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Doxorubicin: An Overview of the Anti-Cancer and Chemoresistance Mechanisms

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Abstract

Cancer remains one of the highest leading causes of morbidity and mortality worldwide. Anthracyclines, specifically Doxorubicin (DOX), have been used for the past three decades as a treatment against a number of cancers. However, its use has been limited due to its severe side effects and toxicity arising during or after treatment. Ample research has already taken place and is still being undertaken in order to understand the mode of action of anthracyclines, including DOX. However, despite the work carried out; the mechanisms proposed remain controversial. Other research has also taken place to get a better understanding of the cell death and growth arrest pathways triggered by DOX. Even though DOX remains one of the most effective chemotherapeutic drugs, resistance development in cancer cells remains a major barrier to effective treatment when using this drug. Apart from the already known mechanisms of DOX chemoresistance, research has shown that post-translational modifications on certain proteins can also contribute to DOX chemoresistance. However, the mechanisms by which DOX resistance arises remain poorly defined. This review tackles some of the currently understood and proposed models for the mode of action of DOX, including the cell death mechanisms triggered by DOX and the DOX resistance mechanisms arising during treatment. By further understanding how DOX functions, its influence on cell biological events and the mechanisms contributing to DOX resistance; it can further help in improving the efficiency and efficacy of the drug, together with decreasing its toxicity.

Keywords: Doxorubicin; Mechanisms; Cell Death; Resistance

Introduction

Doxorubicin (DOX) is an anthracycline antibiotic, which was first extracted from *Streptomyces peucetius var. caesius* and has been used as an effective treatment against a number of cancers [1,2]. When used as primary treatment, it has shown positive results in adult and childhood cancers, including both solid tumors and hematological malignancies [1,3,4]. It is used mostly for breast cancers [5,6], multiple myelomas [7], soft tissue sarcomas [7,8], non-Hodgkin lymphomas [9], childhood solid tumors [10], lung cancers [11] and acute leukemia's [12]. Even though DOX has shown great efficacy in killing rapidly dividing cells and delaying the progression for solid and liquid tumors, drug resistance and several side effects end up developing throughout the DOX treatment, making it a major limitation as an effective cancer treatment [11,13,14].

Despite being used as a chemotherapeutic drug for the past three decades, the molecular mechanisms by which DOX functions, resulting in cell death, still remain unclear. Furthermore, the mechanisms by which chemoresistance arise during DOX treatment are still not properly defined. In addition to already known mechanisms of DOX chemoresistance, research has shown that post-translational modifications on certain proteins contribute to DOX chemoresistance. Understanding the actions of DOX and other related drugs classified as anthracyclines, can help in enhancing cancer cell cytotoxicity reducing the side effects/toxicity, and preventing DOX chemoresistance from arising during treatment.

Chemical structure

DOX is a non-selective class I anthracycline drug, consisting of two different moieties. The aglyconic moiety consists of tetracyclic (anthraquinone) rings having a quinine-hydroquinone adjacent group and a methoxy substituted short chain followed by a hydroxy group. The second moiety is a daunosamine and consisting of a 3-amino-2,3,4-trideoxy-L-fucosyl moiety, which is attached *via* a glycosidic bond to one of the tetracyclic rings [14-16]. A number of active sites including three functional groups: Ketone, amine and hydroxyl are present in the structure (Figure 1). Besides hydrophobic interactions possible through the rings, the functional groups allow

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electrostatic interactions such as hydrogen bonds to occur between DOX and other molecules [4,17,18].

Mechanisms related to cell death

The mechanisms by which DOX acts are common to both the cancer cells that the clinical treatment aims to attack, as well as healthy cells that are affected as a side effect of this treatment [14]. DOX has been found to function through several mechanisms. However, the biological process which is affected depends on a number of factors such as the cancer type, patient genotype, treatment length and DOX dosage used [4]. The following section includes the known mechanisms of action for DOX, without going into which mechanism is the most effective, or which gives rise to toxicity.

DNA alterations: DOX enters the cell *via* passive diffusion and once in the cytoplasm it forms a complex with the 20S subunit of the proteasome (Figure 2) [8,19]. This complex can enter the nucleus *via* nuclear pores, were it interacts with RNA and DNA found in the nucleus. Such interactions occur due to the aglyconic and daunosamine moieties present in the DOX structure (Figure 1), which enable the molecule to intercalate itself into the DNA (Figure 2), resulting in the disruption of DNA repair [20]. Even though the details of this molecular mechanism are still unknown, several in silico studies have been performed and models have been proposed [3,11,13,21,22], with researchers making use of new techniques to study such interactions such as investigating the binding free energy or affinity of DOX once intercalated into the DNA using computational theories and models [15,23-27]. However, the following are some of the current hypotheses suggested for how such DNA alterations occur.

As show in Figure 1, the three functional domains present in the DOX structure can help it interact within the DNA. Intercalation takes place *via* the anthraquinone rings present, which enable the molecule to intercalate itself into the DNA [15,24,28-30]. Furthermore, the daunosamine present is responsible for the formation of a covalent aminal bond (N-C-N) *via* the 3'NH_2 group and the N2 of the guanine base in the DNA minor groove [31-33]. A cellular formaldehyde arising *via* free radical reactions from lipids offers the carbon in the aminal bond [13,31]. Hydrogen bonding between the hydroxyl group of the anchor region and the complementary DNA strand helps stabilize the DOX-DNA mono-adduct created [31,32]. It is proposed that eventually the DNA strands separate, giving rise to condensed chromatin, which triggers apoptosis [4]. However, the exact mechanism of how DOX intercalates itself between the DNA strands is still not clear and remains a debatable subject.

One of the most supported hypotheses for DNA alterations by DOX is through its action on topoisomerase II. DOX traps the topoisomerase II at the cleavage site, resulting in the cleavage complex stabilizing itself, preventing the DNA from resealing, and thus blocking DNA replication and resulting in cell death by apoptosis (Figure 2) [34,35]. Type IIA topoisomerases (TOPO IIα and TOPO IIβ) in particular are the ones targeted by DOX [34]. The increased effectiveness of the drug in DOX-sensitive tumors has been reported to be due to DOX-DNA adducts which form because of DOX intercalating itself in the DNA GC base pairs *via* covalent hydrogen bonds [21,36-38]. This intercalation can destabilize the nucleosomes present due to the torsional stress generated as shown from experiments carried out on mouse squamous carcinoma cells before and after DOX treatment [39,40]. Besides torsional stress, topoisomerase II inhibition also leads to the enhancement of nucleosome turnover downstream of promoters. However, there is still no evidence which confirms the intercalation of DOX into promoters and genes, which results in interference with the nucleosomes during transcription [41].

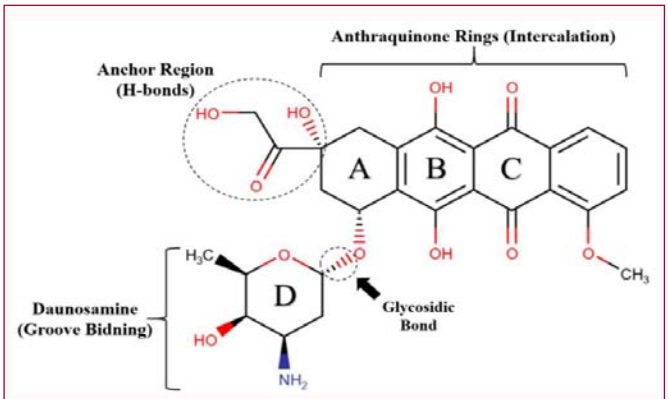

Figure 1: Chemical structure of Doxorubicin in its neutral form. Ring A is bound *via* a glycosidic bond to Ring D known as daunosamine, ring B is the hydroquinone and ring C is the quinone. Retrieved from Micallef, 2020 [18].

Free radicals and reactive oxygen species (ROS) production: Free radicals and ROS can be produced by DOX through several mechanisms (Figure 2). ROS overproduction and reduction of antioxidants gives rise to oxidative stress, which leads to damage of the nuclear material, proteins, and lipids, all of which cause cell damage and death [14,42]. Enzymes required for cell protection can also be damaged and such oxidative damage could be the cause of chromatin and DNA damage [14]. In fact, DOX has been shown to trigger the Liver Kinase B1 (LKB1) enzyme, which is needed for AMP-Activated Protein Kinase (AMPK) activation *via* upstream signaling. Apoptosis due to p53 phosphorylation is initiated by such signaling [14,43]. The following are some of the hypothesized ways by which free radicals and ROS are generated due to DOX.

The quinone in the DOX structure (Figure 1) is oxidized by the mitochondrial complex NAD(P)H-oxidoreductases (Complex I) and NADPH-Oxidases (NOXs). This results in the formation of a semiquinone radical due to the addition of an electron by these two complexes [3,44]. An oxygen molecule reacts with the semiquinone radical generated, forming a Superoxide Anion ($O_2^{\cdot-}$) which helps in the production of other ROS such as Hydrogen Peroxide ($H_2O_2^{\cdot-}$), peroxynitrite and hydroxyl radicals. Research shows that the semiquinone is converted back to its original quinone form by glutathione [4], which enables the DOX molecule to generate larger amounts of superoxide anions in a quinone-semiquinone cycle (Figure 2) [45,46]. NOXs can be activated by DOX too, which initiates the apoptotic pathways in cardiac cells due to the free radicals formed such as peroxynitrite (formed from the reaction of nitric oxide with a superoxide anion) [47,48].

Iron metabolism is also affected due to DOX treatment, as research has shown that DOX interacts with the Iron Regulatory Proteins (IRPs) and ferritin, affecting iron homeostasis which can lead to ROS-dependent or independent damage, as well as cell death by apoptosis [49]. A number of pathways have been proposed with regards to the DOX interaction with the IRPs. Research carried out on cell-free systems showed that after interaction with Doxorubicinol (DOXol - reduced DOX), IRP1 first loses the [4Fe–4S] cluster and then ROS change it to a 'null' protein, missing both RNA-binding and enzymatic activities [50,51]. However, other research showed that the 'null' protein is formed due to a DOX-iron complex [52].

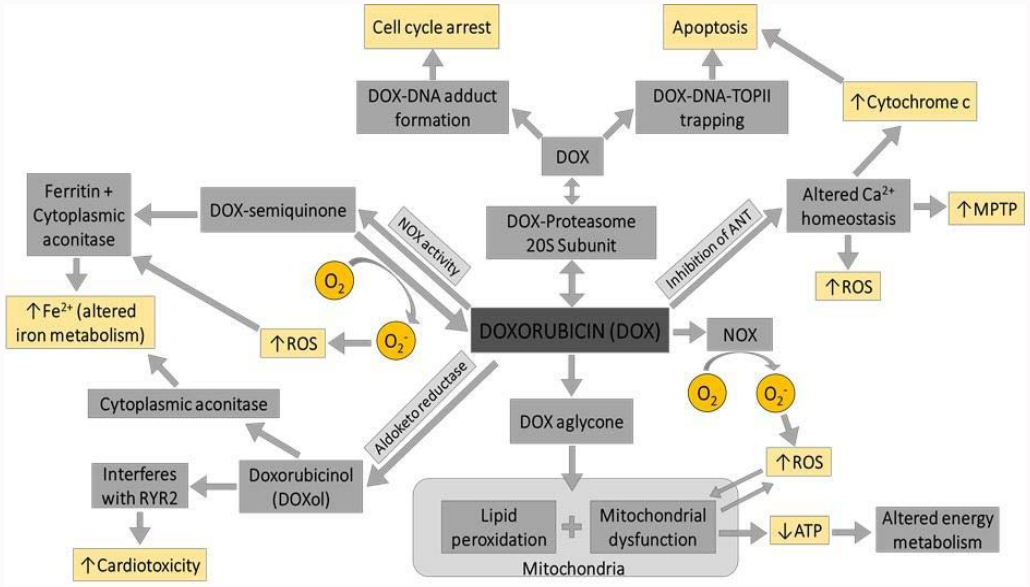

Figure 2: Molecular mechanisms initiated by DOX.
Abbreviations: ATP: Adenosine Triphosphate; Ca2+: Calcium; Fe^{2+}: Iron; NOX: NAD(P)H Oxidase; O2: Oxygen; O2-: Superoxide Anion; MPTP: Mitochondrial Permeability Transition Pore; ROS: Reactive Oxygen Species; RYR2: Cardiac Calcium Release Channel. Adapted from Varela-Lopez et al. [14].

Iron atoms can also be released from the Fe–S cluster of IRP1, which could facilitate iron-mediated free radical formation and apoptosis [49]. Iron metabolism is altered because of an increase in Iron (Fe^{2+}) concentration, released from ferritin and cytoplasmic aconitase due to being promoted by DOX semiquinone and the ROS (H_2O_2 and O_2^-) and due to the release of iron from cytoplasmic aconitase promoted by DOXol (Figure 2) [14].

The Topoisomerase II (TOP II) enzyme involved in DNA replication can also contribute to the production of ROS. In fact, the Topoisomerase 2β (TOPO IIβ) subunit can give rise to the formation of ROS upon DOX administration [53].

Lastly, calcium homeostasis is also hindered by DOX, which can result in a higher permeability of the Mitochondrial Permeability Transition Pore (MPTP) and thus its opening (Figure 2). DOX interferes with calcium homeostasis by inhibiting the calcium-conducting enzyme Adenosine Nucleotide Translocase (ANT). This damages the mitochondria's ability to obtain calcium from the cytoplasm and also increasing susceptibility to mitochondrial calcium-induced depolarization and increased MPTP permeability [54]. Furthermore, DOX can alter calcium conducting ANT by inhibiting calcium homeostasis [4]. Alteration in intracellular calcium levels by DOX treatment also results in the alteration of muscle function, such that due to changes in the response of myotubules, contraction is restricted, and skeletal muscle relaxation is disrupted [55]. DOXol interferes with the cardiac calcium release channel (RYR2) found in the Sarcoplasmic Reticulum (SR), which results in cardiotoxicity (Figure 2) [56]. In addition, Ca^{2+} increases due to an increase in the opening of the Ryanodine Receptor (RYR) in the SR because of DOX-mediated ROS. The higher Ca^{2+} concentration results in further generation of ROS [57]. Lastly, if the intracellular Ca^{2+} level rises, ROS generation is promoted, together with mitochondrial permeability (also regulated due to a transition in the opening of MPTPs), which releases cytochrome c needed for apoptosis (Figure 2) [58-60].

Cell membrane alterations: DOX can decrease the fluidity and lipid organization of reconstituted biological membranes [61]. It does so due to its high affinity for the negatively charged membranes, which enables it to bind to the inner mitochondrial membrane giving rise to lipid peroxidation [62,63]. DOX is converted to lipophilic aglycone (DOX aglycone), which is capable of diffusing through the outer mitochondrial membrane where it then accumulates. Furthermore, the aglycone activates a number of reactions, which release electrons, generating a number of ROS that interfere with the respiratory chain (Figure 2) [46]. Mitochondrial dysfunction due to DOX treatment results in ATP deficiency, especially in cardiomyocytes [61,64]. In addition, cardiac injury also results due to an increase in proteotoxic load, which results due to protein degradation overwhelming the endoplasmic reticulum and mitochondria [14,65]. Lastly, the MPTPs generated due to DOX-induced peroxidation of mitochondria give rise to intrinsic apoptosis and necrotic cell death [66].

Ceramide overproduction: Ceramides are a class of lipids synthesized in the endoplasmic reticulum *via* the condensation of L-serine and palmitoyl CoA by serine palmitoyltransferase producing 3-ketosphinganine, which is then reduced to dihydrosphingosine [67-69]. The dihydrosphingosine undergoes N-acylation followed by desaturation to generate ceramide [69-71]. This class of lipids is involved in apoptosis, growth arrest and senescence of cells and has been shown to increase the cellular uptake of DOX *via* its short chain sphingolipid [72-73]. The level of ceramide has also been shown to increase in patients treated with DOX [74,75] and has been related to DOX resistance [76]. Unfortunately, it is still a poorly explored area and requires further research in order to determine the mechanism by which DOX modulates the formation of ceramide.

Cell death mechanisms due to DOX

Any DOX-induced damage or DNA fragmentation that results by any of the previously mentioned mechanisms or any other mechanism which is still to be understood, may induce a number of cellular events, which lead to growth arrest or cell death [4,77]. Research shows that apoptosis, autophagy, senescence, and necrosis are the cellular events triggered during DOX treatment [78]. However, recently other forms of regulated cell death have been shown to also be initiated by DOX,

in particular necroptosis, pyroptosis and ferroptosis, but how DOX contributes to these pathways is not yet understood [79].

Apoptosis: This pathway is initiated when the cell attempts to repair the damage cause by DOX. However, this is inhibited due to DOX, thus cellular growth is hindered by arrest at the G_1 and G_2 phases. Most of the time, apoptosis is initiated *via* caspase-dependent pathways that can be extrinsic or intrinsic, but apoptosis can also be achieved through a caspase- independent pathway [4].

DOX initiates the extrinsic pathway by regulating the Fas receptor protein which is a type I transmembrane glycoprotein. This receptor present on the surface of several cells can trigger signal transduction pathways which lead to apoptosis [80]. It does so by interacting with the Fas Ligand (FasL), which is a type II transmembrane protein [81]. Both proteins are part of the Tumour Necrosis Factor (TNF) receptor family [81]. The extrinsic apoptotic pathway is triggered by DOX due to this drug down regulating the Fas proteins, which is an inhibitor of FasL [82,83]. However, the FasL protein can also be up regulated due to DOX activating the calcium/calcineurin signaling pathway, which activates the Nuclear Factor-Activated T cell 4 (NFAT-4) [82,84]. DOX can also activate NF-κB (Nuclear Factor Kappa B) *via* ROS which increases the activity of a number of pro-apoptotic genes such as p53 and FasL [4,82,85,86].

DOX initiates the intrinsic pathway by inducing AMPK [43,63], which initiates p53 and c-Jun N-terminal Kinase (JNK) and inactivates the mammalian target for Rapamycin Complex 1 (mTORC1) [4]. The B-cell lymphoma 2/Bcl-2-associated X (Bcl-2/Bax) ratio is altered due to DOX activating AMPK, as this ratio determines whether the cell survives or dies *via* apoptosis [43]. This alteration results in the release of the cytochrome c complex from the mitochondria, which forms a complex with apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9. This leads to the activation of a number of other caspases (-3, -6 and -7) which gives rise to cell death by apoptosis [63,82,87].

Although the extrinsic and intrinsic pathways function differently, the two pathways interlink together, since p53 protein can increase the stimulation of pro-apoptotic receptors present in the extrinsic pathway while caspase 8 can increase the intrinsic function of BAX found in the intrinsic pathway [4,87].

Autophagy: With this pathway the cell protects itself *via* a degradation process, through which it recycles its cellular materials, macromolecules and organelles using lysosomal hydrolytic enzymes [88]. DOX brings about autophagy due to the oxidative stress generated by the ROS which forms in complex 1 (NADH ubiquinone oxidoreductase) of the electron transport chain present in the mitochondria. Autophagy occurs due to AMPK and calmodulin dependent kinase, which are triggered by the ROS damage affecting the calcium-handling proteins and also brings about an increase in the concentration of calcium. Due to the mitochondrial function and energy production disruption, AMPK is up regulated, which leads to the inhibition of mTOR and the up regulation of JNK [89]. JNK up regulation and mTOR inhibition leads to the activation of Unc-51-like kinase 1 (Ulk-1) and dephosphorylation of Autophagy related protein 13 (Atg13) and Family Interacting Protein of 200 kD (FIP200), which are needed for the formation of a pre-autophagosome membrane. In addition, Bcl-2 is dissociated from beclin-1 due to the up regulated JNK, thus a complex made up of Vacuolar protein sorting 34 (Vps34), beclin-1 and Vacuolar protein sorting 15 (Vps15) is formed which is needed for the maturation of the autophagosome [4]. This shows that JNK regulates the maturation of the autophagosome for autophagy to occur [90].

DOX can cause autophagy by triggering poly (ADP-ribose) Polymerase-1 (PARP-1), which then inhibits mTOR when the cell is under stress or lacks the required nutrients in order to survive [63,91,92]. It has been shown that dysregulation in the autophagy pathway leads to DOX-induced cardiac injury and cardiotoxicity [14,93-95]. However, *in vitro* experiments on tumour-bearing mouse models showed that if co-treated with rapamycin, the cardio toxic effects of DOX, autophagy initiation and autophagosome formation were attenuated [96]. Lastly, it has been hypothesized that low levels of autophagy encourage cell survival by preventing apoptosis from occurring, while if autophagy is up regulated, programmed cell death is promoted, as excessive degradation of proteins and organelles disrupts energy homeostasis [97,98].

Necrosis: Necrosis is commonly stimulated when ATP levels are depleted, which make it less likely for the cell to survive. The cytotoxic actions caused by DOX, damage to DNA and oxidative stress, can stimulate this cell death pathway. This is because the ROS generated due to DOX give rise to higher mitochondrial calcium concentrations, resulting in decreased ATP due to the cyclophilin D-dependent MPTP opening and mitochondrial swelling [99]. Most tumors have mutations that hinder apoptosis from taking place, thus allowing cells to continue growing past normal growth cycle checkpoints. Necrosis could explain how chemotherapeutic drugs such as DOX still induce cell death when other pathways are blocked [100]. Thus, if apoptosis cannot be triggered, programmed necrosis gives DNA-damaged proliferating cells another means of death, which is triggered by PARP-1 and H2A histone family member X (H2AX) [99]. Programmed necrosis is also triggered *via* the TNF and TNF-Related Apoptosis-Inducing Ligand (TRAIL) death receptor proteins, which stimulate the Receptor-Interacting Protein (RIP) by inhibiting caspase 8 [99,101]. However, the way in which DOX contributes to this pathway is yet to be understood.

Senescence: DOX can also trigger the cells to stop dividing and proliferating but remaining active, giving rise to the phenomenon of senescence. The pathways involved in inhibiting cell growth are similar to those involved in triggering other cell death pathways. The p53 protein is induced by DOX, which results in the upregulation of the cyclin-dependent kinase (cdk) p21 protein and downregulation of cdc2/cdk1 [78]. Despite DOX upregulating p53, which can result in senescence taking place, this phenomenon may not always contribute to DOX activity [102,103]. Under DOX treatment, senescence showed to be an alternative pathway used by the cells when apoptosis is inhibited [104,105]. However, others showed that autophagy is triggered when the cells cannot undergo apoptosis, with senescence being a secondary downstream response when both are inhibited [78].

DOX chemoresistance

Despite DOX being a suitable therapeutic agent for patients suffering from different cancers, the patient can become resistant to the DOX being administered. Most of the studies carried out on DOX resistance focus on signaling pathways and the ATP-Binding Cassette (ABC) drug efflux transporters [106-108]. However, different non-metabolic pathways and recently, post-translational modifications, have also been shown to be involved in DOX resistance.

Signaling pathways: Signaling pathways stimulate DOX resistance by inducing cell cycle progression, activating replication, and preventing apoptosis and autophagic cell death from taking place [109,110]. Apoptosis and autophagic cell death are not triggered due to down regulation of certain molecules (caspase-3/7/8/9, Bcl-2, p62, microtubule associated protein Light Chain 3 (LC3)-I/–II) responsible for initiating said pathways. Christowitz et al. [110] showed that DOX failed to induce cell death via apoptosis or autophagy, which hinted to drug resistance when treating breast cancer cells with different DOX doses. The signaling pathways involving phosphoinositide 3 kinase (PI3K) and Mitogen-Activated Protein Kinase (MAPK)/ Extracellular-signal-Regulated Kinase (ERK) contribute to DOX resistance [109,110]. Both pathways tend to work in conjugation, but which pathway has a greater effect on DOX chemoresistance is inconclusive and controversial [111]. Jin et al. [112] showed that PI3K/Akt pathway had a greater effect on chemoresistance than the MAPK pathway in breast cancer, while Christowitz et al. [110] showed that the MAPK pathway has a greater effect.

The MAPK/ERK pathway promotes DOX resistance due to its importance in safeguarding cancer cells from oxidative stress, with elevated phosphorylated ERK levels in DOX resistant breast and hematopoietic cells [110,113,114]. ROS production following DOX treatment can trigger Platelet-Derived Growth Factor Receptor α (PDGFRα), which increases the initiation of the MAPK/ERK pathway together with other MAPK pathways, including the p38 and JNK pathways [110,113,115]. The ERK pathway regulates the initiation of apoptosis due to stress created by the cells, such as the DNA-damage arising due to DOX [115].

The PI3K pathway can encourage both tumorigenesis and DOX chemoresistance by upregulating Akt phosphorylation [109,110,116]. Three AKT isoforms (AKT-1,2,3) exist, with all three phosphorylated and un-phosphorylated forms contributing to DOX-resistance in endometrial cancer [117], while AKT1 contributes to DOX resistance in breast cancer [114,118-120].

Multidrug resistance transporters: Over expression and up regulation of ATP membrane transporters are one of the most understood mechanisms of DOX resistance (107,121,122). These transporters are a super family of membrane proteins which make use of ATP hydrolysis to transport exogenous and endogenous substances across membranes against a concentration gradient [123]. Several ABC transmembrane pumps have been discovered, but the ones which contribute to DOX resistance include: multidrug resistance 1 or P-glycoprotein (MDR1 or P-gp), multidrug resistance 3 or 5 (MDR3 or MDR5), multidrug resistance-associated protein 1 or 2 or 3 or 5 or 6 (MRP1/2/3/5/6) and Breast Cancer Resistance Protein (BCRP) [107,123,124]. MDR1 and MRP1 are the transporters mostly implicated in drug resistance [122,125].

The MDR1 (P-gp or ABCB1) pump present at the apical membrane of a number of different cells has a broad range of substrate specificity, with DOX being one of them [124]. It has been proven to cause DOX resistance when unregulated in chronic lymphocytic leukemia, epidermoid carcinoma cells, bladder, endometrial, ovarian, breast, and colorectal cancer [122,124,126-131]. This is due to the P-gp recognizing and removing DOX from the lipid bi layer of the cell membrane using energy obtained from ATP hydrolysis [132]. Furthermore, different leukemia, ovarian, breast and colon cancer cell lines have been shown to become DOX resistance due to up regulation of the MDR3 (or ABCB4) transporter [128,133-135]. The efflux transporter, MDR5 (or ABCB5), specifically expressed in melanoma cells has been shown to confer DOX resistance due to being significantly up regulated under DOX treatment [136,137]. In addition, another study reported DOX resistance due to this transporter when using liver cancer as a cell model [138].

The BCRP (ABCG2 or MXR or ABCP) transport pump, which commonly functions as a defense mechanism against toxins and xenobiotics can control the excretion and absorption of potentially toxic substances, including chemotherapeutic drugs [139]. When up regulated, it gives rise to DOX resistance in breast, osteosarcoma, and prostate cancer [128,130,139-142].

The MRP1 (gene symbol ABCC1) drug transporter was the first cloned ABCC protein to contribute to DOX resistance due to being overexpressed when treating human lung carcinoma cells [123,143]. In addition, other reports have shown that this drug transporter also causes DOX resistance in breast, bladder, colorectal and prostate cancer and acute lymphoblastic leukemia [122,126,144-147]. MRP2 (ABCC2 or cMOAT) has also been shown to contribute to DOX resistance by mediating its efflux when under treatment, such as in oesophageal squamous cell carcinoma, bladder and breast cancer [126,130,148,149]. Upregulation of the MRP3 (ABCC3 or MOAT-D) in lung and bladder cancer gives rise to DOX resistance [126,128,150,151]. Furthermore, Sodani et al. [152] stated that DOX is not a substrate for the MRP5 (ABCC5 or MOAT-C) transporter protein. However, different reports have shown that MRP5 does indeed contribute to DOX resistance in different cancer cells including lung cancer and also in MRP5-transfected embryonic kidney cells [153,154]. Lastly, DOX resistance can also develop when MRP6 (ABCC6 or MOAT-E) is up regulated in ovarian and breast cancer [130,151,152,155].

In the past several years, the scientific community has increased its understanding of the role of most drug transporters in chemoresistance. However, the mechanism by which these transporters act to transport drugs and their inhibition is still poorly understood at a molecular level.

Epithelial-mesenchymal transition (EMT): Throughout the process of EMT, cell-to-cell adhesion in epithelial cells decreases, causing the cell cytoskeleton to alter itself making cells more motile, showing changes towards a mesenchymal phenotype [156,157]. The changes that cells undergo through this process, mainly the reduction in expression of E-cadherin and up regulation of Vimentin and N-cadherin, together with other markers, can contribute to DOX chemoresistance [157]. The EMT process can be induced by anti-cancer drugs and it can give rise to the formation of chemo resistant metastases [158]. DOX resistance due to EMT changes has been shown to arise in a number of cancers, particularly colorectal, gastric, breast and liver cancer [121,158-162].

DNA repair: As previously discussed, DOX forms covalent DNA adducts and alters the function of TOP II. Both these modes of action can eventually give rise to resistance. Thus, DNA damage repair becomes an important contributor to drug resistance. However, if cells lack the proteins involved in the DNA repair pathways, the cells cannot repair the damage present. In fact, Spencer et al. [163] showed that Nucleotide Excision Repair (NER) and Homologous Recombination (HR) play an important role in the repair of anthracycline–DNA adducts. This study showed that cancer cells in which these two DNA damage repair systems work efficiently can overcome the cellular damage induced by DOX.

Specific proteins: Together with AKT, XIAP (X-linked inhibitor of apoptosis protein) is another anti-apoptotic protein which can give rise to DOX chemoresistance, in certain cancers [164]. Inhibitors of Apoptosis Proteins (IAPs) block apoptosis either by binding and inhibiting specific caspases, or through caspase-independent mechanisms [165]. XIAP engages in the PI3K/AKT pathway to safeguard the cells by acting as an AKT promoter through its interaction with Phosphatase and Tensin Homolog (PTEN), as an E3 ubiquitin ligase. Thus, it negatively regulates the PTEN protein and its cytosolic/nuclear localization, preventing the completion of apoptosis in resistant cells [116,166]. In fact, XIAP was up regulated in epidermoid carcinoma cells, breast, and endometrium cancer, with results showing that XIAP contributed to DOX resistance [116,129,164].

The Estrogen Receptor (ER) together with ERα/β, responsible for promoting cell proliferation and tumorigenesis [167] can also give rise to DOX chemoresistance in certain cancers [168]. High concentrations of ERα in breast cancer have been shown to contribute not only to DOX resistance but also to other chemotherapy treatments [168,169].

DOX-mediated DNA damage can also trigger cell cycle arrest [170] due to the p53 tumour suppressor being activated. However, p53 can also regulate the transcription of the p16 and p21 genes, which are needed for DNA repair, cell cycle control and apoptosis [170,171]. DOX can fail to induce cell cycle arrest, causing DOX resistance, either due to the p53 or p21 protein being mutated or due to failure of upstream pathways that stabilize and post-translationally initiate wild-type p53 [110,172,173].

Mutations or abnormal expression of the TOPO IIα subunit, cytoplasmic rather than nuclear localization of TOPO IIα and suppression of TOPO IIα-mediated apoptotic signaling can all contribute to DOX resistance [174]. How DOX resistance is linked to increase TOPO IIα in relation to tumour growth is still not understood. One hypothesis is that for the cells to survive high expression of this enzyme, they down regulate the apoptotic program commonly triggered by DNA strand breaks [175]. Another proposed hypothesis is that the high expression levels are linked to the development of mutations in this enzyme, which result in decreased DOX sensitivity [176]. An additional mechanism of DOX resistance could be through the reduction in TOPO IIα expression and increase in the β-isoform of TOPO II that is less sensitive to DOX [35,175]. Certain DOX resistant hepatocellular carcinoma cell lines have shown an increased TOPO IIa expression as opposed to the proposed down regulated expression [177]. However, breast cancers with depleted TOPO IIα have an increased resistance to DOX [178].

Post-translational modifications: By examining post-translational modifications of proteins, one can determine whether a patient is benefiting from the chemotherapy regimen being administered. In addition, it is possible to study if certain modifications are dominant upon resistance. Certain post-translational modifications have been shown to potentially contribute to DOX chemoresistance.

The p21 protein has been shown to be methylated by Protein Arginine N-Methyltransferase 6 (PRMT6) at arginine 156 under both *in vitro* and *in vivo* conditions, which helps increase the cytoplasmic localization of p21. When treating Hela and 293T cell lines, DOX chemo sensitivity was reported to have decreased due to PRMT6-mediated methylation, which increased the cytoplasmic localization of p21 through enhanced phosphorylation [179]. This result showed that the methylation-mediated p21 translocation appears to affect the regulation of cell cycle progression and apoptosis in response to DNA damage. Thus, p21 translocation promoted by PRMT6-mediated methylation appears to reduce DOX chemo sensitivity [179].

In certain tumour types, the protein chaperone Hsp60 is pro-carcinogenic by interfering with apoptosis and tumour cell death [180]. When treating NCI-H292 lung cancer cell lines with DOX, Gammazza et al. [181] reported a significant increase in HSP60 lysine acetylation. This post-translational modification hinders the formation of the HSP60/p53 complex and promotes its dissociation, which resulted in an increased level of free p53. They proposed that this free p53 activated the p53-dependent pathway, inducing the cell senescence detected by the Senescence-Associated beta-galactosidase (SA-β-gal) activity assay. In this state, the cells cannot divide and become unresponsive to growth signaling and resistant to apoptosis.

Liu et al. [182] showed that post-translational modifications on histones H3 and H4 can contribute to DOX resistance in the acute and chronic leukemia cell lines HL60 and K562 respectively. An increased level of H3K9 methylation, H3K14, H3K18 and H3K23 acetylation, and potentially H4K20 methylation, are associated with drug resistance in both cell lines. They proposed that despite H4K20 and H3K9 losing their methylation marks during tumorigenesis; they may re-gain some of this methylation pattern, together with the deactivation of certain genes to give rise to DOX-resistance. Despite Liu et al. [182] observed increased levels for the respective epigenetic modifications; Vasyl et al. [183] reported a loss of histone H4K20 methylation and a loss of histone H3K9 acetylation when studying DOX resistance using the MCF-7 breast cancer cell line. In addition, Vasyl et al. [183] also detected an increase in phosphorylation of histone H3S10, in DOX resistant MCF-7 when compared to the parent cell line. While both studies provide insights on epigenetic changes taking place as the cells gain DOX resistance, the mechanisms and genes underlying such histone modifications remains unknown.

Conclusion

DOX can effectively treat a number of cancers through its different modes of actions. However, it has been limited in its use due to the severe toxicity arising during and after treatment. Toxicity arises due to the different modes of actions DOX utilizes in order to inhibit the growth of the tumour. In addition to this, different cellular events can be triggered by DOX, all of which are responsible for cell cycle arrest or cell death. Furthermore, just like any other cytotoxic agent, different mechanisms triggered by the cells throughout treatment can give rise to chemoresistance. Numerous studies have been carried out on all these factors, but despite the current knowledge, further research is still required. This will help in better understanding this chemotherapeutic drug, together with potentially uncovering mechanisms that are still unclear and unknown. Apart from the areas tackled in this review, various other work not discussed here has also been carried out, particularly the genes involved in controlling the response of DOX [11,130,184], DOX cardiotoxicity [185,186,187] and other mechanisms of DOX resistances such as due to the proteins Jagged-2 (JAG2) [188], NF-κB [175] and FOXO3 [175,189,190], due to microRNA's [175] and due to certain metabolic pathways [132]. Most of the research currently taking place focuses on further understanding all these areas. In addition, importance is also being given to decrease DOX toxicity, developing inhibitors which prevent DOX resistance from arising and also developing efficient ways to

improve DOX efficacy throughout treatment.

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