Understanding anthracycline action: molecular insights to improve cancer therapy

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CHAPTER 1

Introduction to Anthracycline Biology

Anthracyclines comprise a class of chemotherapeutics that are among the most effective antineoplastic agents currently in clinical use. Daunorubicin (originally named daunomycin) was first discovered as a natural compound produced by *Streptomyces peucitus* in 1960.[1,2] Shortly thereafter, doxorubicin (also known as adriamycin) was isolated from a randomly mutagenized strain of *S. peucitus*.[3] Daunorubicin is highly effective in treating leukemias[4], and doxorubicin exerts even broader anti-cancer activity that extends to lymphomas, sarcomas, and a wide range of solid tumors.[5] Although these anthracyclines were discovered decades ago, they remain among the most potent and widely used anticancer drugs and are therefore a cornerstone of cancer treatment today.

Anthracyclines are composed of a tetracyclic anthraquinone aglycone linked to a sugar moiety by a glycoside bond.[6] Daunorubicin and doxorubicin are the archetypal anthracycline drugs, and due to their remarkable effectivity thousands of analogues have been produced in attempt to discover equally or more effective compounds. Numerous anthracycline analogues have been generated using modified bacterial strains, of which aclarubicin (produced in *S. galilaeus*) is the only variant in current clinical use, although its application is limited to China and Japan.[7] Many other anthracyclines have been produced through semi-synthesis, notably epirubicin[8] and idarubicin[9] which are used

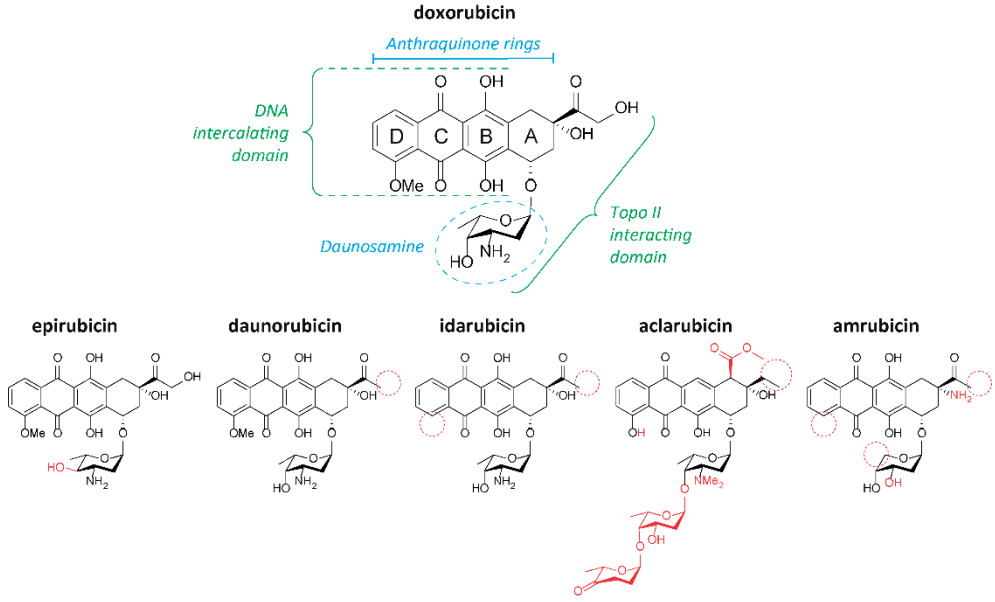

Figure 1 – Chemical structures of doxorubicin and related anthracyclines. The aglycone is numbered in accordance with Brockmann (1963).[6] The daunosamine is circled in blue. Anthracycline domains relevant for binding to DNA and topoisomerase II are indicated in green. Structural differences compared to doxorubicin are indicated in red. This figure is adapted from van der Zanden *et al.*, FEBS Journal (2020).

worldwide. Additionally, the completely synthetic anthracycline amrubicin is used for cancer treatment in Japan[10] (Figure 1).

Molecular mechanisms of action

Of all the anthracycline analogues discovered, synthesized and produced in the past decades, only six variants have made it into clinical practice[11] (Figure 1). One might wonder why we still dedicate research efforts to synthesizing more variants and improving our understanding of anthracycline biology. Interestingly, regardless of the decades of clinical use, new discoveries regarding the molecular mechanisms of these drugs still emerge to date.[12] Not all anthracyclines share the same modes of action, and our understanding of the different effects exerted by these structurally closely related drugs is still incomplete. Although the exact mechanisms through which anthracyclines exert their anti-cancer activity are not fully understood, it is generally accepted that multiple mechanisms and pathways are involved.[13]

Anthracyclines are mostly taken up by cells through passive diffusion, although ATP-dependent transporters can also contribute to drug uptake.[14] Once inside the cell, anthracyclines accumulate in many cellular compartments but most notably exert their cytotoxic effects in the nucleus and mitochondria. Their inherent capability to intercalate into double stranded DNA is at the basis of many modes of action. The anthraquinone aglycon intercalates between DNA base pairs while the sugar moiety is pointed into the DNA minor groove (Figure 1 and Figure 2A). Anthracycline intercalation is not mediated by active processes in the cell but happens because of affinity and occurs in the absence of the cellular context. DNA intercalation inhibits both DNA and RNA synthesis[15,16], thereby altering replication and transcription processes in the cell.

Inhibition of topoisomerase II (Topo II) is the best described molecular mechanism leading to anthracycline-induced cell death.[17] Topo II is an enzyme critical for managing torsional DNA stress and facilitating transcription, replication and repair. It creates transient double-stranded DNA breaks to release tension in the DNA created during replication and subsequently closes the initial break by re-ligating the DNA strands.[18] Most anthracyclines (such as doxorubicin, epirubicin, daunorubicin and idarubicin) intercalate into the DNA and form a stable anthracycline-DNA-Topo II ternary complex (Figure 2B). The enzyme is halted in its catalytic step after inducing the initial DNA breaks, which prevents Topo II from re-ligating the broken DNA strands. This leads to activation of the DNA damage response and p53 pathways. However, when the DNA repair process fails, the accumulation of lesions results in cell cycle arrest and cell death.[19]

Some anthracyclines (like aclarubicin) interrupt the binding of Topo II to DNA, and do not induce DNA double stranded breaks, despite inhibiting the enzymatic activity (Figure 2C).

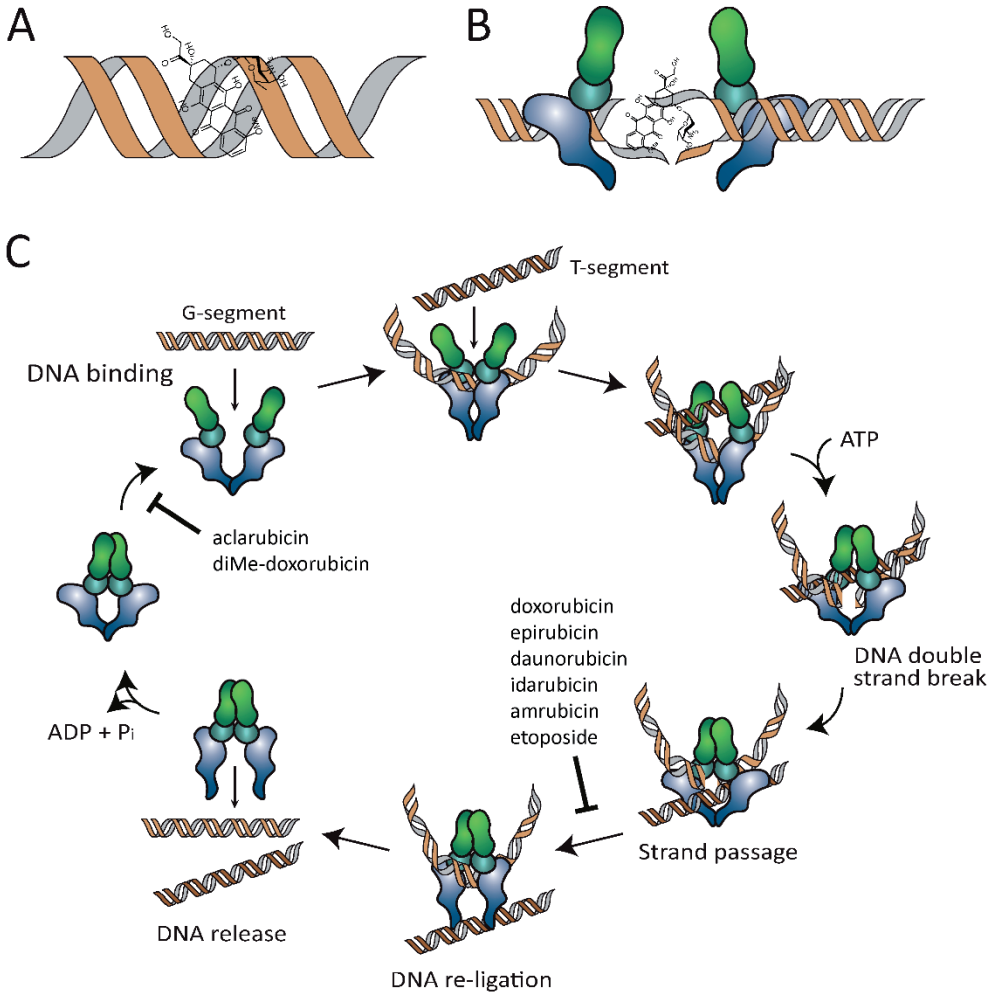

Figure 2- Schematic representation of the Topo II inhibition mechanism of anthracyclines. To release tension and DNA supercoils Topo II binds to the DNA and introduces a transient double strand break in one of the strands (the G-segment), allowing the second strand (the T-segment) to pass through. After religation of the DNA break, Topo II is released from the DNA. Most anthracyclines (doxorubicin, daunorubicin, epirubicin, idarubicin and amrubicin), as well as etoposide (having a chemotype distinct from the anthracyclines), stabilize the Topo II-DNA complex after the induction of double strand breaks and prevent the break from being resealed. The anthracycline variants aclarubicin and dimethyl-doxorubicin inhibit the enzymatic activity of Topo II by preventing its binding to DNA. This figure is adapted from van der Zanden *et al.*, FEBS Journal (2020).

Within the cell's nucleus, DNA is compacted at several levels. Genomic DNA is wrapped tightly around histones and the resulting DNA-protein complex is called chromatin. The repeating structural unit of chromatin is the nucleosome, which contains eight histone proteins.[20] One of the most recently discovered mode of action of anthracyclines involves the disruption of the chromatin structure. When anthracyclines intercalate into DNA the sugar moiety competes for space with histones, causing histones to dissociate from the DNA and nucleosomes to collapse.[21]

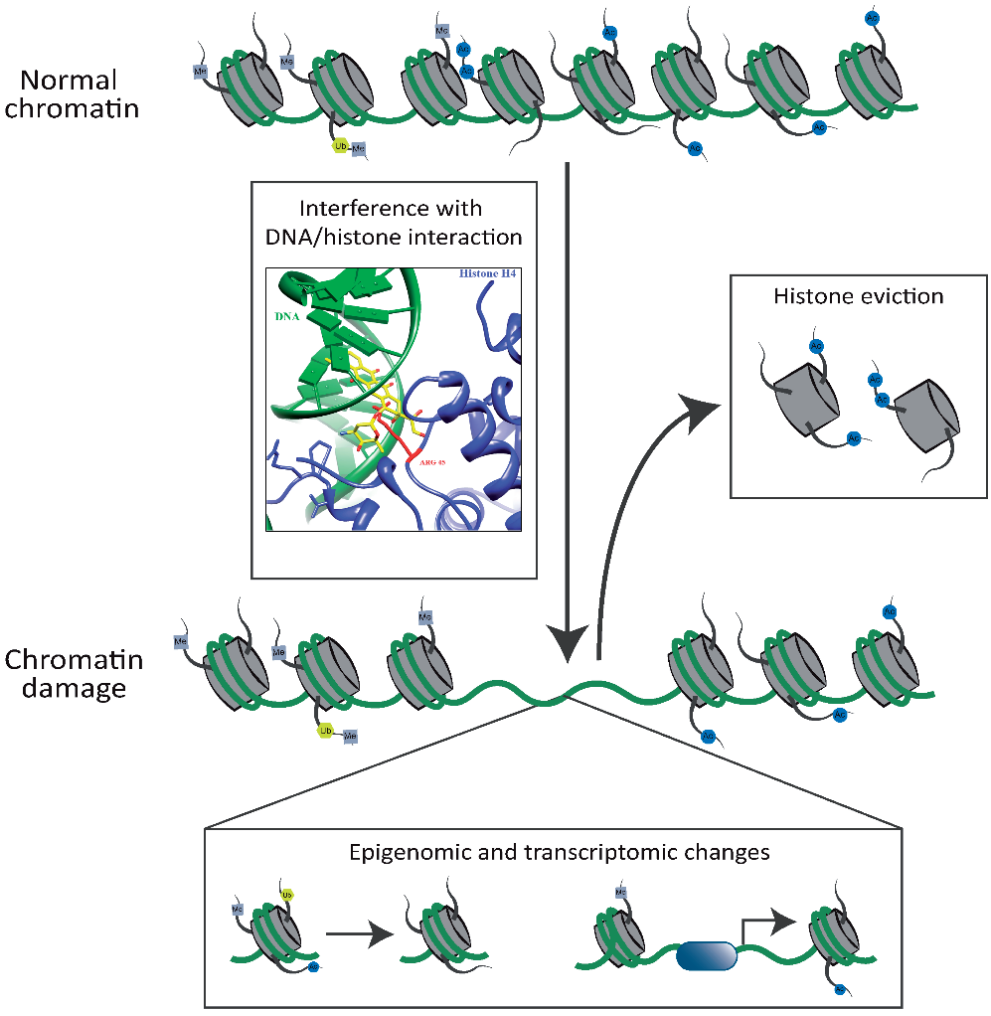

Figure 3 – Schematic representation of chromatin damage induced by doxorubicin. Doxorubicin intercalates into the DNA, where the sugar moiety destabilizes nucleosomes by competing for space with histones. Histone eviction results in epigenetic and transcriptomic alterations and altered DNA damage repair, collectively referred to as chromatin damage. This figure is adapted from van der Zanden *et al.*, FEBS Journal (2020).

This process has been shown to be independent of ATP, suggesting it is a drug-intrinsic action mediated by DNA intercalation of the anthraquinone aglycone and the competition for space between the sugar moiety and histones.[21] Histone eviction is not observed for all anthracycline variants[22–24], but this unique mode of action has only been observed for anthracyclines and one other class of compounds named curaxins.[25] Other DNA intercalating drugs (such as mitoxantrone) or non-intercalating Topo II poisons (for example etoposide) do not induce histone eviction.[26] The disruption of chromatin structures results in delayed DNA damage response and epigenetic and transcriptomic alterations, collectively referred to as chromatin damage (Figure 3). The molecular pathways that lead from histone eviction to programmed cell death are not known, but the capacity to evict histones appears to be a better indicator of the anticancer activity of anthracyclines than their DNA-damaging activity.[22]

The accumulation of anthracyclines in the cells mitochondria directly affects their energy management. Mitochondrial impairment is caused through direct interactions of anthracyclines with respiratory chain complexes and other proteins involved in oxidative phosphorylation[27], as well as through the generation of reactive oxygen species (ROS).[28] Extensive studies have shown that doxorubicin alters the respiratory chain in ways characteristic of chemicals that accept and redirect electrons, known as redox

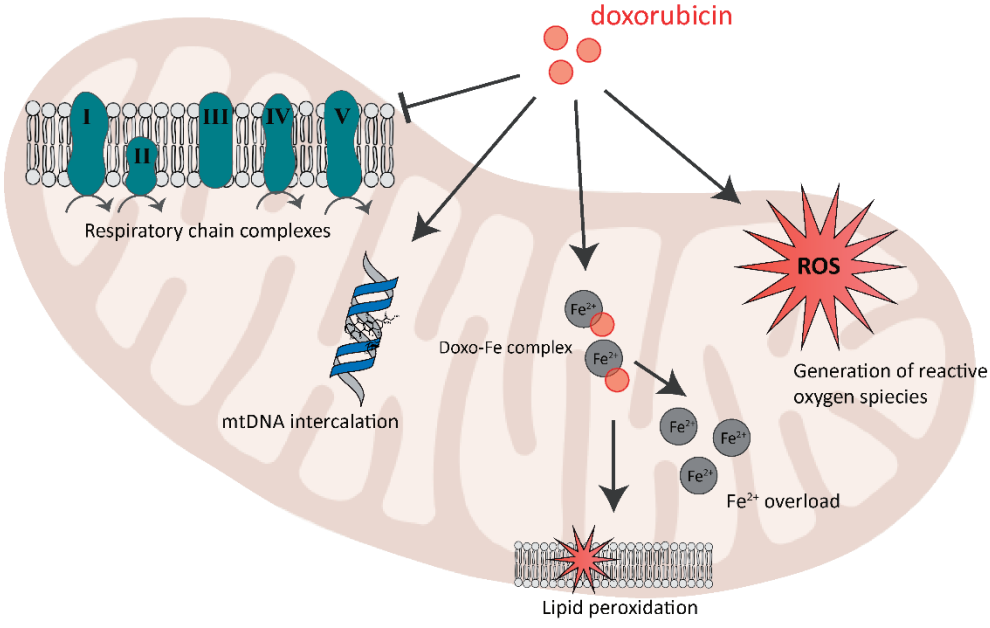

Figure 4 – Schematic representation of the mitochondrial processes that are that are disrupted by doxorubicin. Doxorubicin accumulates in the mitochondria, where it intercalates into mitochondrial DNA, disrupts oxidative phosphorylation and iron homeostasis, and induces an excessive production of reactive oxygen species.

cycling agents. The redox cycling capacity of anthracyclines is a major contributor to ROS generation.[29] Additionally, anthracyclines drive an iron overload in mitochondria which further contributes to excessive ROS production.[30] High levels of ROS can induce lipid peroxidation which results in damage to membranes and cellular structures, ultimately leading to cellular stress and apoptosis.[31] Another mechanism through which anthracyclines can induce mitochondrial damage is again linked to the inherent capacity to intercalate into DNA. Within the mitochondria the intercalation of anthracyclines leads to the formation of 8-hydroxydeoxyguanosine (8OHdG) adducts[32] (Figure 4). Similarly to genomic DNA lesions, these adducts affect the transcription and translation of mitochondrial genes. Lastly, mitochondrial dynamics are altered in response to anthracyclines, which are disrupting the balance between mitochondrial fusion and fission events.[33] This imbalance can lead to mitochondrial fragmentation and eventual cell death.

Side effects and toxicities

Tumor cells typically exhibit altered cellular features which make them distinct from healthy cells.[34] Some of these features contribute to anthracycline selectivity towards tumor cells, such as increased metabolism and rapid cell division. Anthracycline toxicity is however not limited to tumor cells, and the severe side effects that accompany anthracycline treatment can be both life threatening and treatment limiting. The difference in relative toxicity toward tumor cells versus healthy cells creates a therapeutic window, allowing for doses that effectively kill tumor cells while remaining within acceptable toxicity levels for normal cells. Still, anthracycline treatment is associated with various toxicities affecting normal cells. Anthracycline treatment induced-side effects include acute and reversible issues such as nausea, alopecia and leukopenia and long-term side effects such as cardiotoxicity, gonadotoxicity and therapy-related tumorigenesis. Overall, anthracycline treatment has a significant impact on patients' quality of life, and the associated adverse events restrict the clinical use of anthracyclines. The emergence of side effects is difficult to predict for individual patients and depends on multiple variables such as chemotherapeutic dose, the number of treatment cycles and individual risk factors.

Anthracycline-induced cardiotoxicity is one of the most severe side effects, encompassing a wide array of symptoms including contractile dysfunction, ventricular dysfunction, cardiomyopathy, arrythmias and heart failure.[35] Currently, no treatment exists for anthracycline-induced cardiotoxicity, leading to the exclusion of patients at higher risk such as the elderly or those with existing cardiac issues. The onset of anthracycline-induced cardiotoxicity is dose-dependent, irreversible and may occur early or late during

treatment. Although it is one of the best-studied side effects of anthracycline treatment, the underlying molecular mechanisms remain incompletely understood. They are likely to overlap with those underlying toxicity towards tumor cells, such as DNA intercalation, Topo II inhibition, and disruption of the respiratory chain and mitochondrial function. Previous studies have demonstrated that the combination of DNA damage and chromatin damage, as exerted by doxorubicin, may drive its cardiotoxicity.[26] Clinical observations reveal that aclarubicin, which only induces chromatin damage, is less cardiotoxic for cancer patients than doxorubicin treatment.[36,37] Specific characteristics of heart tissue may also increase its sensitivity for anthracycline-induced toxicity compared to other tissues. Due to their high energy demands, cardiac cells contain a large number of mitochondria and have a high respiratory rate. Previous studies suggest that the effects of anthracyclines on cardiac mitochondrial function may underlie cardiac dysfunction. Various strategies have been attempted to manage anthracycline-induced cardiotoxicity, including counteracting excessive levels of ROS[38], novel delivery methods[39,40] and the synthesis of less cardiotoxic anthracycline variants.[26,41]

Another drawback of anthracycline treatment resides in the toxicity towards rapidly dividing normal cells, such as those found in gonadal tissues. Damage to gonadal tissue can lead to a shortened reproductive lifespan and infertility, which are associated with significant psychosocial stress. For patients of reproductive age, fertility preservation can be achieved by cryopreserving gametes or embryos before anthracycline treatment.[42] However, this method is not applicable for prepubescent patients, even though doxorubicin treatment during childhood is known to affect adult fertility.[43] Several compounds have been proposed to be combined with anthracycline treatment to limit gonadotoxicity, including hormone agonists[44], antioxidants[45,46], proteasome inhibitors[47] and DNA damage repair inhibitors.[48] However, no clinical studies have yet validated the effects of these combination treatments on gonadal function and anthracycline efficacy. A preferable approach would be to develop anthracyclines that are less toxic to normal cells while retaining their antitumor effectivity. Previous studies have indicated that gonadotoxicity is largely attributable to the anthracycline-induced generation of DNA double-strand breaks.[48–53] Therefore, the development of anthracyclines with reduced DNA-damaging activity could offer more tolerable treatment options.

Among all long-term side effects, anthracycline-dependent tumorigenesis is considered one of the most detrimental due to the associated morbidity and mortality. Currently, 17-19% of all newly diagnosed primary tumors occur in cancer survivors.[54] Anthracycline treatment can cause transformation and mutagenesis in healthy cells[55], increasing the risk of secondary tumor formation. The susceptibility of cancer survivors to develop therapy-related malignancies depends on various risk factors, including genetic predisposition, carcinogenic exposures (such as tobacco and alcohol use), host effects

(like age, gender, immunodeficiency or obesity), and combination therapies with other mutagenic chemotherapeutics (such as alkylating agents, etoposide, or radiotherapy).[56–59] Consequently, the exact molecular mechanisms through which anthracyclines contribute to the development of therapy-related malignancies remain unclear. However, previous studies suggest that the generation of DNA double-strand breaks by anthracyclines may significantly contribute to the development of these malignancies. A better understanding of the structure-activity relationship of anthracyclines is required to eliminate their DNA-damaging activity and possibly prevent associated toxicities.

Resistance to anthracycline treatment

Resistance to chemotherapeutics poses a critical barrier to treatment and is one of the leading causes of chemotherapy failure. Several mechanisms contribute to anthracyclines resistance, and not all are fully understood. One key mechanism is mediated by ATP-binding cassette (ABC) transporters. These membrane transporters are responsible for the efflux of a wide range of chemotherapeutics across the plasma membrane, leading to decreased intracellular drug levels and treatment resistance.[60] ABCB1 is the best studied ABC transporter in the context of anthracycline resistance, and all anthracycline variants currently in clinical use are known substrates for ABCB1.[61] Numerous studies have observed increased ABCB1 expression in tumor cells in response to anthracycline chemotherapy, and correlations between ABCB1 expression levels, drug resistance, and poor prognosis have been reported for many tumor types.[62] Significant research efforts have focused on blocking ABC transporters with small molecule inhibitors to enhance chemotherapy efficacy.[63] However, none have made it to clinical practice due to off-target toxicities.[64] A more straightforward approach may be the development of anthracyclines which are not transported by ABC-transporters, but despite previous efforts such anthracyclines have not made it into clinical practice either.

The results of research on other cellular mechanisms regulating anthracycline resistance are more ambiguous. Early research suggested that Topo II expression might play an important role in anthracycline resistance, but the correlation between anthracycline sensitivity and Topo II expression is supported by conflicting evidence. Since the most widely used anthracyclines (doxorubicin, daunorubicin, epirubicin and idarubicin) induce DNA damage, it has been proposed that regulators and effectors of the DNA damage response may also be involved in anthracycline resistance. Genome-wide screening for factors driving resistance to doxorubicin revealed not only the expected involvement of ABCB1, but also multiple proteins associated with the DNA damage response.[65] Alterations in the expression and activity of DNA damage response regulators may alter the pathways leading to cell death, allowing tumor cells to continue proliferating. There

may be potential value in combining inhibitors of DNA damage repair kinases with anthracycline treatment.[66] But to date, such combinations have only been tested in pre-clinical settings and the development of anthracycline variants that do not cause DNA damage could potentially bypass the resistance mechanisms related to the DNA damage response altogether.

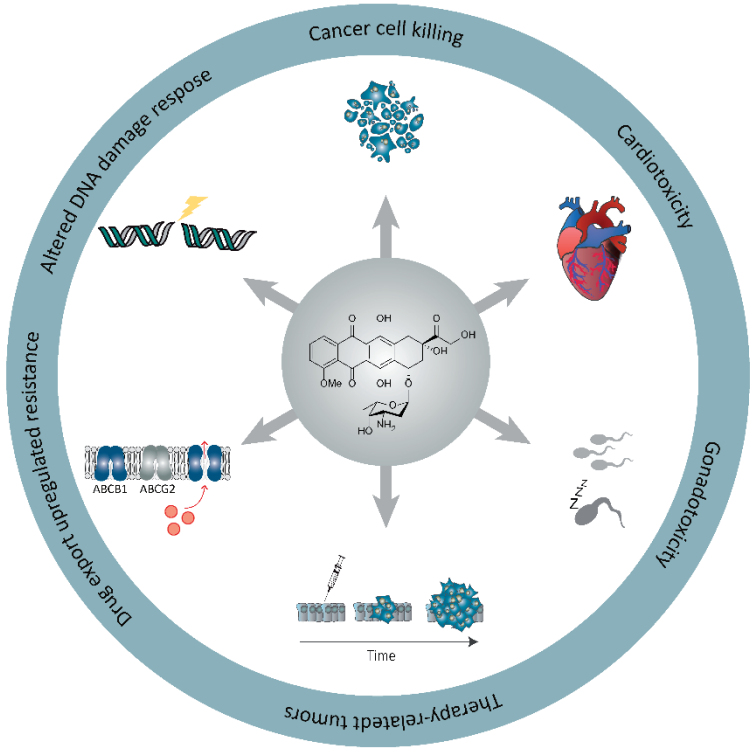

Figure 5 – Schematic overview of the different activities and toxicities of doxorubicin. The different activities and toxicities play a crucial role in identifying future limitations and opportunities of anthracycline development.

From past achievements to new developments

Anthracyclines have been in clinical use for decades, and their effectiveness against many tumor types has outweighed their limitations. However, it remains crucial to address these limitations because of significant impact on the quality of life for cancer patients. The use of anthracyclines is hindered by severe side effects and toxicities that occur during or after treatment. Additionally, the development of resistance poses a major

barrier to successful anthracycline treatment (Figure 5). Various approaches have been explored to improve the efficacy and reduce the toxicity of anthracyclines, but none have established a permanent position in cancer treatment.

In this thesis, we focus on one strategy to improve the efficacy of anthracyclines and overcome resistance. We report the design, synthesis and biological evaluation of structural variations of the archetypical anthracyclines (doxorubicin, daunorubicin, epirubicin, idarubicin and aclarubicin). The aim of this work is to gain a better understanding of the structure-activity relationships of anthracyclines, with the ultimate goals of identifying anthracycline variants that may elicit fewer adverse events and circumvent the molecular mechanisms underlying drug resistance.

In **chapter 2**, we demonstrate that different anthracycline variants have distinct molecular mechanisms. In this extensive structure-activity relationship study we show that small chemical modifications can significantly impact the cytotoxicity and biological activities of these drugs. In addition, we determined the ability of novel anthracycline variants to induce both DNA- and chromatin damage. To gain a better understanding of the molecular pathways leading to chromatin damage-induced cell death, we performed a genome-wide CRISPR screen in **chapter 3**. Here, we identified p53 as an important regulator of cell death in response to aclarubicin. We show that aclarubicin activates a p53-dependent transcriptional program that leads to apoptosis, like traditional DNA-damaging anthracyclines, but in the absence of DNA lesions. In **chapter 4**, we identified several anthracycline variants that are still effective in drug transporter overexpressing, doxorubicin-resistant cells. These variants exhibit improved cytotoxicity and enhanced nuclear localization compared to their clinically used counterparts, while retaining their canonical anthracycline functions of DNA intercalation and Topo II targeting. In **chapter 5**, we further explored anthracycline function by comparing the effects of different anthracycline variants on mitochondrial DNA intercalation, transcription and translation. Finally, we present some directions for future research regarding the development of effective but less toxic anthracycline variants.

In conclusion, the extensive structure-activity relationship studies on novel anthracycline variants presented in this thesis enhance our understanding of the molecular mechanisms involved in anthracycline activity and resistance and may aid in the development of more effective and more tolerable anthracyclines in the future.

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