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COMMENTARY

DOES BULK DAMAGE TO DNA EXPLAIN THE CYTOSTATIC AND CYTOTOXIC EFFECTS OF TOPOISOMERASE II INHIBITORS?

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Topoisomerase II is a nuclear enzyme which permits chromosomal dysjunction at the termination of DNA synthesis by the breakage and religation of DNA with an intermediary strand passing event [1]; topoisomerase II is covalently bound to the 5′ termini of the broken strands in the course of DNA cleavage [2, 3]. Topoisomerase II is also thought to be involved in DNA replication [4], in chromosomal condensation [5], and in maintenance of the chromosomal scaffold [6]. A number of clinically important antitumor drugs may express their cytostatic or cytotoxic effects by interfering with the activity of topoisomerase II. The anthracycline antibiotics, the anthracenediones, the epipodophyllotoxin derivatives, the ellipticines and drugs of the aminoacridine class are thought to interfere with the DNA religation (reunion) step via stabilization of the covalently linked complexes formed between topoisomerase II and the 5′ cleaved termini of the DNA molecule [7–10]; these "cleavable" complexes of topoisomerase II and DNA are observed as DNA strand breaks after dissociation of the homodimeric subunits of topoisomerase II in protein denaturants, such as detergents [11–13]. Stabilization of the cleavable complexes and the concomitant expression of both single-strand and double-strand breaks in DNA (i.e. bulk DNA lesions) are thought to be the initial events mediating the antitumor effects of these antineoplastic agents [14, 15].

RELATIONSHIP BETWEEN LESIONS IN BULK DNA AND TOXICITY OF TOPOISOMERASE II INHIBITORS

Concentration-dependent induction of DNA lesions

The concept that formation of cleavable complexes (and the concomitant induction of DNA strand breaks) [13] accounts for the antiproliferative and cytotoxic activity of the topoisomerase II inhibitors is based, to a large extent, on correlative studies for agents such as the epipodophyllotoxins, VP-16 and VM-26, and the aminoacridine, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA) [16–18]. While some investigators have shown a similar relationship for the anthracycline antibiotic, Adriamycin® [19, 20], other studies have failed to discern significant strand breakage at IC_{50} values for the anthracyclines [21–23] or the anthracenedione, mitoxantrone [24]; i.e. drug toxicity frequently fails to correspond with bulk DNA damage. This anomaly could, in part, be explained by prolonged retention of Adriamycin and mitoxantrone and the *persistence* of low levels of DNA lesions [23, 24]. It should be emphasized that a correspondence between DNA strand breaks and cytotoxicity does not prove causality. Nevertheless, for structurally similar analogs within a given class of agents, such as the epipodophyllotoxins or the aminoacridines, drug toxicity generally correlates well with induction of DNA strand breaks [17, 25]. In addition, a correlation between the cytotoxicity of anthracycline derivatives and the intensity of topoisomerase II mediated DNA breakage *in vitro* has been reported recently [26].

Studies in tumor cells resistant to topoisomerase II inhibitors

The implication of topoisomerase II as an antineoplastic drug target and the role of protein-associated DNA damage in mediating cytotoxicity are also supported by various studies in cells selected for resistance to topoisomerase II poisons, where a reduction in drug-induced DNA strand breaks and/or DNA–protein cross-links has been observed [27–33]. Alterations in the levels or drug sensitivity of topoisomerase II in both drug-"resistant" and drug-hypersensitive cell lines as compared to the drug-sensitive parent cell line have also been reported [18, 29–31, 34–36]. A careful study by Bellamy *et al.* [37] using a doxorubicin-resistant human myeloma cell line where drug accumulation was modulated by verapamil, demonstrated a close correlation between intracellular accumulation of doxorubicin, double-strand breaks in DNA, and drug toxicity. In addition, using T-4 infected *Escherichia coli*, it has been possible to demonstrate that a single mutation bestows drug resistance and abrogates drug sensitivity of phage topoisomerase II, consistent with this enzyme being the primary target for m-AMSA [38, 39].

Collateral modulation of DNA strand breaks and drug toxicity

Different chemical modulators have been shown to produce corresponding alterations in the induction of DNA strand breaks, DNA–protein cross-links, and drug toxicity. For instance, in studies using L1210 cells, the intercalator, ethidium bromide, produces a concurrent reduction in the cytotoxicity

of VP-16 and the capacity of VP-16 to induce both single-strand and double-strand cleavage in DNA [40]. Conversely, the DNA synthesis inhibitor, hydroxyurea, potentiates m-AMSA cytotoxicity, and enhances m-AMSA-induced DNA strand breaks and DNA–protein cross-links in the L1210 cell line [41].

Despite the general acceptance of the paradigm that DNA strand breaks and DNA–protein cross-links (i.e. bulk DNA damage) play a predominant role in the antiproliferative and cytotoxic effects of topoisomerase II inhibitors, there are many examples in the literature of a dissociation between these bulk DNA lesions and the capacity of topoisomerase II poisons to kill the tumor cell or to inhibit tumor cell growth (as described below). A primary thrust of this commentary is to assess whether these exceptions are encompassed by the accepted paradigm, whether it is necessary to modify this paradigm, or whether an alternative hypothesis for drug action should be invoked. A possibility worthy of consideration is that certain subsets of breaks at select sites in cell DNA may be more toxic than breaks throughout the genome, *per se*, either because (i) these breaks are not repaired, (ii) these breaks are misrepaired, or (iii) these breaks occur at sites critical to the maintenance of normal cell function.

DISSOCIATION BETWEEN DNA LESIONS AND DRUG CYTOTOXICITY

Cell sensitivity to topoisomerase II inhibitors as a function of the cell cycle

Estey *et al.* [42] have reported that the DNA of HeLa cells is hypersensitive to m-AMSA-induced cleavage during mitosis without a corresponding phase-specific susceptibility to drug cytotoxicity. Similarly, Chow and Ross [43] demonstrated maximal sensitivity to etoposide-mediated DNA cleavage in BALB/c3T3 cells during the G_2M phases, while maximal cytotoxicity is expressed during S-phase. Schneider *et al.* [44] demonstrated temperature-dependent differences in tumor cell sensitivity to induction of lesions and toxicity of topoisomerase II inhibitors. In these studies, the bulk of detectable DNA damage appears to be dissociable from drug toxicity, although other explanations, such as the requirement for additional biochemical processing of these lesions (see below), may be sufficient to reconcile these findings.

Disparate levels of DNA lesions

If it is assumed that bulk DNA damage (i.e. throughout the genome) is equivalent, then a given level of DNA damage incurred by the cell would be expected to produce a consistent degree of toxicity, regardless of the drug utilized or the cell line under study. However, at a given level of toxicity, topoisomerase II inhibitors of different classes, such as m-AMSA, 5-iminodaunorubicin and 2-methyl-9-OH-ellipticine, produce disparate levels of DNA strand breaks [45]. One possible explanation for this observation is that half-lives of cleavable complexes formed by the different drugs may differ. As discussed by Kohn *et al.* [46], another possible explanation would be that different topoisomerase II inhibitors may induce lesions at different sites on the genome; in this context, Riou *et al.* [47] have demonstrated preferential cleavage in c-*myc* by VM-26 and m-AMSA; interestingly, both Adriamycin and 9-OH-ellipticine apparently failed to induce specific cleavage in c-*myc* [48], consistent with the intriguing possibility that these agents interact preferentially with other genomic sites. Differential genomic damage would also be consistent with the different cleavage patterns produced by different chemical classes of topoisomerase II inhibitors with DNA *in vitro* [7], and the local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin [49].

Lack of collateral modulation of bulk lesions in DNA and drug activity

While certain chemical modulators, such as ethidium bromide, and hydroxyurea have been shown to produce corresponding alterations in drug-induced DNA cleavage (or DNA–protein cross-links) and cytotoxicity, other agents have been shown to dissociate these events. For instance, both α-difluoromethylornithine (α-DFMO, an ornithine decarboxylase inhibitor) and dimethyl sulfoxide (DMSO) have been shown to increase DNA cleavage induced by m-AMSA in L1210 leukemia cells without a corresponding increase in tumor cell kill [50, 51]; DMSO also enhances strand breakage produced by mitoxantrone in H-35 rat hepatoma cells without an increase in antiproliferative activity [52]. Dinitrophenol was shown recently to increase DNA double-strand breaks and DNA–protein cross-linking in Chinese hamster cells while reducing cell killing by m-AMSA [53]. 17β-Estradiol, an estrogen, was shown to concomitantly increase DNA damage produced by m-AMSA, VM-26, Adriamycin and mitoxantrone in MCF7 breast tumor cell lines; yet, cytotoxicity was enhanced only for m-AMSA and VM-26 [22].

A number of investigators have also demonstrated that the protein synthesis inhibitor, cycloheximide, prevents expression of the cytotoxic effects of drug-induced lesions in DNA without altering the extent of these lesions [54–56]. This finding indicates that these lesions, while necessary, are apparently not sufficient for cell killing, and that other intervening biochemical events are required for expression of drug toxicity.

Dissociation of cytotoxic and antiproliferative effects of topoisomerase II inhibitors from DNA strand breaks and DNA–protein cross-links (resistant cells)

A dissociation between the cytotoxic or antiproliferative effects of topoisomerase II inhibitors and the induction of DNA strand breaks or DNA–protein cross-links appears to be most pronounced in drug-resistant tumor cells. Several derivative cell sublines have been isolated which fail to show any detectable DNA damage at concentrations in the range of drug IC_{50} values [32, 33, 57, *]. Other sublines show resistance to DNA cleavage, but not

* Ellis AL, Munger CE, Bunch RT, Woods KE, Randolph JK, Boise L, Swerdlow PS, Zwelling LA, Hinds M, Yanovich S and Gewirtz DA, manuscript in preparation.

to killing by topoisomerase II inhibitors [58]. Still other cell lines are resistant to cell killing by various topoisomerase II inhibitors, but fail to show a corresponding decrease in drug-induced cleavage [29, 59, 60]. In the case of cell killing in the absence of DNA strand breaks, one might argue for an alternative mechanism of drug action in the resistant cells or for the induction of lesions below the sensitivity limits of currently available assays. In the case of DNA strand breaks in the absence of cell killing, one possible explanation for the apparent lack of toxicity of the strand breaks *might* be that these breaks are occurring primarily at genomic sites which have little influence on cell growth or viability.

Reversibility of DNA lesions

One of the most intriguing observations in the study of topoisomerase II inhibitors is that DNA strand breaks and DNA–protein cross-links induced by drugs such as the epipodophyllotoxins and m-AMSA are readily reversed when drug is removed from the cellular milieu, while antiproliferative or cytotoxic effects are sustained [61–63]. This observation would be consistent with the idea that transient lesions in DNA effectively compromise cellular function such that cell growth and viability are compromised. Evidence supporting the capacity of transient lesions in DNA to mediate cell injury has been presented for camptothecin, a drug which inhibits the enzyme topoisomerase I by the stabilization of enzyme–DNA complexes which are converted to single-strand DNA breaks [64]; in this case, cell killing via the induction of DNA–protein complexes appears to be related to interference with DNA replicative function [65, 66]. The involvement of replicative function in the activity of the topoisomerase II inhibitors appears less certain [65, 66], although a recent report by Kaufmann [67] clearly suggests an important role for both DNA and RNA syntheses in mediating the toxicity of etoposide.

A POSSIBLE RESOLUTION TO THE PROBLEM

Without proposing alternative mechanisms of action for drugs such as the epipodophyllotoxins and m-AMSA (although one cannot rule out this possibility at the high drug concentrations required for expression of toxicity in resistant cells), many of the apparent inconsistencies described above could be explained by the hypothesis that DNA damage produced by the topoisomerase II inhibitors at different genomic sites fails to yield equivalent cytotoxic consequences to the tumor cell. The observation that different classes of topoisomerase II inhibitors give rise to unique levels of DNA damage at equivalent toxicities may be related to DNA damage occurring at different genomic sites. Low (essentially undetectable) levels of gene-specific lesions which fail to be repaired (or lesions which are misrepaired) might permit expression of the cytotoxicity of topoisomerase II poisons despite the fact that most if not all of the damage detected in *bulk* DNA is reversed. *Sustained* or unrepaired breaks at genomic sites which are intimately involved with proliferative function could lead to compromised cell growth and/or loss of viability more readily than similar levels of DNA strand breaks at genomic sites which code for (nonessential?) structural proteins. A low level of underlying damage sustained at critical sites on the genome may mediate drug action through common effects on gene expression by interaction with promoter or enhancer regions. In this context, a number of DNA-interactive drugs have been shown to modulate expression of genes, such as c-*myc* and c-*fos*, which are thought to be intimately associated with the regulation of cellular proliferative function [68–73]. Consequently, it may prove to be beneficial to focus research efforts on the capacity of topoisomerase II inhibitors to induce damage to *specific* functional genes as well as to modulate gene expression and thereby alter ordered cell growth and cell-cycle progression.

OTHER ISSUES TO BE RESOLVED

Certain additional critical questions, which limit our understanding of the nature of drug interaction with topoisomerase II remain to be resolved:

(A) Drugs which bind to DNA by intercalation such as the anthracyclines, anthracenediones and aminoacridines as well as non-DNA binders such as the epipodophyllotoxins (but, see Ref. 74) are effective topoisomerase II inhibitors; however, it is not understood how these agents actually prevent the enzyme-mediated religation step since a hypothetical ternary complex between drug, topoisomerase II and DNA has not been identified.

(B) While inhibition of religation and stabilization of the "cleavable-complex" are clearly linked, the molecular relationship between these two events remains to be elucidated; that is, it is not understood how the drug actually changes the relationship between topoisomerase II and DNA, such that dissociation of the topoisomerase II from DNA is compromised.

(C) A major issue to be resolved relates to the mechanism by which cleavable complex formation results in cell killing. The studies which dissociate DNA strand breaks from cytotoxicity (e.g. using cycloheximide) indicate that certain biochemical events downstream of "cleavable-complex" formation are required in order for this lesion to express lethality in the tumor cell. Studies have indicated a role for calcium in the final pathway leading to cell death [75] and for cdc2 kinase in the inhibition of cell proliferation by topoisomerase II inhibitors [76]. In addition, it has been proposed that topoisomerase II inhibitors may express cytotoxicity via induction of sister chromatid exchange [60, 77, 78].

In summary, while a great deal of progress has been made in understanding the role of topoisomerase II as a critical target for select antineoplastic drugs, certain fundamental issues relating to the molecular and biochemical mechanisms which mediate the cytotoxicity of these agents remain to be resolved. It is possible that research directed at identification of gene-specific sites of DNA damage may provide the insights necessary for a deeper understanding of the mechanisms by which topoisomerase II inhibitors compromise tumor cell growth and viability.

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