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Review

Doxorubicin, DNA torsion, and chromatin dynamics

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ABSTRACT

Doxorubicin is one of the most important anti-cancer chemotherapeutic drugs, being widely used for the treatment of solid tumors and acute leukemias. The action of doxorubicin and other anthracycline drugs has been intensively investigated during the last several decades, but the mechanisms that have been proposed for cell killing remain disparate and controversial. In this review, we examine the proposed models for doxorubicin action from the perspective of the chromatin landscape, which is altered in many types of cancer due to recurrent mutations in chromatin modifiers. We highlight recent evidence for effects of anthracyclines on DNA torsion and chromatin dynamics that may underlie basic mechanisms of doxorubicin-mediated cell death and suggest new therapeutic strategies for cancer treatment.

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1. Introduction

Doxorubicin (also called adriamycin) belongs to a class of compounds with similar structures, called anthracyclines. Like daunorubicin, the first anthracycline compound to be described, doxorubicin was isolated from *Streptomyces peucetius*, a soil bacterium [1,2]. Doxorubicin has shown great efficacy in cancer cell killing for both solid and liquid tumors, but the emergence of drug resistance and potential side effects such as heart muscle damage after doxorubicin treatment are major limitations for successful cancer treatment [3]. Despite the extensive usage in the clinics, the molecular mechanism(s) by which doxorubicin

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causes cell death or cardiotoxicity remains unclear. Thus, understanding the action of doxorubicin and related anthracycline drugs may provide clues for enhancing cancer cell killing and reducing side effects. A number of models have been proposed for doxorubicin-mediated cell death, including topoisomerase II poisoning, DNA adduct formation, oxidative stress, and ceramide overproduction [4–6]. However, the models remain disparate and controversial. A deeper understanding of the basic molecular interactions of doxorubicin within the cell is required to understand how doxorubicin kills cancer cells and causes side effects.

Anthracycline drugs such as doxorubicin are mostly planar molecules that preferentially intercalate between neighboring DNA base pairs, anchored on one side by one or more sugar moieties that sit in the DNA minor groove (Fig. 1). When DNA is topologically constrained, as in the case of plasmid circles, the strand separation that occurs during intercalation unwinds the double helix and produces DNA supercoils, resulting in increased torsional stress. Linear genomes of eukaryotes are partitioned into independent topological domains by protein factors such as insulator binding protein CTCF [7], so each domain is topological constrained. In vitro studies suggest that torsional stress can affect the structure and dynamics of nucleosomes, the repeating unit of chromatin composed of DNA wrapped around octameric histone cores [8,9]. Interestingly, recent in vivo studies implicate doxorubicin in nucleosome eviction and replacement [10,11]. Taken together, torsion-induced nucleosome destabilization is emerging as a significant molecular mechanism for the action of doxorubicin and related anthracycline drugs.

2. Models for doxorubicin-mediated cell death

A number of mechanisms have been proposed for doxorubicin-mediated cell death. However, some of these such as inhibition of DNA and RNA synthesis are only seen at doses higher than the clinical dose (~40 to 60 mg/m^2) [4] (Table 1). Here, we examine the proposed mechanisms for doxorubicin action in clinically relevant drug doses.

2.1. Topoisomerase II poisoning

Topoisomerases are highly conserved enzymes that are present in virtually all life forms, from bacteria to humans, and they regulate DNA topology to facilitate DNA replication, transcription, and other nuclear processes. Many anticancer and antibacterial drugs target topoisomerases for cell killing, such as camptothecins, etoposide, and quinolones [12]. The most parsimonious model for doxorubicin action involves topoisomerase II poisoning, resulting in double-strand DNA breaks and cell death at clinically relevant drug concentrations [3,4]. Topoisomerase II is an ATP-dependent enzyme that exists in two isoforms in humans, topoisomerase IIα and topoisomerase IIβ. The enzyme binds DNA supercoils and entangled DNA, breaks both strands of one DNA duplex, passes the other duplex through the resulting gap and reseals the break. This process results in the release of torsional stress formed during biological processes such as DNA replication and transcription (discussed below) [12]. In addition, topoisomerase II is essential for decatenation of DNA during mitosis, and deficiency in topoisomerase II prevents normal cytokinesis resulting in cell death [13]. Etoposide, a topoisomerase II poison, traps topoisomerase II at breakage sites, stabilizes the cleavage complex and impedes DNA resealing [14]. Doxorubicin has been hypothesized to function in a similar way [15] and it has been shown that topoisomerase II levels determine the effectiveness of doxorubicin treatment in a mouse model of lymphoma [16]. However, there are many examples in which doxorubicin-mediated cell killing is independent of topoisomerase II. For example, doxorubicin was shown to cause cell death independent of topoisomerase II in a promyelocytic leukemic cell line [17]. In addition, doxorubicin as well as another anthracycline drug, aclarubicin, which does not trap topoisomerase II, evicts histones independent of topoisomerase II leading to cell death [10,18]. These findings suggest that anthracycline-induced topoisomerase II poisoning by trapping topoisomerase II at

Table 1
Actions of doxorubicin and their corresponding drug dose.

	Doxorubicin dose [a]	Reference
Topoisomerase II poisoning	0.4 μM	[4]
DNA adduct formation	0.025 μM	[25]
Free radical formation	0.1 μM	[36]
Ceramide overproduction	0.3 μM	[44]
Histone eviction	0.34 μM	[11]
Cardiomyocyte apoptosis	0.1 μM	[78]
Ovarian cancer cell apoptosis	0.5 μM	[79]

[a] The drug dose varies between different cell types.

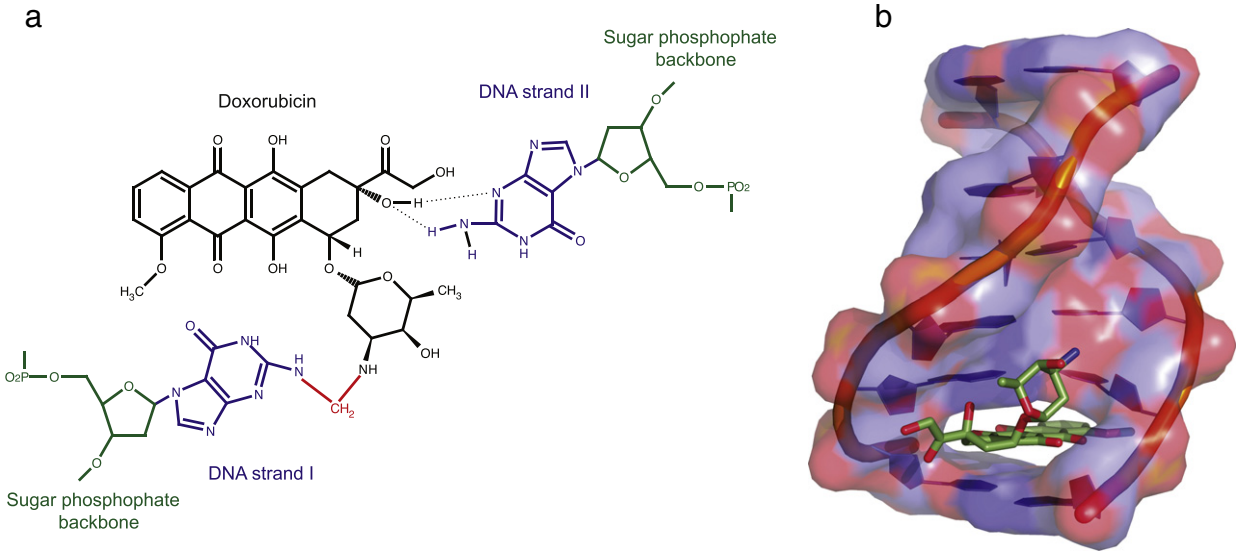

Fig. 1. Structure of the doxorubicin-DNA complex. (a) Doxorubicin forms a covalent bond (shown in red) with guanine on one strand of DNA mediated by formaldehyde and hydrogen bonds with guanine on the opposing strand [77]. (b) A structure of intercalation of doxorubicin into DNA. Doxorubicin intercalates into DNA and pushes apart the flanking base pairs with the sugar moiety sitting in the minor groove.

cleavage sites is unlikely to be the only mechanism of cancer cell killing by anthracycline drugs.

The anti-cancer activity of doxorubicin is attributable to killing of dividing cells, where topoisomerase IIα is the major form of the enzyme. However, heart muscle failure is a side effect that results from damage to non-dividing cells, where topoisomerase IIβ is the major form. Indeed, cardiomyocyte-specific deletion of topoisomerase IIβ has been shown to protect mice from developing doxorubicin-induced heart failure [19]. Inhibitors of topoisomerase II have also been shown to protect cardiomyocytes from doxorubicin-induced toxicity [20]. These findings suggest that trapping topoisomerase IIβ by doxorubicin in non-dividing heart cells underlies doxorubicin-induced cardiotoxicity.

2.2. DNA adduct formation

As a DNA intercalator, doxorubicin prefers the intercalation site containing adjacent GC base pairs, probably due to specific hydrogen-bond formation between doxorubicin and guanine (Fig. 1a) [21–23]. Formation of doxorubicin-DNA adducts has been shown to activate DNA damage responses and induce cell death independent of topoisomerase II [17,24]. Importantly, doxorubicin-DNA adducts are detectable at clinically relevant drug concentrations, suggesting that doxorubicin-DNA adducts form during chemotherapy [25]. The interaction between doxorubicin and DNA can be stabilized by a covalent bond mediated by cellular formaldehyde that is generated by free radical reactions from carbon sources such as lipids and spermine [26,27]. This interaction involves formation of a covalent bond between doxorubicin and guanine on one strand of DNA mediated by formaldehyde and of a hydrogen bond between doxorubicin and guanine on the opposing strand (Fig. 1a). Interestingly, higher levels of formaldehyde have been detected in doxorubicin-sensitive tumor cells compared to resistant tumor cells and normal cells [28,29]. The formation of more doxorubicin-DNA adducts might contribute to increased effectiveness of the drug in doxorubicin-sensitive tumor cells.

The discovery of the covalent doxorubicin-DNA adduct has led to a new approach to improve the anticancer activity of doxorubicin. Compounds that release formaldehyde upon hydrolysis, such as pivaloyloxymethyl butyrate (AN-9), butyroyloxymethyl-diethyl phosphate (AN-7), and hexamethylenetetramine (HMTA), have been developed and utilized in combination with doxorubicin. AN-9 showed a synergy with doxorubicin in cancer cell killing as well as in overcoming doxorubicin resistance to varying degrees by increasing DNA adduct levels [30,31]. In addition, the combination of AN-7 and doxorubicin has been shown to enhance its anticancer activity, to protect against doxorubicin-induced toxicity in neonatal rat cardiomyocytes, and to prevent weight loss in mice [32,33].

Despite the evidence that DNA adducts form during doxorubicin treatment, DNA adduct formation is unlikely to be the major mechanism of doxorubicin action, because clinical doses result in only 4.4 ± 1.0 adducts/10^7 base pair DNA, which accounts for just a small fraction of total doxorubicin [25].

2.3. Oxidative stress

The quinone structure of doxorubicin can be oxidized to a semiquinone radical through addition of one electron, mediated by a number of NAD(P)H-oxidoreductases [5,34]. Semiquinone radicals quickly react with oxygen to generate superoxide and hydrogen peroxide causing DNA damage. Additionally, doxorubicin is an iron chelator and the doxorubicin-iron complex catalyzes the conversion of hydrogen peroxide to highly reactive hydroxyl radicals [35]. Thus, doxorubicin-induced release of free radicals may cause oxidative stress, resulting in DNA damage and cell death [3].

A study using gas chromatography/mass spectrometry has shown that after 72 to 96 h of slow intravenous infusion of doxorubicin with a steady level of 0.1 μM in the plasma, DNA base oxidation increased up to 4-fold in peripheral blood mononuclear cells from breast cancer patients [36]. Oxidized DNA bases were also detected in a cardiac cell line and normal breast epithelial cells [37,38]. These findings suggest that free radical-induced DNA damage indeed happens early after doxorubicin treatment. If free radical formation accounts for doxorubicin-mediated cell killing, free radical scavengers would rescue cell death after doxorubicin treatment. Free radical scavengers have been used in the clinic as protectants against doxorubicin-induced cardiotoxicity but many of them failed, suggesting that free radical formation is not the only mechanism of cardiotoxicity [39].

In addition to direct DNA damage by free radical formation, administration of low doses of doxorubicin can result in increased levels of oxidative metabolism [40], which might have multiple effects on components of the chromatin landscape. For example, 2-oxoglutarate is involved in redox cycling reactions in cellular metabolism, and is also the cofactor for JmjC domain-containing histone lysine demethylases [41]. Another enzyme co-factor that is regulated by oxidative metabolism is NAD(P), which is the substrate for ADP-ribosylation reactions, including ADP-ribosylation of histones [42]. Although it is an intriguing possibility that the effects on chromatin by alterations in these enzymes resulting from doxorubicin-induced oxidative changes can lead to DNA damage, other possibilities need to be considered. For example, when a histone acetyl group is removed by a histone deacetylase (HDAC), the acetate anion that is released is co-exported with a proton out of the cell, thus incrementally raising the intracellular pH (pH_i) [43]. The hyperacetylation of histones that results from administration of histone deacetylase inhibitors prevents release of acetate and protons, thus lowering pH_i and potentially decreasing cancer cell survivability in an acidic environment. So although HDAC inhibitors have dramatic effects on global histone lysine acetylation levels and can alter the chromatin landscape, these chromatin effects might not be relevant to the anti-cancer effect of the drug. By the same reasoning, potential effects of doxorubicin on histone modification might have as-yet undiscovered metabolic effects that are independent of their effects on the chromatin landscape.

2.4. Ceramide overproduction

In addition to the generation of free radicals and the increase in oxidative metabolism, doxorubicin treatment increases ceramide levels [44–47]. Ceramide is a lipid molecule consisting of a sphingosine and a fatty acid that is involved in a variety of cellular processes including growth arrest, apoptosis, and senescence [6]. Interestingly, exogenous cell-permeable ceramide sensitizes cancer cells to doxorubicin-induced cell death [48]. In addition, doxorubicin treatment increases ceramide levels in doxorubicin-sensitive MCF-7 cells but not in doxorubicin-resistant MCF-7-AdrR cells, suggesting that ceramide levels might mediate doxorubicin resistance. Indeed, up-regulation of glucosylceramide synthase (GCS), an enzyme that converts ceramide to glucosylceramide, is associated with cellular resistance to doxorubicin, whereas suppression of GCS restores sensitivity to doxorubicin resulting in cell death [49–52]. Recently, doxorubicin has been reported to have a new role in blocking proliferation of cancer cells through stimulation of ceramide synthesis and enhancement of proteolysis of a membrane-bound protein CREB3L1 [53]. Interestingly, CREB3L1 expression level is associated with cellular sensitivity to doxorubicin, although its expression is not required for other anti-cancer drugs such as etoposide, bleomycin, or paclitaxel to inhibit cell growth. Surprisingly, no cell death was observed after doxorubicin treatment in this study [53]. It is possible that inhibition of cell proliferation makes cells less susceptible to doxorubicin, as doxorubicin preferentially kills dividing cells. These findings suggest that ceramide overproduction might be specifically involved in sensitizing cancer cells to doxorubicin treatment, but this upstream effect is unlikely to involve changes in the chromatin landscape.

3. Doxorubicin, DNA torsion, and chromatin structure

The disparate models described above underscore the controversies surrounding the multiple modes of doxorubicin action within the cell. Next we consider the possibility that the intercalation of doxorubicin between DNA bases has a direct effect on chromatin that ultimately results in cancer cell killing. Specifically, we focus on the consequences of doxorubicin intercalation into DNA and ask what effect if any this event might have on chromatin structure and dynamics.

3.1. DNA topology and chromatin

When an anthracycline molecule enters the cell, it diffuses into the nucleus and intercalates into DNA. Anthracyclines are bound to DNA so stably that daunorubicin autofluorescence has been used by cytogenetics for Q-banding chromosomes, whereby AT-rich regions fluoresce brightly and GC-rich regions quench [54]. Intercalation of anthracyclines pushes apart the flanking base pairs (Fig. 1b). Because each base pair is stacked in 36° counterclockwise rotation relative to the pair below, intercalation has topological consequences. In covalently closed circular DNA, the two strands are wound around each other a certain number of times, called the linking number (Lk). When the DNA circle is relaxed, Lk is equal to the number of turns in the double helix, or the twist (Tw), which is roughly 1 turn per 10.5 base pairs. Changes in the relaxed value of Lk results in torsional stress that manifests itself as changes in Tw and/or writhe (Wr), which is the number of times the double-stranded DNA crosses itself, commonly known as supercoiling. Writhe can be described as positive or negative, depending on whether supercoiling occurs in right- or left-handed direction, respectively.

These topological aspects are most evident in bacteria, whose circular genomes exist in a tightly regulated topological state. However, linear genomes of eukaryotes can also be described using the same topological terms, as the genome is partitioned into independent topological domains whose borders are restricted [7]. Thus, changes in Lk, Tw, and Wr in one domain do not transfer to another. The wrapping of the DNA around the octameric histones in a left-handed direction generates one negative Wr per nucleosome. Thus, nucleosomes constrain negative supercoiling.

3.2. Torsional stress and nucleosome destabilization

Cellular processes that require access to DNA inevitably alter its topological state, producing torsional stress. These processes include DNA replication, transcription, recombination, and repair. During replication, helicases that separate the two strands alter the Lk and produce torsional stress, resulting in waves of positive supercoiling downstream of the helicase. Similarly, transcription by RNA Polymerase II (RNAPII) results in denaturation of DNA at the transcription bubble. The subsequent rotation of the DNA relative to RNAPII during transcription creates domains of positive and negative supercoils downstream and upstream, respectively, as predicted by the twin-supercoiled domain model [55,56]. Furthermore, enzymes that remove or replace nucleosomes alter the supercoiling levels. For example, the nucleosome remodeler SWI/SNF is known to generate negative supercoils [57,58].

Torsional stress can be detrimental, affecting the overall structure and integrity of DNA. Furthermore, changes in Tw and/or Wr can deregulate processes such as replication, transcription, and nucleosome stability (discussed below). For example, accumulation of positive torsion leads to transcriptional inhibition in over 80% of all genes in yeast [59,60]. To counteract torsional stresses generated during various DNA-based processes, cells utilize topoisomerases, thereby altering Lk. As discussed above, doxorubicin can inhibit topoisomerase II in the cleaved form and thus directly cause double-strand breaks. However, as a DNA intercalator, doxorubicin primarily alters DNA topology. Single molecule measurements show that intercalation of one doxorubicin molecule relaxes the natural twist of the double helix by −27° [61], suggesting significant underwinding in the presence of the drug. Such a change in Tw in the negative direction introduces compensatory positive torsional strain on the DNA. Increases in torsional stress can affect many different processes, but because over 80% of eukaryotic DNA is complexed in nucleosomes, perhaps the most immediate effect is directly on nucleosome structure and dynamics.

Nucleosomes are quite sensitive to torsional changes. Single molecule in vitro studies shows that nucleosome assembly stalls when DNA is under positive torsional stress [62] whereas negative supercoiling promotes assembly [63]. Another in vitro study showed a preferential exchange of nucleosomes from positively- to negatively supercoiled DNA [64]. Furthermore, the presence of positive torsional stress seems to induce a structural change in the nucleosome, presumably resulting in a more open complex where the H2A/H2B dimers somewhat dissociate from the tetramer core [8,9]. This kind of restructuring may render the nucleosome less stable.

3.3. Doxorubicin and nucleosome dynamics

The unwinding of DNA upon doxorubicin intercalation may produce sufficient positive torsional stress to destabilize nucleosomes, which would serve as a direct mechanism for action of the drug. Indeed, a study from our laboratory tested this hypothesis using a recently developed strategy to measure nucleosome turnover, the disassembly and subsequent reassembly of nucleosomes. Using metabolic labeling of newly synthesized proteins followed by affinity purification, newly incorporated H3/H4 core particles can be analyzed by tiling array or next generation sequencing [65,66]. The method is called covalent attachment of tags to capture histones and identify turnover, CATCH-IT. By comparing nucleosome turnover profiles in mouse squamous cell carcinoma cells before and after doxorubicin treatment at a concentration of 0.34 μM, we found that doxorubicin enhances nucleosome turnover around active gene promoters, despite a minor effect on gene expression level. This enhancement is independent of the DNA damage response proteins, p53 and ATM. This latter point is important as p53 is mutated in many human cancers and both ATM and p53 have been implicated in the response of tumors to doxorubicin therapy [67,68]. Interestingly, a similar effect on nucleosome turnover was observed by treating the cells with another anthracycline drug, aclarubicin, which inhibits topoisomerase II without causing DNA double strand breaks [18] indicating that anthracycline drug intercalation into DNA may play a direct role in enhancing nucleosome turnover [11]. Another study showed that anthracycline drugs including doxorubicin and aclarubicin, but not etoposide, evict histones from regions of accessible chromatin in both human melanoma cell lines and acute myeloid leukemia blasts from patients, leading to impairment of DNA repair and apoptosis [10]. Additionally, this study found that 9 μM doxorubicin evicts histones in topoisomerase IIα-depleted cells, suggesting that topoisomerase IIα is not required for doxorubicin-induced histone eviction [10]. Taken together, these studies suggest that inhibition of topoisomerase II could further exacerbate torsional strain by anthracycline drug intercalation, leading to enhancement of nucleosome turnover downstream of promoters.

Topoisomerase inhibitors can have dramatic effects on chromatin-associated processes, including replication and transcription. The positive torsion generated ahead of RNA polymerase [55] can potentially unwrap nucleosomes, which are negatively supercoiled, and destabilize them [69]. We have found that inhibitors of both topoisomerase I and II cause both increased positive torsion and increased nucleosome turnover in gene bodies and vice-versa in intergenic regions of *Drosophila* cells [70]. The net effect of topoisomerase-mediated enhancement of torsion in gene bodies is to further destabilize nucleosomes during RNA polymerase transit, thus increasing exposure of DNA to processes that can cause DNA breaks, such as free radicals, and ultimately cell death. Nucleosome destabilization might also be promoted by SWI/SNF-class remodelers which act to evict nucleosomes at promoters,

countered by ISWI/CHD-class remodelers that act to stabilize nucleosomes in gene bodies [71]. In this way, doxorubicin intercalation into promoters and genes might act by interfering with the balance between maintaining promoters free of nucleosomes while preventing loss of nucleosomes during transcription. This model may account for the observation that anthracycline drugs, including aclarubicin, increase somatic recombination in a *Drosophila* in vivo assay for DNA damage [72].

4. Future directions

Over the last several decades, many different modes of doxorubicin action have been reported, which is consistent with the broad spectrum of activity of the drug in cancer treatment. As discussed above, intercalation of doxorubicin into DNA leading to torsional stress and nucleosome destabilization may account for the basic mechanism of anthracycline-mediated cell killing. Surveying the effects of other intercalating drugs on chromatin structure and dissecting the mechanistic link between this and cancer cell killing may lead to the development of better anticancer drugs. Interestingly, another intercalating drug, voreloxin, a quinolone derivative, has been recently found to act similarly to anthracyclines in killing cancer cells, and intercalation is required for voreloxin-mediated cell death [73]. Voreloxin holds promise as an alternative to anthracyclines, as it does not generate high levels of reactive oxygen species, which can contribute to anthracycline-mediated cardiotoxicity [39,73].

The fact that relatively high doses of anthracyclines (9 μM) used in one study caused histone eviction and apoptosis [10], whereas low sublethal doses (0.1 –0.4 μM) used in another study nevertheless caused global nucleosome turnover and a DNA damage response [11], raises the possibility that downward dosage adjustments might be considered for current clinical protocols based on the maximum tolerated dose. The effect of anthracyclines on destabilizing nucleosomes also suggests that combining anthracyclines and other drugs that destabilize nucleosomes may have synergistic effects in cancer cell killing. For example, valproic acid, an HDAC inhibitor approved for clinical use as an anticonvulsant and mood-stabilizing drug, increases the activity of doxorubicin and leads to tumor regression and chromatin decondensation when used in combination with another anthracycline drug, epirubicin [74–76]. The development of new drugs that destabilize nucleosomes have the potential of enhancing the therapeutic efficacy of anthracycline drugs while reducing side effects.

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