Chapter

Molecular-Level Understanding of the Anticancer Action Mechanism of Anthracyclines

Manish Shandilya, Shrutika Sharma, Prabhu Prasad Das and Sonika Charak

Abstract

Anthracyclines drugs are used as a treatment regime to combat cancer owing to their great chemotherapeutic potential. They are characterized by the presence of a wide range of derivatives, the most famous are doxorubicin and daunorubicin. The proposed action mechanism of anthracyclines and their derivatives to exert cytotoxic effect involves the intercalation of the drug molecule into nucleic acid and inhibition of the activity of topoisomerases. These events consequences in halting DNA replication and transcription mechanisms of the cell. Understanding of the structural and conformational changes associated with nucleic acid after binding with drugs provides significant knowledge for the development of more effective drugs. A comprehensive elucidation of the molecular mechanism(s) of action of anthracyclines drugs plays a significant role in the rational drug designing to obtain an effective, selective, and safe anti-cancer drugs.

Keywords: anthracycline, anti-cancer activity, DNA, molecular mechanism

1. Introduction

Anthracyclines were primarily recognized as antibiotics due to their anti-bacterial properties in 1939 [1]. However, the chemical characterization of the anthracyclines which includes a rigid planar aromatic ring that remains bound to an amino-sugar by a glycosidic bond (**Figures 1** and **2**). Quinone and hydroquinone groups of these molecules on adjacent rings allow gain and loss of electrons in the conversion of quinone to the semiquinone radical [2, 3]. This semiquinone free radical converts back to quinine under aerobic conditions resulting in the formation of superoxide anion and hydrogen peroxide. The excessive formation of these free radical consequences in lipid peroxidation within cell membranes, DNA damage and finally cell death. This makes them the potent non-selective anti-cancer drugs i.e., they are used in the treatment of a wide range of cancer like small cell lung cancer, breast cancer, lymphoblastic and myeloblastic leukemia, etc. [1, 4–6]. There is a high probability that a cancer patient will be administered with anthracycline at some stage of their chemotherapy session. Daunomycin and doxorubicin were the earliest anthracyclines isolated from *Streptomyces peucetius* and were effective against a wide range of human cancers. Owing to significant antitumor potential, the World Health Organization (WHO) has included daunomycin and doxorubicin

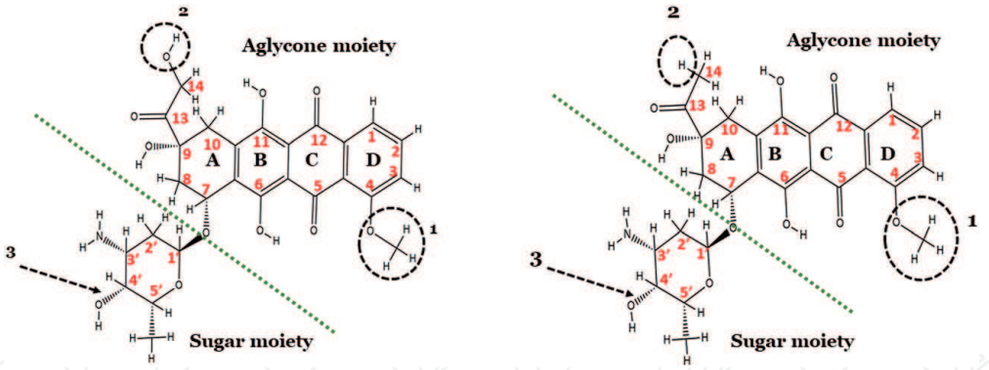

Figure 1.
Chemical structure of doxorubicin and daunorubicin. Dotted circle and dotted line arrow represents probable substitution position in anthracyclines. Doxorubicin and daunorubicin differ at C_{14} position encircled with 2. Green dotted line is used to depict aglycone and sugar moieties of anthracycline drugs. Chemical structures were rendered using ChemDeaw software.

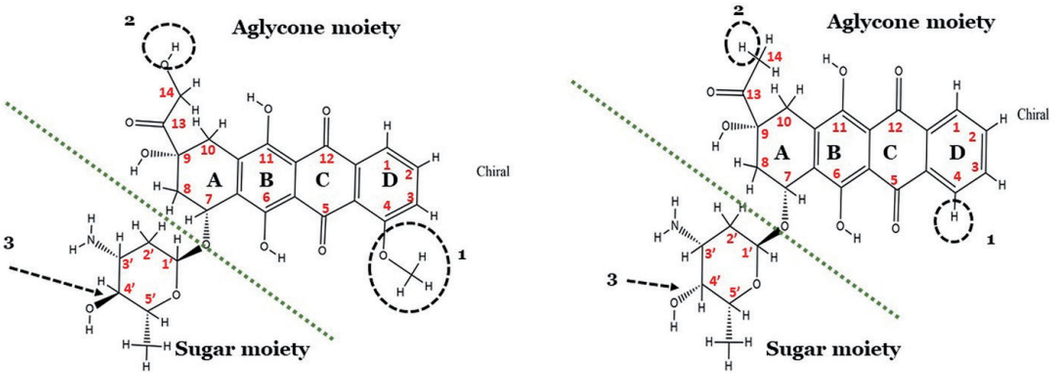

Figure 2.
*Chemical structure of epirubicin and idarubicin. Epirubicin and doxorubicin (**Figure 1**) differs at position no. 3 (C4′ of sugar moiety, stereoisomer). Idarubicin differs from daunorubicin (**Figure 1**) at position 1 by absence of methoxy group. Chemical structures were rendered using ChemDraw software.*

in the model list of medicines [7]. But it has been discovered that repeated administration of these drugs can impart chemotherapy-resistance to the tumors and cardiotoxicity [4]. To reduce or subside these side effects, major efforts are being done to find better alternatives. Therefore, the study of more than 2000 analogs has been done so far [8].

Regarding the chemical structure of daunomycin $C_{27}H_{29}NO_{10}$ and doxorubicin $C_{27}H_{29}NO_{11}$, they share the same carbon skeleton (**Figure 1**). The difference in their chemical structure comes at the side chain at C-14 position; daunomycin has a hydrogen atom whereas doxorubicin has an alcohol group (**Figure 1**) [5, 6].

2. Proposed action mechanisms

2.1 Anthracyclines as DNA intercalators

The exact mechanism of the anthracycline in the body is not known and still under investigation. However, DNA is recognized as the prime target of well-known anthracycline like doxorubicin. The primary mechanism involves the intercalation of planar tetracyclic chromophore between the DNA base pairs subsequently affecting the transcription and translation of DNA. The binding affinity of the drug to DNA is not only the factor contributing to the cytotoxic activity of anthracyclines but other factors like binding mode and binding site of anthracycline also play important role in exerting cytotoxic effects.

The specificity, binding affinity, and the binding mode of every anthracycline differ with the difference in the sequence of DNA bases (**Figures 3–5**). Structural, computational and solution studies on the daunomycin-DNA complex have provided the information that daunomycin has preferential binding where AT is present between two GC base pairs i.e. GCATGC [9, 10]. Equilibrium binding and DNase footprinting methods were utilized to study site and sequence specificity of daunorubicin to DNA. The results of these experiments demonstrate that daunomycin indeed recognizes specific DNA sequences and its binding affinity with DNA increases with an increase in GC content [11]. Moreover, the effect of daunorubicin in cleaving the linear pBR322 DNA by restriction endonuclease *Eco*RI and PvuI was investigated to assess the sequence-specific binding of daunorubicin [11]. The recognition sequence of *Eco*RI and PvuI are 5′-GAATTC-3′and 5′-CGATCG-3′ respectively. Chaires et al. observed that PvuI inhibits the rate of digestion of pBR322 DNA more than the *Eco*RI suggesting preferential sequence specificity of daunorubicin. Similar results were evident from the crystal structure analysis of daunomycin-DNA d (CpGpTpApCpG) complexes (**Figure 4**) [10]. The specificity for the GC base pair is due to the hydrogen bonds formed during the interaction. The hydroxyl group on C9 of daunorubicin interact with N2 and N3 of guanine with two hydrogen bonds. This preference to GC base pair also explains the increase in binding affinity of the drug with an increase in GC content of DNA [10]. Similarly, crystal structures of anthracyclines like doxorubicin, epirubicin, and idarubicin show sequence-specific intercalative binding mode between DNA bases (**Figures 3** and **5**).

Moreover, several experiments were done to study the intercalation mechanism of anthracyclines with DNA. A spectrofluorometric method was also developed for the estimation of anthracycline intercalation in living cells and DNA solutions [12]. Belloc et al. have done measurement of anthracyclines (daunorubicin and idarubicin) intercalation in the DNA of living cells by flow cytometry [13]. Ashley et al. has demonstrated the intercalative property of anthracyclines in nuclear as well as in mitochondrial DNA using picogreen (fluorescent DNA binding dye) [14]. The intercalation of anthracycline into mitochondrial DNA has a significant impact

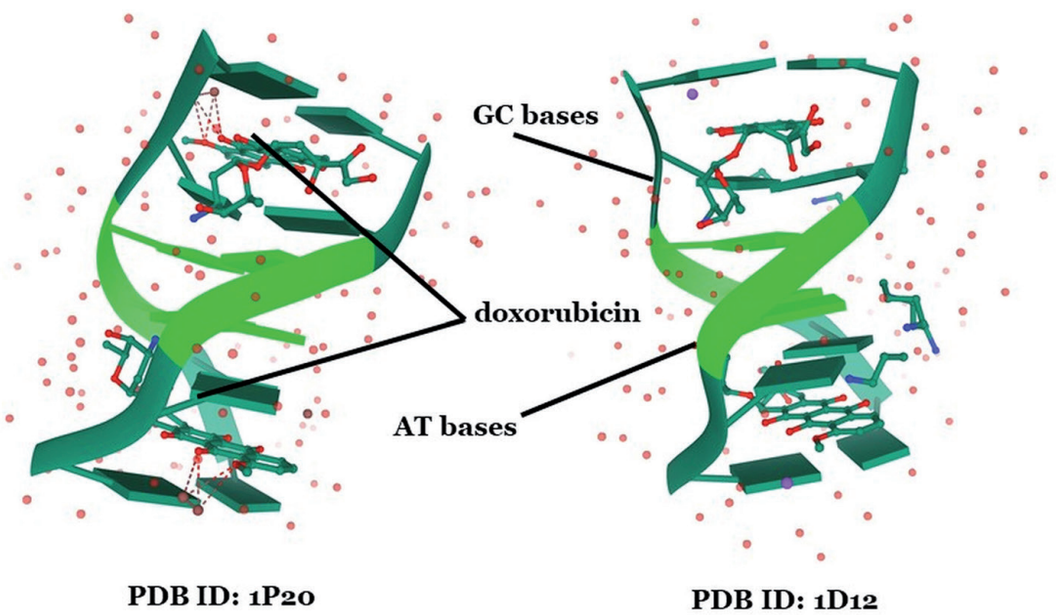

Figure 3.
Intercalation of doxorubicin between GC basepairs. Crystal structure of doxorubicin getting intercalated between GC bases. Structures are taken from protein data bank. Light green color depicts AT baseparing and dark green color shows GC bases. Red dots represent crystallized water molecules. Figure clearly depicts intercalation of doxorubicin in GC bases of DNA and preferential binding with nucleotide sequences.

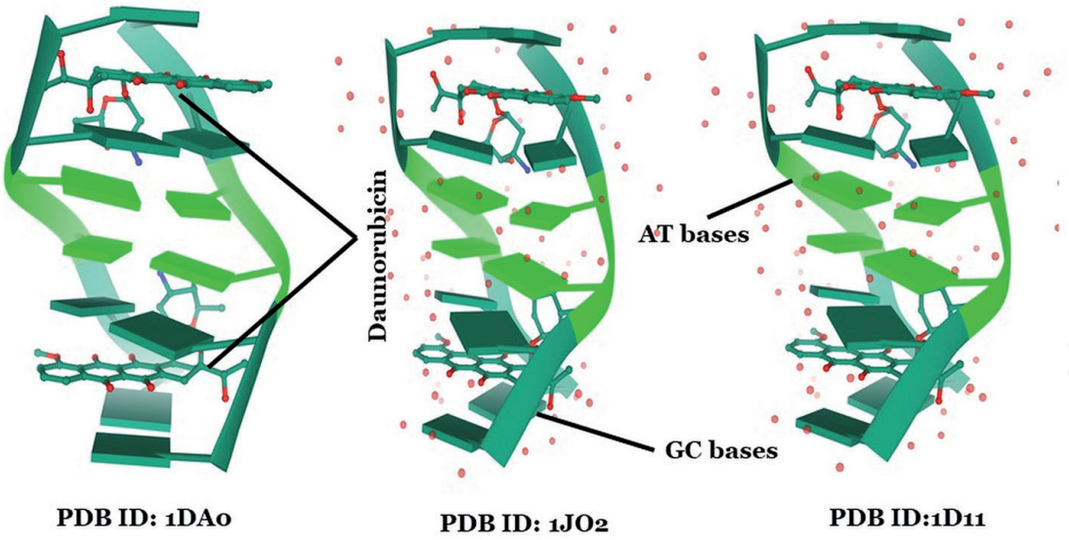

Figure 4.
Intercalation of daunorubicin between GC basepairs. Crystal structure of daunorubicin getting intercalated between GC bases. Structures are taken from protein data bank. Light green color depicts AT baseparing and dark green color shows GC bases. Red dots represent crystallized water molecules. Figure clearly depicts intercalation of doxorubicin in GC bases of DNA and preferential binding with nucleotide sequences.

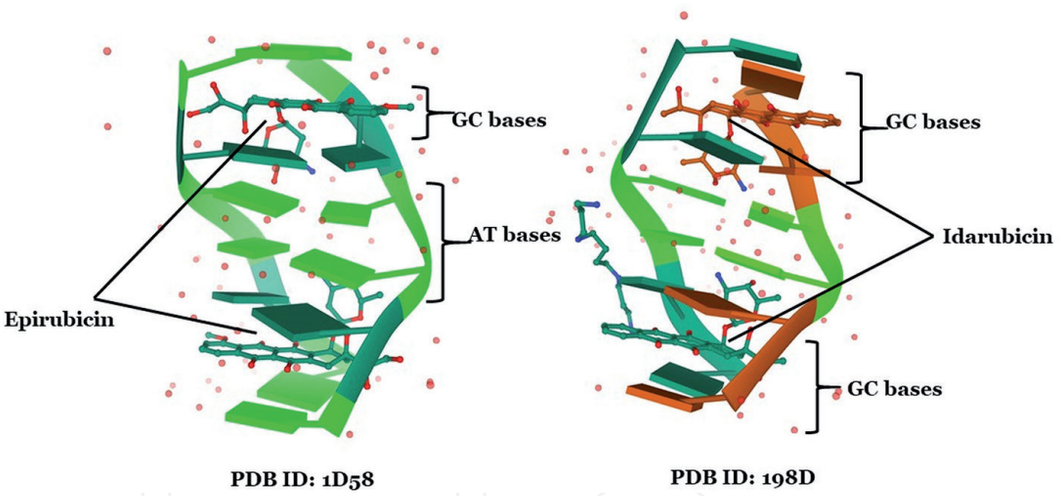

Figure 5.
Intercalation of epirubicin and idarubicin between GC basepairs. Crystal structure of epirubicin getting intercalated between AT and GC basepairs. Crystal structure of idarubicin getting intercalated between GC bases. Structures are taken from protein data bank. Red dots represent crystallized water molecules. Figure clearly depicts intercalation of epirubicin and idarubicin in GC bases of DNA and preferential binding with nucleotide sequences.

on mitochondrial toxicity. The effect of doxorubicin binding on the morphology of the single stranded DNA was further quantitatively analyzed using Atomic Force Microscopy (AFM). AFM studies strengthen the probable mechanism of intercalative binding mode as consequences of doxorubicin interaction with DNA [15].

Other studies found that B-DNA is preferred over Z-DNA by the daunorubicin for binding. Allosteric conversion of the Z form into B form has also been observed in some cases. Ionic concentration in which usually Z form of DNA is present changes to B form on the binding of daunorubicin to poly dGdC or resist to change from B form to Z form [16]. There are several pieces of evidence suggesting drug binding to DNA results in the inhibition of specific DNA function contributing towards their therapeutic effects.

2.2 How anthracyclines damage DNA?

Anthracyclines are known to damage DNA by several mechanisms which include topoisomerase-II poisoning, free radical formation, and DNA-anthracycline adduct formation. The semiquinone radical of anthracycline can intercalate between DNA base pair resulting in DNA damage by forming reactive oxygen species (ROS).

2.2.1 Via topoisomerase II poisoning

Along with DNA intercalation, topoisomerase II is also considered as the primary target of anthracyclines [17]. Topoisomerases help in solving the topological problems like supercoiling, knotting, and catenation of DNA during replication, transcription, and recombination by creating single and double stranded breaks and subsequently rejoining the breaks. Based on structure and function, mammalian cells have two types of topoisomerases which are topoisomerase I and II. Topoisomerase I is monomeric and forms single strand breaks in DNA whereas topoisomerase II is dimeric and introduces double stranded breaks in DNA. Anthracyclines interfere with the normal functioning of breaking and rejoining of DNA strands by topoisomerases, particularly topoisomerase II consequence in the formation of an abortive anthracycline- DNA-topoisomerase ternary complex, hence poisoning the enzyme action. This ternary complex impends the religation of breaks in the dsDNA. Hence, anthracyclines act on topoisomerase II and stabilize the DNA-topoisomerase II complex. Due to this topoisomerase II which otherwise is essential for the normal functioning of the cell now acts as a lethal toxin to the cell and leads the cell to apoptosis. During intercalation, the planar ring of the aglycone and sugar moiety remains in contact with DNA bases while the A ring and the substituents present the C9 which are present in the minor groove of DNA interact with the enzyme (**Figures 1** and **2**). Perhaps that's why modifying the C9 substituent changes the activity of the drug. Cleavage does not occur on all the sites recognized by the Topoisomerase II and depends on the specific base sequence where the drug interacts with the enzyme. An increase in drug activity is seen when the 4-methoxy group is removed and the sugar moiety is substituted on 3′. 3′ substitution also has a significant role in the determination of specific sites for anthracycline associated cleavage of DNA.

Several lines of evidence have shown that these anthracyclines induce irreversible DNA damage by forming a ternary complex with DNA topoisomerase which introduces permanent double stranded breaks which ultimately lead to apoptosis in rapidly dividing cells [18, 19].

2.2.2 Via oxidative stress

Anthracyclines also causes the production of free radicals inside the cell which are responsible for the cytotoxic effect of these drugs. Though the mechanism of this process is still unclear increased number of reactive oxygen species (ROS) and the presence of deoxyaglycone in the urine after the administration of drugs indicates the possibility of this mechanism [5]. Oxidative stress is the imbalance between the reactive nitrogen species and reactive oxygen species in the cell. Mitochondria are believed to take part in this process. Quinone ring of the anthracyline aglycone act as electron acceptor (**Figures 1** and **2**). In the electron transport chain (ETC), one electron is transferred from NADPH to flavoprotein and then to the aglycone due to which quinone gets reduced to form semiquinone free radical. This reaction is catalyzed by NADPH cytochrome P-450 reductase [20]. From

the semiquinone, this electron gets transferred to oxygen and semiquinone gets converted into stable hydroquinone in this redox cycle. Due to this electron transfer oxygen gets reduced to superoxide which readily gets converted to other reactive oxygen species like hydroxyl radical which is harmful to cells. These reactive oxygen species can lead to DNA damage and lipid peroxidation which finally result in cell apoptosis.

When oxygen becomes limiting, semiquinone free radical transfer the electron to other electron acceptors, and rearrangement in anthracyclines takes place. The aglycone ring remains connected to sugar moiety by a glycosidic bond which is an ether containing linkage with oxygen in it which accepts the unpaired electron and due to this cleavage of glycosidic bond takes place. As a result, the formation of 7-deoxyaglycone takes place which either directly passes through urine or as conjugates in the bile [5]. There are several hypotheses explaining why superoxide dismutase (SOD), catalase, and antioxidants do not work effectively after the administration of anthracyclines. Studies have shown that anthracyclines can alter the glutathione (GSH) level and the enzymes involved in its redox pathway. According to another hypothesis free radicals are formed on the cell surface or internally in the cell and then get transferred to the cell surface due to which SOD and catalase fail to act upon these ROS [17].

2.2.3 Via forming DNA-anthracycline adduct

Formation of the anthracycline-DNA adduct in the presence of formaldehyde is another proposed mechanism of anthracycline action. There are studies that show the formation of doxorubicin-DNA at clinically relevant concentrations of drugs [21]. Anthracycline forms covalent bond with one DNA strand and hydrogen bond with other DNA strand resulting in DNA damage. The reaction between anthracycline and cellular formaldehyde is the foremost step to form activated Schiff base which then forms aminal linkage with the exocyclic amino group of guanosine. Formaldehyde in the cells is derived from the various carbon sources like lipids and spermine by iron mediated free radical reactions. In addition, Kato et al. have found increased formaldehyde levels in the cancer cells as compared to normal cells [22].

Phillips et al. using the bidirectional transcription footprinting technique demonstrate that adriamycin-DNA adduct formation induces transcription inhibition with alkylation at specific DNA sites [23]. However, Bilardi et al. have demonstrated that anthracycline-DNA adduct induces breaks in DNA via a mechanism independent of topoisomerase-II [24]. Moreover, DNA breaks formation occurs mainly due to the stalled or collapsed replication fork and these DNA breaks are repaired by homologous recombination dependent process. Forrest et al. investigated the activation of DNA damage response pathways after doxorubicin-DNA adduct formation and found that the downstream processing is dependent on various stages like recognition of adduct during replication, transcription, or any other stage of the cell cycle. Ataxia telangiectasia mutated (ATM) and ATM and Rad3 related protein kinase (ATR) are the DNA damage recognition proteins that get activated in response to double and single DNA strand breaks respectively. Forrest et al. demonstrate that both ATM and ATR proteins have the capability to react with intermediates produced as a result of doxorubicin-DNA adduct formation [25].

DNA-anthracycline adduct formation probably follows a similar pattern as followed by cisplatin-DNA adduct (**Figure 6**). The same process also takes place in presence of xanthine oxidase which is present in the living cells [23, 26]. Other studies revealed that formaldehyde is also an important factor for the DNA adduct formation which is formed in the reaction media due to oxidation of different components in media by hydrogen peroxide. Formaldehyde has the carbon through

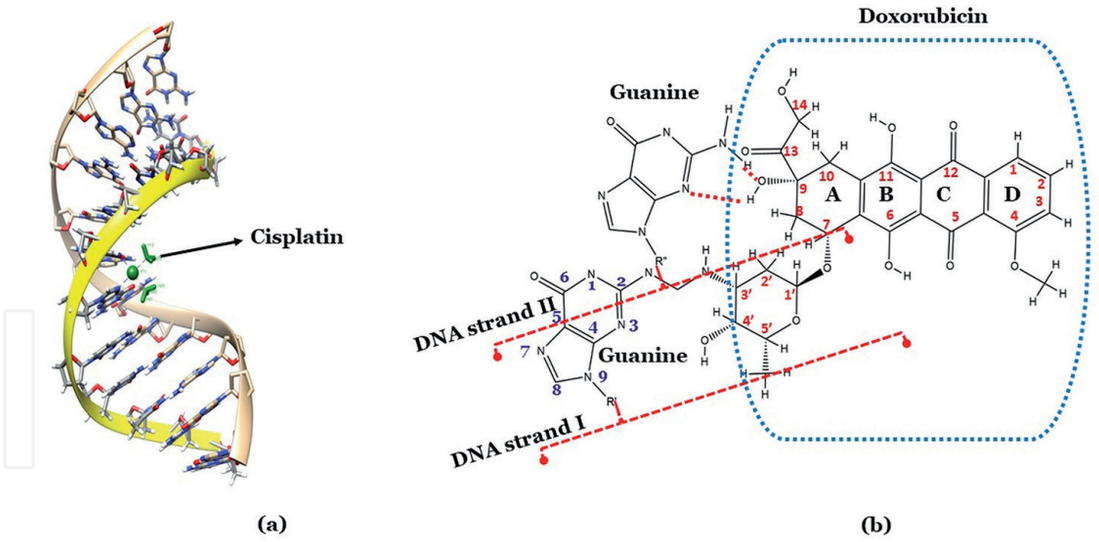

Figure 6.
DNA-drug adduct formation. DNA cisplatin structure was visualized and rendered using Chimera software. Chemical structures were rendered using ChemDraw software. (a) DNA-cisplatin adduct (PDB ID: 2NPW). (b) Schematic representation of doxorubicin-DNA adduct.

which N-C-N i.e. covalent bond forms between 3′ NH_2 of amino sugar of doxorubicin/daunorubicin and 2-NH_2 of guanosine residue of DNA backbone (**Figure 6**) [25]. Quinone methide 1 is present as a transient form in this reaction. Further, it is stabilized by the hydrogen bond formed between two strands of double helical DNA. Though the structure of the adduct is known but the mechanism by which it promotes cell death is not clear [27].

Doxorubicin forms DNA adduct with the assistance of formaldehyde and the concentration of formaldehyde in the cell is low. So, to overcome this, some prodrugs are administered which gets converted to formaldehyde inside the cell for e.g. pivaloyloxymethyl butyrate give rise to formaldehyde inside the cell when cleaved by esterase. Due to DNA-anthracycline adduct formation cytotoxicity of anthracyclines readily increases [21].

3. Side effects

Cardiotoxicity is one of the main side effects caused by anthracyclines. Types of cardiotoxicity are Type I and Type II, Type I cardiotoxicity results irreversible cell death, while Type II cause reversible damage. At the beginning, cardiotoxicity thought to directly correlate with the cumulative drug dose, but later on, it was discovered that cardiotoxicity can occur even during the treatment [28, 29].

Increased ROS level in cardiac cells is considered as one of the factors contributing to anthracycline mediated cardiotoxicity. Administration of anthracyclines consequences in increased ROS involving the transfer of an electron to oxygen during the reduction of quinone to semiquinone which further gets converted to hydroquinone to complete the redox cycle. This process takes place in mitochondria and the number of mitochondria is mostly higher in cardiomyocytes as compared to other cells [30]. Moreover, enzymes such as SOD, catalase, glutathione transferase, and cytochrome P450 which can reduce the level of ROS in cells are comparatively less in cardiomyocytes resulting in an increased cardiotoxic effect [31]. According to some reports, doxorubicin also increases NADPH oxidase level and enzymes containing flavin like nitric oxide synthase, p450 reductase in cardiomyocytes. These enzymes increase the level of reactive oxygen species in the cell which leads to oxidative stress [31, 32].

Doxorubicin has a strong affinity for Fe^{+3} ions and the reaction of ferric ions with hydroxyl and ketone groups of doxorubicin results in a free radical complex of doxorubicin-Fe^{+2} [33]. The interaction between the negatively charged cell membranes and as this positively charged doxorubicin-Fe complex consequences in lipid peroxidation [33, 34]. Normally the level of iron is quite low in cells and usually, at this low level, doxorubicin cannot interact with iron [29]. The balance of iron is maintained by some transport proteins in the cell and according to some recent studies, doxorubicin disturbs this balance and as a result accumulation of iron occurs in mitochondria [34, 35]. Doxorubicin also interacts with the Fe/S group of iron regulatory protein IRP1 and affects its post-translational modification due to which function of IRP1 is affected and its binding affinity to IRE (iron response element) becomes low resulting in an increased amount of iron in the cell [33]. Mitoferrin, a mitochondrial carrier protein that helps in the entry of iron into the mitochondria and another transfer protein mABC1 export out the iron from mitochondria. Doxorubicin affects the functioning of mABC1 protein due to which it stops the export of iron out of the mitochondria while mitoferrin functions normally and as a result amount of free iron increases inside mitochondria [36].

4. Anthracycline resistance

Along with cardiotoxicity, anthracyclines treatment also induce anthracycline resistance even at the desired cumulative dose [37]. Resistance to drugs can be either natural or can be acquired. Natural resistance is detected in some cells even before the administration of the drug. While the acquired one occurs after the administration of the drug. Several mechanics of drug resistance responsible for the incidence of drug-resistance which are: change in ATP- binding cassette [ABC] related drug efflux and accumulation, qualitative and quantitative changes in topoisomerase II, p53 activity, overexpression of ROS scavenging enzymes, etc. ABC (ATP- binding cassette) transporter proteins are considered as the primary cause of anthracycline related drug resistance [R]. P-glycoprotein (Pgp) is one of the ABC proteins believed to induce anthracycline resistance by drug efflux, inhibition of influx, and drug accumulation inside the cell [38]. Anthracycline interacts with Pgp which causes the active efflux of anthracycline from the cell through its transmembrane domain. Pgp is encoded by the mdr1 gene which becomes active in case of cell differentiation under any chemical or environmental effect. As the anthracycline interacts with the plasma membrane, Pgp recognizes it and exports it out. So, the increased level of Pgp creates an imbalance between the export and import of the drug.

Moreover, alteration in topoisomerase II activity either quantitative i.e. decrease in the number of enzyme or qualitative i.e. alteration in the normal activity of enzyme due to mutation or other reason can give resistance to cell against further doses of anthracyclines. Anthracycline mediated cell apoptosis also depends on the expression of p53. So, inactivation or down regulation of p53 can give rise to drug resistance [38]. SOD, GSH, catalase are the scavengers of ROS and their over expression can also impart anthracycline resistance during cytotoxicity [39]. An increase in the repair of the DNA damage caused by anthracyclines also contributes towards anthracyclines resistance in cells.

5. Anthracycline analogs

Several studies were conducted to understand the effect of structural changes in anthracyclines on their antitumor efficacy. Modification of anthracyclines has

a significant impact on their antitumor activity as well as on their side effects. Therefore, a comprehensive study on understanding the effect of specific structural modifications in anthracyclines to its antitumor potential and efficacy leads to the development of better analogs. Subtle modifications in the chemical structure of anthracyclines had a significant impact on the rate of drug penetration into the nucleus [40]. Doxorubicin and it semisynthetic analog epirubicin prepared by epimerization at C-4′ sugar display different antitumor potential (**Figures 1** and **2**). Moreover, epirubicin causes less cardiac damage as compared to its parent compound. Similarly, the removal of the methoxy group in the anthraquinone structure of daunorubicin results in the formation of idarubicin, considered to a better analog with a broad spectrum of antitumor potential as compared to daunorubicin (**Figures 1** and **2**).

Numerous studies were undertaken in preparation for better analogs. One such study was done with the aim of preparing a better daunorubicin analog that was prepared and their level of cytotoxicity, DNA damaging property, cellular uptake of daunorubicin analogs was investigated to find an anthracycline that can overcome drug resistance. Anthracycline derivatives display different action mechanisms in causing DNA lesions in various human cancer cell lines as well as in their resistant sublines [41]. It was hypothesized that the replacing primary amino group at the C3 position of the daunosamine moiety by a trisubstituted amidino group might help in overcoming drug resistance [41].

Shaul et al. investigated the subcellular localization as well as their cytotoxic effect of anthracyclines and their analogs in various cell lines [42]. Association between the chemical structure of different anthracyclines and their subcellular distribution and their function was investigated in cancer cell lines. Confocal microscopy experiments were done to study subcellular localization of anthracyclines and their analogs. Fluorescent DNA intercalator displacement experiments conducted for studying the intercalative properties demonstrated that the DNA intercalation property of anthracycline was not related to their cytotoxic effect. Structural information on the binding of anthracycline drugs with the target molecule helps in the development of effective drugs.

X-ray crystallographic and NMR (nuclear magnetic resonance) spectroscopic studies on anthracyclines-target complex have been conducted in the past and provide significant information that helps in the rational designing of drugs. Proceeding in similar lines, Yan et al. investigated the interactions of doxorubicin and its derivatives with DNA using resonance Raman and surface-enhanced resonance Raman scattering spectroscopy and provide significant details on anthracycline binding with DNA [43]. Spectroscopic techniques like Fourier transform infrared spectroscopy (FTIR), circular dichroism, fluorescence provided significant information binding properties of anthracyclines with nucleic acid [44–48]. Conformational studies on anthracycline-nucleic acid complex using computational methods like molecular dynamics (MD) simulations also contributes significantly to the development of the better anthracycline alternative.

6. Conclusion

Anthracyclines are widely used as antineoplastic agents owing to their great anticancer potential. There are several mechanisms proposed by which anthracyclines exert their cytotoxic effect. These drugs mostly act as DNA intercalators and halt vital functions like transcription and replication of cells. DNA damage by topoisomerase II poisoning, oxidative stress, and by forming anthracycline-DNA adduct are other proposed mechanism of anthracycline action. Despite their

widespread usage in cancer treatment, their administration consequences in certain adverse side effects including cardiotoxicity that limits their clinical use. Therefore, further elucidation of the mechanism by which anthracycline drugs exert their cytotoxic effect becomes extremely important. Structural, *in silico* molecular docking/MD simulations studies and their correlation with the cytotoxic effect provide significant information for the development of structure-based analogs. The synthesis of novel structure-based anthracycline analogs should be continued to get an analog with better efficacy and minimum side effects. The development of an analog that can reverse the effect of drug resistance as well as reduce dose-dependent toxicity is essential.

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Conflict of interest

The authors declare no conflict of interest.

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