for improved efficacy. The addition of a second sugar moiety at the 4′ position in DOX, DNR, and IDA appears to have little impact in improved activity. Topo I inhibitory activity of IDA is not altered by the addition of a 4′-aminosugar and its topo II inhibitory active is modulated more by the presence of an amine at either the 3′ or 3″ positions.53 Given the impact of aminosugar substitution on anthracycline pharmacology, these studies are notable for their analysis of 4′-substitution using bulky additions. However, these results suggest that the 4′-disaccharide congeners of DNR, DOX, and IDA offer no clearly established therapeutic advantage over the parental compounds.

An alternate approach to nuclear targeting of anthracyclines has been the development of congeners with bulky aminosugar substitutions at the 3′-site. It has been established that while substitution of the 3′-amino with a hydroyx group on DOX does not alter the ability of the compound to stimulate topo II-mediated DNA cleavage,54 3′-acyl substitutions reportedly reduce topo II interaction and cytotoxicity.58 However, cyclic substitutions at the 3′ site not only preserve drug cytotoxicity, but also alter the mechanism of cytotoxicity. Included in this approach are the 3′-morpholinyl-substituted anthracyclines which arose from the studies of Acton and colleagues who demonstrated that increased cytotoxicity and circumvention of P-gp mediated resistance could be achieved by cyclic-alkylation of the aminosugar nitrogen.65 In contrast to DOX, morpholinyl-, cyanomorpholinyl- and methoxymorpholinyl-doxorubicin covalently bind DNA though a mechanism by which the morpholino ring undergoes rearrangement/opening and is bound to DNA with a structure reported to resemble N-(2-hydroxyethyl)doxorubicin, based upon crystallographic analysis.66 In the case of cyanomorpholino-doxorubicin, the cyano group is released.66 The interaction of this structure aligns the amino group in the minor groove as a requirement for DNA alkylation. The resulting cytotoxicity is not associated with topo II-mediated DNA cleavage but rather with enhanced topo I-mediated DNA cleavage67 and DNA crosslinking.68,69 The result is a class of congeners that demonstrate potency 10-1000-fold higher than DOX, in vitro.31 Of the variety of morpholinoanthracines synthesized in the past two decades, only 3′-deacetoxy-3′-morpholinyl-13-deoxo-10-hydroxy-11,13-guanyl-DNR (MX2) is currently undergoing clinical testing. MX2 is more lipophilic than DOX and DNR and shows improved diffusion across the blood/brain barrier. In patients diagnosed with either glioblastomas, anaplastic astrocytomas and malignant oligodendroglialoma, intravenous MX2 produced a complete response in 30% of the patients. Dose limiting toxicity was myelosuppression in 30% of the patients and, significantly, no cardiotoxicity was observed.70

As with the 3′-morpholinyl substitution, 3′-benzyl substitution on the aminosugar preserves nuclear targeting and potency, but alters the cytotoxic mechanism of the anthracycline. In a recent report, Lothstein and co-workers demonstrated that N-benzyl-adriamycin (AD 288) is a potent catalytic inhibitor of topo II based upon the ability of AD 288 to: 1) inhibit topo II activity without stimulating DNA strand scission in a dose-dependent manner, 2) inhibit the formation of stable cleavable complexes by etoposide in a dose-dependent manner, and 3) circumvent drug resistance conferred by altered topo II activity.59 Intercalating topo II catalytic inhibitors, such as ACR, appear to modify DNA duplex topology in a manner that prevents topo II from achieving the initial non-covalent complex with DNA.71 Likewise, N-benzylation of DOX eliminates dose-dependent stabilization of the topo II/DNA ternary complex without decreasing cellular cytotoxicity. This effect is likely produced by enhanced distortion of the DNA helix through intercalation and minor groove binding. The aminosugar of DOX extends outside the nucleotide base stack, so despite the addition of the N-benzyl moiety to the aminosugar, AD 288 has DNA intercalative strength comparable to that of DOX.72 Therefore, it appears that the N-benzyl moiety would have little effect upon the characteristics of anthracycline intercalation into DNA, but may alter helix conformation through minor groove interaction, as reported for ACR.73 Despite the ability of AD 288 to circumvent p-glp, prior studies74 report that AD 288 is a substrate for p-glp mediated drug extrusion while more recent findings indicate that Bcl-2 expression markedly delays AD 288-induced apoptosis in both hematopoietic and solid tumor cell lines.75

Perhaps the bulkiest 3′-amino substitution that has been performed on DOX or DNR has been the “bisanthracine” WP631 described by Martin.76 WP 631 is composed of two DNR molecules crosslinked at the 3′ amines by an p-xylyl spacer. The result is a dimerized anthracine that intercalates into DNA with a binding affinity approaching that of protein binding to DNA. Further, the apparent DNA sequence selectivity of intercalation of WP631 results in the high affinity for Sp1 consensus sequences and the inhibition of protein binding to these sequences in vitro. Thus WP631 appears to function principally as a transcriptional inhibitor.

Other bulky substitutions at the 3′-amine include guanouridone and peptide moieties attached to DOX that produce a targeted DOX produg. For example, DeFeo-Jones describes L-377,202, a DOX-N-glutaryl-(4-hydroxyprolyl)-AlaSer-cyclohexaglycyl-GlnSer-Leu-CO2H conjugate that is hydrolyzable by the serine protease activity of prostate specific antigen.77 Targeting of DOX to prostate is demonstrated by the non-cytotoxicity of L-377,202 in non-prostate tumor cell lines relative to LNCap human prostate cancer cells. Comparable in functional strategy is a series of benzoyloxycarbonyl DOX and DNR pro-drug congeners used as part of a gene-directed prodrug therapy. These agents rely on the activity of the bacterial enzyme carboxypeptidase G2 transfected into target tumor cells to catalyze the scission of amidic, urethanic, or ureidic bonds linking benzyl and L-glutamic moieties to release DOX and DNR.78 While these prodrugs represent covalent modification of DOX and DNR, they nevertheless are cytotoxic through the action of parental DOX and DNR and, as such, do not represent mechanistically novel anthracyclines.

CYTOPLASMIC TARGETING BY ANTHRACYCLINES: CHROMOPHORE SUBSTITUTIONS

In addition to modification of the daunosamine sugar, substitution on polycyclic chromophore has been a rich source of experimental anthracyclines, not the least of which is DOX, which is a C-14-hydroxylated derivative of DNR. As an extension of the prodrug strategy pursued through
aminosugar substitution, DA-125 is a 3'-deamino-3'-hydroxy-2'-fluoro-doxorubicin-14-propylamine prodrug for the delivery of 3'-deamino-3'-hydroxy-2'-fluoro-doxorubicin through ester hydrolysis of the C-14 akyamine moiety. Although the mechanism of action of DA-125 has not been identified, characteristics similar to 4-demethoxy-3'-desamino-3'-hydroxy-2'-iodo-doxorubicin (annamycin; ANN) might be expected: topo II-mediated DNA cleavage, circumvention of P-gp and MRP-mediated drug resistance, and reduced cardiotoxicity. However, pre-clinical and clinical trials with DA-125 have been disappointing, with DA-125 cross-resistance developing in DOX-resistant gastric carcinoma and pulmonary adenocarcinoma cells and significant bone marrow and testicular toxicities.

In an effort to synthesize active 14-substituted congeners, the addition of alkylesters at C-14, particularly 5-carbon moieties, has produced a family of congeners that are unique among anthracyclines in activity and cytotoxicity targeting. Two lead compounds in this structural class are N-benzyladriamycin-14-valerate (AD 198) and N-trifluoroacetyladriamycin-14-valerate (valrubicin; VLR; AD 32). The valerate moiety in combination with either the benzyl or trifluoroacetyl substitutions results in a marked increase in lipophilicity and rapid cellular penetration.

The valerate substitution also alters the subcellular localization of the anthracyclines, with both AD 198 and VLR rapidly and persistently localizing in the perinuclear cytoplasmic region. Despite the absence of nuclear localization, both compounds exhibit cytotoxicity that is equal to or greater than DOX in an array of mammalian tumor cells. This has lead to an intense investigation of the cytotoxic targets and mechanism of action of these two agents. Early studies by Bodley reported the poor ability of AD 198 to either intercalate into DNA or interfere with topoisomerase activity. While initial interpretations of these observations included the notion that AD 198 was simply a prodrug for its cellular metabolite, N-benzyladriamycin (AD 288), more recent evidence indicates that the cytotoxicity of AD 198 does not require its biotransformation to AD 288. In 32D murine myeloid leukemia cells, CEM human lymphocytic leukemia cells, and 3T3 murine fibroblasts, apoptosis following brief AD 198 exposure is readily detectable prior to the biotransformation of AD 198 to AD 288. Parallel studies with N-benzyladriamycin-14-pivalate (AD 445), a congener that also localizes in the cytoplasm and which is resistant to esterase-mediated hydrolysis of the 14-alkylester sidechain, demonstrates equal potency to AD 198. Third, AD 198 and AD 445 cytotoxicities are unimpeded by the anti-apoptotic effects of Bcl-2 and Bcl-XL, while expression of these mitochondrial proteins produces marked delays in both DOX- and AD 288-induced apoptosis. Thus, in contrast to previously characterized anthracyclines, the cytotoxic target(s) of AD 198 and AD 445 appear to be localized to the cytoplasm.

The novel targeting of AD 198 and AD 445 is attributed to the alkylester moiety at C-14. In combination with the A and B ring components, AD 198 shares a spatial homology with the phorbol ester, PMA, and diacetylglycerol, both activators of the conventional and novel isozymes of the C protein kinase (PKC) family of signaling enzymes. Because of this structural homology, AD 198 specifically binds to the C1 regulatory domain in a manner that competitively inhibits phorbol ester binding. These studies have further suggested that AD 198 caps the hydrophilic region of C1 to allow anchoring to cellular membranes. C1-ligands, including AD 198, are amphipathic molecules that interact with their receptors at the aqueous-lipid interface of phospholipid membranes. A polar region of the ligand interacts with membrane lipid head groups and water, while also providing hydrogen-bonding partners for amino acid residues within the binding pocket of the C1-domain. Hydrophobic moieties serve to position the ligand within the membrane as well as to anchor the ligand/receptor complex to the membrane. The 14-O-valerate sidechain and N-benzyl function of AD 198 are highly flexible. Random incremental pulse search analysis has identified a low energy conformation in which the hydrophobic anthraquinone ring system, N-benzyl group and 14-O-valerate sidechain are oriented above an arbitrary plane below which polar oxygen atoms are present for interaction with the aqueous phase and for hydrogen bonding to the C1 domain. This theoretical membrane orientation is consistent with previous studies of AD 198 interactions with the phospholipid bilayers. Docking simulations of AD 198 to the PKC-delta ( ) C1 domain

**Fig. 2** Cellular targets of nuclear-targeted and cytoplasmic-targeted anthracyclines.
demonstrates a pharmacophore for AD 198 that is similar to that of the phorbol esters and DAG.\textsuperscript{89}

The spatial conformation of AD 198 results in modulation of PKC activity in cell-free studies and in intact cells. AD 198 displays the ability to displace \textsuperscript{3}H-PdBu from its C1 binding site on PKC in a dosedependent manner. Consequently, AD 198 inhibits phorbol ester-mediated activation of purified PKC in a dose-dependent manner. This observation could be interpreted either as inhibition of PKC activity by AD 198 or displacement of phorbol ester combined with less potent activation of PKC compared with phorbol ester. In intact cells, AD 198 induces the rapid translocation of PKC- and PKC-in several cell lines just prior to the detection of apoptosis.\textsuperscript{75,85}

In 32D myeloid leukemia cells, the induction of apoptosis by AD 198 or AD 445, but not DOX or AD 288 is marked by the rapid caspase-mediated cleavage of PKC- to release the constitutively active catalytic fragment and is inhibited by the selective PKC inhibitor, rottlerin.\textsuperscript{75} These observations are consistent with the concept of AD 198 and AD 445 inducing cytotoxicity through rapid novel PKC activation. The activity of AD 198 and AD 445 in inducing apoptosis through a PKC-mediated mechanism is supported by the progressive loss of the C1 regulatory domain, as found in PKC-, AD 198 has no effect.

AD 198 and AD 445 are further noteworthy in their ability to circumvent multiple mechanisms of drug resistance as a result of both structural and functional drug characteristics. Since neither compound targets topo II, reduced topo II activity does not reduce drug cytotoxicity.\textsuperscript{72,86} The degree of lipophilicity conferred by both the N-benzyl and the 5-carbon 14-alkylester moieties\textsuperscript{84} is sufficient to entirely avoid extracellular transport by either Pgp or MRP.\textsuperscript{89} Interestingly, the presence of either the N-benzyl moiety alone (AD 288) or the valerate sidechain along, as in adriamycin-14-valerate, still permits Pgp-mediated transport.\textsuperscript{89} The most promising aspect of AD 198 and AD 445 is the observation that the cytotoxicity of both drugs is unaffected by the expression of the anti-apoptotic members of the Bcl-2 family of mitochondrial proteins. Expression of these proteins in human tumors is linked to poor prognosis of chemotherapy. In experiments performed by Barrett,\textsuperscript{79} expression of transfected human Bcl-2 or Bcl-X\textsubscript{L} in murine 32D cells resulted in a 4-fold delay in cell kill following DOX or AD 288 treatment, but had no effect on the cytotoxicity of either AD 198 or AD 445. Bcl-2 and Bcl-X\textsubscript{L} do not appear to be inactivated by AD 198 or AD 445 either through hyperphosphorylation or caspase-mediated cleavage. Rather, functional Bcl-2 appears to be overwhelmed by drug-induced apoptotic signaling through PKC-cleavage and activation. This reported mechanism of action for AD 198 and AD 445 would characterize these agents not as drugs that trigger apoptosis by inflicting cellular damage, but as drugs that directly trigger apoptosis. Consistent with this notion is the inability of cellular p53 status to affect AD 198 and AD 445 cytotoxicity. The binding of AD 198 and AD 445 to the C1 regulatory domain poten-}

### CONCLUSIONS AND FUTURE DIRECTIONS

We have described current design strategies aimed at developing anthracyclines that represent a significant therapeutic improvement over DOX and DNR. Within the past two years, two distinct paths of anthracycline design and development have become evident. Both paths share the common goal of reduction of dose-limiting toxicities such as cardiotoxicity and myelosuppression, and circumvention of resistance. However, one path continues to focus exclusively on aminosugar substitutions to potentiate DNA-related damage, while the other path encompasses C-14 acylester modifications in combination with aminosugar modification to produce congeners that no longer directly target DNA, but rather may potentiate apoptotic signaling through modulation of PKC activity. These two strategies are likely to lead to profound differences in the future of anthracycline drug development. For nuclear-targeted anthracyclines, the anthraquinone ring system, although responsible for cardiotoxicity and potential carcinogenicity, is essential for cytotoxicity and will likely be preserved. However, the cytoplasmic-targeted anthracyclines such as the 14-O-acylanthracycline, freed of the requirement to intercalate DNA in order to be cytotoxic, may no longer require the anthraquinone structure. Thus, at the fork in the road of anthracycline development, one road will likely lead to additional anthracyclines, while the other may lead to the evolution of...
anthracyclines into a new structural and functional class of antitumor agents.

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Nuclear and cytoplasmic anthracyclines


