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Drugs Against Cancer: Stories of Discovery and the Quest for a Cure

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Chapter 8: The Story of Doxorubicin: a star with a nearly fatal flaw.

Introduction

Doxorubicin (also known as adriamycin) is a DNA intercalator (Chapter 4) and a topoisomerase II blocker (Chapter 9). It became one of the most useful anticancer drugs; it was found effective, although not curative, for many cancers. Its usefulness however was bedeviled by toxic effects on the heart, brain, liver, and kidney. Of those toxicities, the most serious was damage to the heart; patients often died of congestive heart failure if the cumulative amount of the drug administered was above a certain limit, (Von Hoff et al., 1979) (Figure 7.1).

The drug sometimes caused the cancer to disappear, but the remissions lasted only a few months before the tumor reappeared and was resistant to the drug (Benjamin et al., 1974). Some breast cancer patients who were successfully treated by surgery followed by a period of doxorubicin as "adjuvant treatment" still had some degree of heart damage even 10 years later (Murtagh et al., 2016). Thus, the heart damage was irreversible, and it could be so severe that the only remedy was transplantation of a new heart. The potentially lethal damage to the heart prevented administration of higher doses that might have cured the cancer.

Enormous effort was made therefore to find out exactly how the drug damaged the heart. Although the mechanism of the heart damage was clarified, no preventative was found other than to limit the amount of drug administered. Moreover, it was not fully determined exactly how doxorubicin suppressed cancers, although the general opinion was that its action on topoisomerase II was the main therapeutic mechanism.

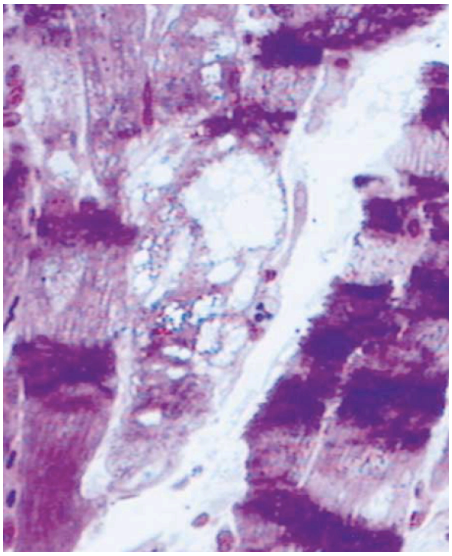

Figure 7.1. Heart tissue damaged by doxorubicin after treatment for breast cancer in a 63-year old woman (40X). The normal fibrils in a heart muscle cell (lower left corner) are disrupted in many of the muscle cells. A disintegrating heart muscle cell with many blebs is seen in the center. It is mostly white, since it lacks essential proteins that would stain dark. This was from a small heart biopsy from an ailing patient (Singal and Iliskovic, 1998). (*Permission needed.)*

An anticancer drug is discovered by tracking down a red antibiotic.

The doxorubicin story began with a search for antibiotics made by microorganisms in the soil. Researchers found organisms that were associated with a red color. As chemists often like to do, the researchers wanted to isolate the substance that had the color. In 1963, the researchers, led by Aurelio Di Marco at Farmitalia Research Institute in Milan, Italy isolated a red antibiotic from *Streptomyces peucetius,* which they called "daunomycin"; later they renamed it "daunorubicin" in view of its red color.

Di Marco's group was struck by finding that their new antibiotic not only killed bacteria but inhibited cancer growth in mice (Di Marco et al., 1964; Di Marco et al., 1965). Lengthy investigations eventually disclosed that daunorubicin inhibited DNA synthesis and bound to DNA by intercalation between the DNA base pairs (Pigram et al., 1972) (see Chapter 4).

As an aside, coming to mind in the context of intensely colored anticancer drugs is a rather amusing, but instructive, talk at a Cancer Chemotherapy Gordon Conference many years ago given by Daniel von Hoff, in which he showed a series of slides that

suggested that color was as good way as any to predict whether a compound might have anticancer activity!

Indeed, many anticancer drugs are intensely colored, because their chemical structures often have multiple rings with alternating double bonds (aromatic structure). This is particularly true for DNA intercalating agents, because to fit between the base pairs, the molecule (or at least a part of it) has to be planar (*i.e.* flat), which requires a multi-ring structure with alternating double bonds ("aromatic" rings). Moreover, the charge distribution over the ring system, which depends on the nitrogen atoms that most intercalators have in their ring system, helps the molecule to stack firmly against particular base pairs.

Then, in 1967, Frederico Arcamone and his coworkers at Farmitalia isolated another red antibiotic from a mutant strain of the organism that produced daunorubicin (Arcamone et al., 1969). The new antibiotic was very similar to daunorubicin in chemical structure, as well as in chemical and biological properties, so they named it "doxorubicin"; they found that the only structure difference from daunorubicin was that doxorubicin had a hydroxyl group added at position 14 (Figure 7.2). It is notable that useful variations of a drug can sometimes be obtained from mutant variants of a particular organism. Doxorubin was superior to daunorubicin in its pattern of antitumor activity relative to toxicity (Bonadonna et al., 1970). Great effort was then made to find the cause of the toxicity and how to combat it. Although the former objective was achieved, the latter was recalcitrant.

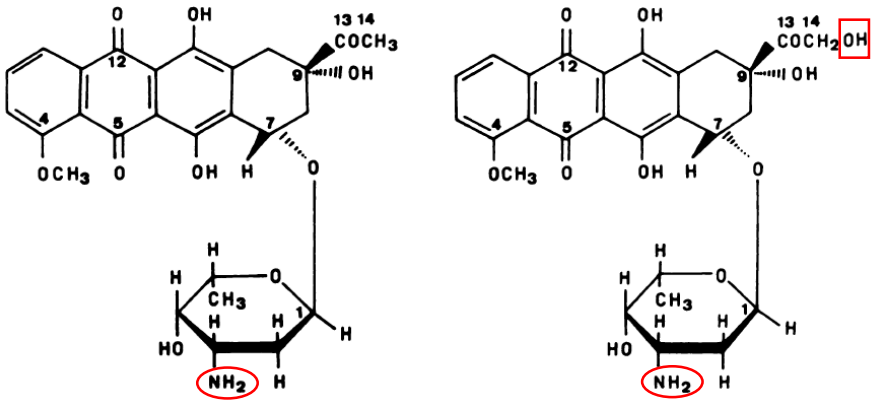

Figure 7.2. Chemical structures of daunorubicin (left) and doxorubicin (right). The only difference is that doxorubicin has an OH group added at position 14 (red box). The four rings with their double-bonds have the size, shape, and electronic structure to bind stacked against a DNA base-pair, as occurs in intercalation and in DNA-topoisomerase II trapped complexes (Figures 7.3). The amino group (circled red) confers a positive charge to the six-membered ring that lies near the negatively charged DNA backbone (Figure 7.4).

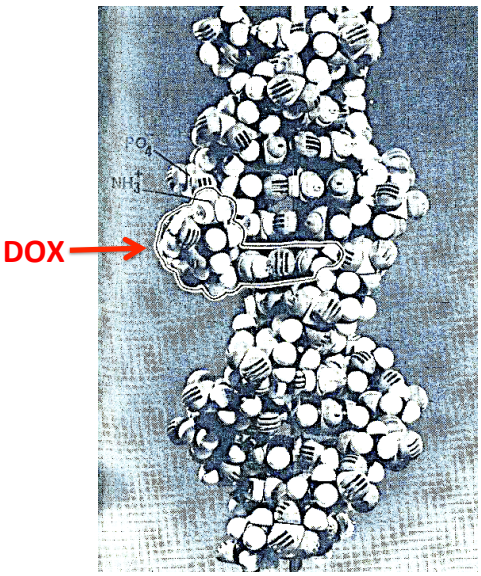

Figure 7.3. Molecular model showing how doxorubicin (DOX) intercalates between base-pairs in DNA (Pigram et al., 1972) (*permission needed)*.

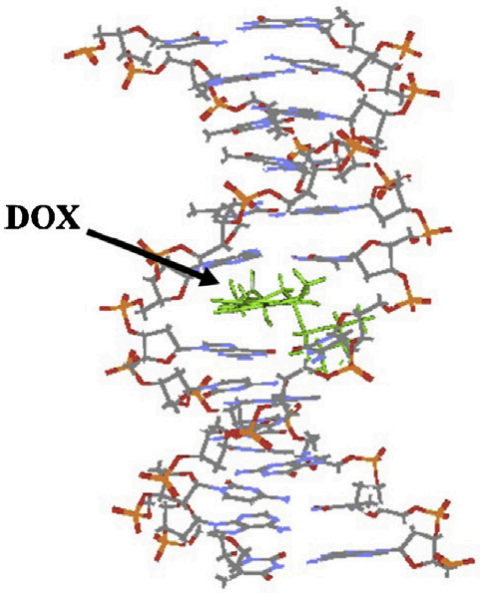

Figure 7.4. A computer-generated model of how Doxorubicin (DOX) intercalates between base-pairs of a DNA double helix (Agudelo et al., 2014). Doxorubicin is show in green. Note that doxorubcin's flat ring system intercalates, while its side-chain with its positive charge is located outside of the stack of base-pairs and close to the negatively charged DNA backbones. (*Permission needed.*)

How doxorubicin damages the heart

Since heart damage was so prominent in doxorubicin's action, we will discuss first what was found out about it and then consider how doxorubicin exerts its anticancer action.

After decades of inconclusive speculation, the chemical culprit that damages the heart was finally identified (Mukhopadhyay et al., 2009) (Pacher et al., 2007). In accord with one of the leading hypotheses, the culprits were found to be "free radicals" (to be explained in a moment) (Sinha et al., 1987) (Rajagopalan et al., 1988). As long suspected, the toxic free radicals are produced by mitochondria as they use oxygen to make ATP, the energy currency of the cell. Doxorubicin was found to increase the production of free radicals during the energy production process in mitochondria. It turned out, however, that most of the damage is due to a particular free radial product, as will be explained below.

As electrons flow through the chain of proteins (cytochromes) in the membranes of mitochondria, they generate ATP, and also some free radicals as a side effect. The process is rather complicated and is described in standard biochemistry textbooks. Here, the main thing to know is that some of those electrons flowing through this cytochrome chain occasionally go astray and produce the aforementioned free radicals; doxorubicin, as we shall see, facilitates free radical production. Also important is that heart muscle cells are particularly rich in mitochondria, which supply the high energy needs of the heart. Therefore, the heart's high energy needs entail high electron flow through the mitochondrial cytochrome chains with likely production of damage-inducing free radicals, especially in the presence doxorubicin.

Here are a few details about how that happens: Doxorubicin's aromatic ring system easily picks up an electron as it flows through the cytochrome chain, and the doxorubicin molecule then easily transfers the electron to an oxygen molecule. The extra electron on the oxygen molecule makes it highly reactive, because the odd electron desperately wants to pair up with another electron. One way it does that is to grab an electron off of some important protein in the heart, which, in the end, damages the heart muscle. More explicit detail in a moment.

Another factor making doxorubicin particularly dangerous to the heart is that it tends to bind to lipid membranes, including membranes where the cytochrome chain is located in the mitochondria: the drug then is in position to pick up an electron flowing through the cytochromes. As already said, heart cells are particularly vulnerable, because of their unusually large numbers of mitochondria – which they have in order to provide for the high energy requirement of the heart.

Tissue cells, including those of the heart, do have enzymes (superoxide dismutase and catalase) that rapidly destroy free radicals. In the presence of doxorubicin,

however, the increased free radical production can overwhelm the capacity of those protective enzymes.

A little more about free radicals: A free radical is a molecule or atom with an odd number of electrons. Molecules are stable only if they have an even number of electrons (except for some molecules that have a heavy metal atom, such as iron). The chemical structure of doxorubicin, with its multiple aromatic rings (Figure 7.2), easily picks up an extra electron, forming a 'semiquinone' type of free radical (Keizer et al., 1990). The doxorubicin semiquinone gets some stability from its odd electron being distributed over the alternating single and double bonds in the ring system. The odd electron eventually transfers to oxygen (O_2), forming superoxide (O_2^-), which engages in further reactions producing highly reactive oxidants that can damage many essential molecules in the cell.

Intensive investigation revealed more detail about how doxorubicin damages the heart by way of free radicals (Mukhopadhyay et al., 2009) (Pacher et al., 2007). As already said, doxorubicin binds to the inner membrane of mitochondria in heart muscle cells, where it can pick up an electron from the electrons flowing through the energy-generating electron transport chain. The resulting doxorubicin semiquinone free radical then transfers its extra electron to a molecule of oxygen to produce superoxide (O_2^-), which is an O_2 molecule with an extra electron added (which gives the molecule a negative charge). At this point, another reactive biologically important molecule with an odd number of electrons comes into play: nitric oxide (NO).

Nitric oxide consists of an atom of nitrogen bound to an atom of oxygen. The two together have an odd number of electrons; hence NO is a free radical; however, it is relatively stable, as free radicals go, having a half-life in tissues of a few seconds. That is a long time for a free radical in tissues: long enough for the free radical to move around among the cells to find a vulnerable target. Its high reactivity and relatively short life span allows it to be an important signaling molecule in cells and tissues.

It was recently found that the entity that actually causes most of the heart damage is a chemical combination of nitric oxide (NO) and superoxide (O_2^-); the combination forms the highly reactive peroxynitrite ($O{=}N{-}O{-}O^-$) molecule (Mukhopadhyay et al., 2009) (Pacher et al., 2007). Peroxynitrite production is favored when superoxide is generated in increased amounts through the ability of doxorubicin to shuttle unpaired electrons, and when NO levels are also high. The production of peroxynitrite from nitric oxide and superoxide is highly efficient: the two molecules combine every time they come in contact. Peroxynitrite is a strong oxidant with a short half-life, but long enough to reach targets where it can cause trouble. Its short half-life is countered by a high and sustained production rate (Szabo et al., 2007) (Pacher et al., 2007). Peroxynitrite can react with many different constituents in the cell; however its major toxic action in the heart is thought to be at mitochondria, where it can enhance the production of superoxide, thus producing a positive

feedback loop of problems (Szabo et al., 2007) (Pacher et al., 2007) (Mukhopadhyay et al., 2009).

Another aspect of the path to heart cell destruction is that peroxynitrite stimulates mitochondria to produce molecules that cause damaged cells to die in a cell suicide process (apoptosis). Thus, mitochondria are involved both in the doxorubicin-facilitated production of superoxide and in the cell-killing effect of peroxynitrite in the heart.

Over extended periods of treatment with doxorubicin, increasing numbers of heart muscle cells die. Since adult heart muscle cells are not replaced, the damage is irreversible and progressive. That is why the extent of damage to the heart depends on the cumulative amount of doxorubicin a patient receives over time, and the damage persists for many years (Singal and Iliskovic, 1998).

However, some investigators thought that a free radical may also contribute to doxorubicin's extraordinary anti-cancer action (Keizer et al., 1990).

How do the molecular actions of doxorubicin produce anti-cancer activity?

After being submerged in discussion of heart-damaging free radicals, we come at last to the question of how doxorubicin produces its anticancer action.

We have noted that doxorubicin binds to DNA by intercalation (Figures 7.3 and 7.4) and blocks DNA and RNA syntheses. Several other DNA-intercalating drugs share these actions, but lack doxorubicin's remarkable anti-cancer activity. Hence DNA intercalation and inhibition of DNA synthesis by themselves were thought insufficient for the anticancer action.

Our laboratory had found that DNA intercalation is often associated with a blocking action on topoisomerase II (Pommier et al., 1985) (Zwelling et al., 1981), which became top-of-the-list of likely causes of doxorubicin's anticancer activity. Drug actions on topoisomerases is the subject of Chapter 9.

Nevertheless, there was doubt as to whether the actions of doxorubicin on topoisomerase was the full answer, because there are other drugs that have similar actions, but lack the broad anti-cancer activity of doxorubicin (Burden and Osheroff, 1998). It was proposed that doxorubicin's unusually strong anticancer action results from a combination of (1) topoisomease II blocking and (2) free radical damage in mitochondria with consequent apoptosis (programmed cell death) of cancer cells (Mizutani et al., 2005).

Topoisomerase II transiently cleaves both DNA strands in the double helix, so as to allow one double helix to pass through another, thereby disentangling the DNA in order to allow the chromosomes to separate during mitosis (discussed in Chapter 9). Doxorubicin was found to prevent that action by binding to the topoisomerase II-DNA complex while the DNA strands are cleaved, which would prevent the strands from rejoining. This cleaved DNA-drug complex apparently was lethal to the cell unless the cleavage complex reversed spontaneously or was resolved by a molecular repair process.

The DNA-topoisomerase II complexes trapped by doxorubicin thus would kill the cell if the trapped complexes were not repaired before an encounter with a replication or transcription process occurred. The cell has several mechanisms that could repair the trapped DNA-topoisomerase complexes; the relevant repair processes are discussed in Chapter 8.

Liposomal preparations of doxorubicin for better delivery to tumors

Attempts were made to improve the delivery of the poorly soluble doxorubicin into cancer cells by putting the drug molecules into microscopic lipid vesicles called liposomes {Uziely, 1995 #10353}, and such preparations were approved for clinical use. A further improvement was to coat the liposomes with polyethylene glycol (Papahadjopoulos et al., 1991); such forms of liposomes were said to be "pegylated" (Figure 7.5). Pegylation prevented phagocytic cells from taking up and inactivating the drug. Doxorubicin in pegylated liposomes somehow reduced the toxicity to the heart (Markman, 2006) (Thigpen et al., 2005) (Gabizon, 2001).

Delivering drug-liposome combinations into cancer cells was also enhanced by the high permeability of blood vessels in tumors, which allowed liposome-sized bodies to exit from the blood vessels and reach cancer cells. Blood vessels in cancer tissue tend to have increased permeability; consequently, normal tissues tend to be spared. Once inside the cancer tissue, the drug tended to be retained for a relatively long period of time, because of poor drainage through lymphatic vessels in tumors (Torchilin, 2011). Thus, various forms of liposomes were developed and used clinically to improve drug delivery into cancers.

A further improvement was to link folic acid molecules to the poly(ethylene glycol) layer on the surface of the drug-containing liposomes. The idea was to take advantage of the relatively large number of folic acid receptors on the surface of many cancer cells. The folic acid on the liposome's surface would bind the receptors on the tumor cell surface and increase the amount drug-containing liposome that gets into the cell (Sriraman et al., 2016).

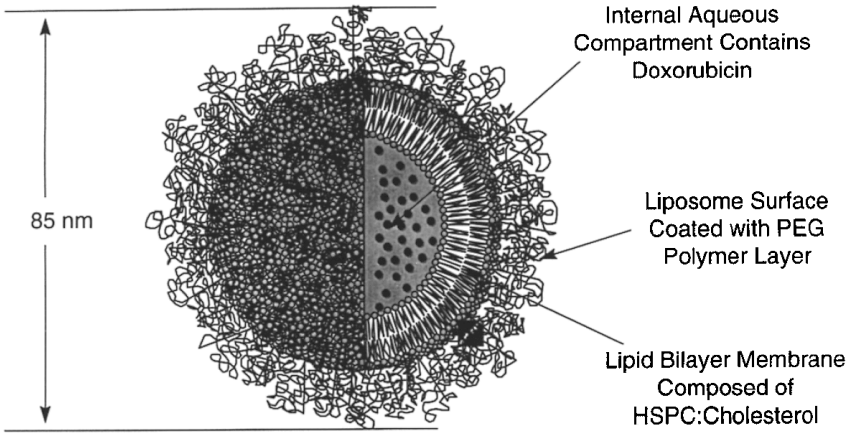

Figure 7.5. The structure of a doxorubicin-containing pegylated liposome. Doxorubicin was encapsulate in the center, surrounded by a double-layered lipid membrane with poly(ethylene glycol) (PEG) chains on the surface (Gabizon, 2001) (*permission needed).*

Synopsis

Doxorubicin is one of the most useful drugs in cancer chemotherapy, but is plagued by toxicity, particularly to the heart, which limits the cumulative amount of drug that can safely be administered. The anti-cancer activity of doxorubicin was thought to be due mainly to its ability to trap DNA-topoisomerase II complexes, perhaps combined with its tendency to generate free radicals in mitochondria, leading to programmed cell death "apoptosis." The free radical action was found to be the cause of the toxicity to the heart, but preventing this toxicity was not fully achieved.

The anti-cancer usefulness of doxorubicin was enhanced by incorporating the drug in liposomes, which selectively delivered the drug to cancer cells. That action was enhanced by coating the liposomes with poly(ethylene glycol (PEG). Selectivity for delivery to some tumors was further enhanced by attaching folic acid to the surface of the PEG-coated liposomes molecules, in order to favor selective uptake by cancer cells that have large amounts of folic acid receptors on their surface.

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