Prospects of benzoquinone-containing high-throughput screening hits in drug discovery

Abstract

Pan assay interference compounds (PAINS) are compounds that can bind nonspecifically to biological targets or assays during high-throughput (HTS) screening. These compounds can be redox-active, metal chelating, nucleophilic or cause membrane perturbation, among others. Often unknowingly to the medicinal chemist, they can cause false positives and hinder research. Multiple structural filters have been created to screen for these PAINS, although it is still not possible to eliminate all false positives. One of these problematic substructures is the quinone_A substructure, which corresponds to benzoquinone. Although problematic, there is a fair share of approved drugs that contain this substructure. In this article, we investigate the quinone_A substructural alert and propose a possible extension. We also investigate the mechanisms of action of these drugs, their toxicity and prospects of quinone derivatives. Lastly, we define a new online repository for PAINS. The quinone_A substructural alert was found to be justified and one should be wary of quinone hits in HTS. The main mechanisms of quinone-based drugs are cytotoxicity by reactive oxygen species (ROS) formation and DNA intercalation. They can also play an important role in the electron transport chain. These mechanisms correspond to their nonspecific binding capacity and are also the reason for their toxicity. The online repository will combine different PAIN filters, their definitions and structure-activity relationship (SAR) data to maximize aid to the medicinal chemist. It is believed that the quinone_A filter could be extended by accounting for substituted functional groups on the quinone ring. Quinone-based drugs have great anticancer efficacy and drugs affecting the electron transport chain show favorable toxicity and could be further developed if incentive allows it.

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Abbreviations

Adenosine triphosphate - ATP
Deoxyribonucleic acid – DNA
High-throughput screening - HTS
Pan assay interference compounds – PAINS
Pneumocystis jirovecii pneumonia - PCP
Reactive oxygen species – ROS
Structure-activity relationship - SAR

Drug discovery in the World

In a fast-evolving world that contains more and older people than ever before (1), new ways of drug discovery are paramount to sustain our way of living. A greater population means that there is more room for sickness to develop. Although sickness across the world has been greatly reduced in the last years, the need for new medicines is still present (2). Traditionally, drugs came from natural sources and were often found unintentionally. Salicin, for instance, has been used for centuries in Chinese medicine. Extracted from willow tree bark, it is a precursor of salicylic acid and acts therefore as a mild analgesic (3). Since this way of drug discovery is based on chance and as there is more knowledge of pharmaceutics in general, new paradigms have come to life.

Modern-day approaches

Nowadays, methods such as high-throughput screening (HTS) are used in drug discovery. These are *in vitro* methods that can perform large amounts of assays simultaneously. Typical HTS implementations screen up to 100.000 compounds per day (4). It is therefore essential to have a relatively simple assay design, robotic-assisted sample handling and automatic data processing (4). The underlying assays are often target-based, but many other types exist. They measure the excitation or inhibition of biological targets such as receptors or proteins by candidate compounds.

One of these target-based assays is the Amplified Luminescent Proximity Homogeneous Assay (AlphaScreen^{TM}) method (Figure 1) (5). This method is based on the proximity of two fluorescent active beads. One bead is covalently bound to a biological target, the other bead is bound to the compound of interest. If the compound has selectivity for the target, it will bind and pull the beads closer together. The method works by exciting the donor bead with light at 680nm. Subsequently, this bead excites present oxygen molecules into a higher energy state. This high-energy oxygen then passes on its energy to the acceptor bead, which in turn emits light at a wavelength of between 520nm to 620nm. This signal is then detected and processed and registered as a hit.

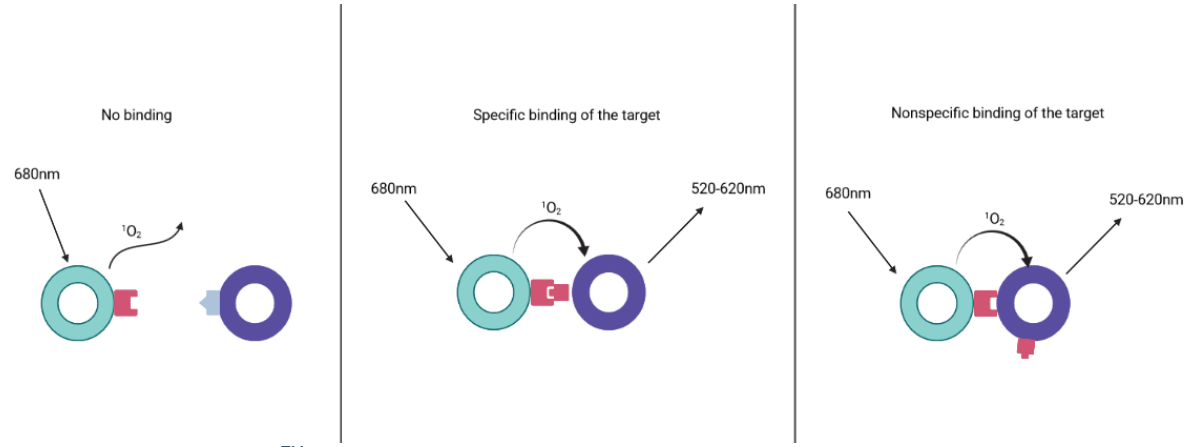

Figure 1. The AlphaScreen^{TM} detection method. The left (cyan) bead is the donor bead and is bound to a biological target. The right (purple) bead is bound to the compound of interest. When the beads are bound and in proximity, the emitted light travels into the detector as seen in both specific binding and nonspecific binding of the target. Created with BioRender.

The assay signal is interpreted, and drug candidates are defined. If a drug-like compound is found, medicinal chemists will begin to optimize the candidate to increase its drug-like properties, such as solubility, permeability, and metabolic stability. These properties are important pharmacological properties to maximize in vivo activity and minimize toxicity (6).

Problems with high-throughput screening

The expenses and time constraints often make HTS a one-shot deal (7). A good, thorough high-throughput screen is quite expensive because it requires a very big chemical library, as well as robotics and automated systems (4). Diversity among the compounds is also needed because analogs will show similar selectivity (8). It is therefore important to utilize a big, high-grade library with high diversity as it will make for higher quality research. Libraries of less than 50.000 compounds can be used as they are relatively inexpensive but will mostly be useful in assay validation or to confirm known active compounds (7).

Pan assay interference compounds

Another recurring problem in HTS is the emergence of frequent hitters. These are compounds that give positive signals in a variety of different assays (8). They are often called promiscuous compounds. The reason for their promiscuity is that they have nonspecific binding abilities. Multiple mechanisms are defined including chemical aggregation, metal chelation and redox cycling (9). Of these mechanisms, aggregation counts for 88% to 95% of false positives (10) in HTS. In 2010, Baell & Holloway coined the term pan assay interference compounds (PAINS) (11). The Australian research team described around 30 chemical substructures that can bind nonspecifically to either the biological target or to the assay structure itself (Figure 1).

Since then, Baell & Holloway have been pushing for global awareness of these structures, as medicinal chemists do not yet always recognize these compounds as problematic (12). There are hundreds of publications that excitingly describe a new potential drug candidate, even though the compound would be flagged by multiple PAIN filters. Further investigation of these compounds will waste resources and time because these compounds are non-optimizable and will most likely not become approved as they bind nonspecifically. Baell & Holloways PAIN substructures have been criticized though (13). The use of a nonpublic chemical library makes the research non-reproducible. Also, their use of only one assay method (AlphaScreen^{TM}) makes only a crude estimation of the PAIN substructures. To this day, multiple studies have been conducted that provide a stricter definition of PAIN filters, that are tested across multiple assays (13).

Multiple chemical databases have now integrated PAIN filters to aid medicinal chemists in their search for optimizable drug candidates. Most of them are open to the public, such as the ZINC15 and ChEMBL databases. Besides alerting for PAIN substructures, these databases contain information about compound pharmacological properties, vendors and current research, among others.

Quinones

One of the most promiscuous compounds according to Baell & Holloway are compounds with the quinone_A (benzoquinone) substructure (11). This is also verified by the Lilly Research Group (14). Quinones have multiple ways of nonspecific binding, namely by redox cycling and Michael addition. These mechanisms are responsible for their high promiscuity. Interestingly, multiple FDA-approved drugs contain this substructure. These are mostly natural products or derivatives of natural products (15). These include various anti-cancer drugs such as doxorubicin, mitoxantrone and mitomycin C. It should be noted though, that these drugs have been developed before the emergence of HTS.

Baell stated: *"The PAINS behavior more or less universally exhibited in quinones should render them deemed to be unprogressable as low-micromolar potency screening hits […]."* (15)

This begs the question: what are the prospects for new quinone derivatives in HTS drug discovery? In this paper, we will try to answer that question by looking deeper into approved quinones, their toxicology, their reaction mechanisms and their structural properties. We will also look at the prospects of the quinone_A filter, its validity and future. Lastly, we will define a new online repository for PAINS.

An overview of chemical substructural filters – what has been done

To start answering the research questions, it is important to understand how these structural filters came to exists in the first place. In this part, we will look at different quinone substructural filters by Baell & Holloway, and the Lilly research group. A variety of other filters exist, such as the Glaxo or BADAPPLE filters, but we will only go over the first two.

Baell & Holloway

Baell & Holloway analyzed a chemical database of around 93.000 compounds (11). They tested these compounds on 6 different AlphaScreen™ assays and determined hits by measuring inhibition. The cutoff value of inhibition was a readout of < 50%, anything below this result would not be a hit. Of the 370 compounds that contain a quinone_A substructure, 228 derivatives were registered as hits in 2 of the 6 of the assays, as opposed to 86 that were not found to be promiscuous at all. The enrichment value of this quinone_A filter was 265%. A generic calculation for the enrichment value is found below:

$$Enrichment = \frac{positive\ hits}{no\ hits} \times 100\%$$

The way that a positive hit is defined may differ across studies, so that can influence the enrichment value. Baell & Holloway defined the enrichment value as the quotient of the amount positive hits in more than 2 assays and the amount that did not hit at all. They held an enrichment value of 30% as a basis for a good filter. This was based on the following process: they compared 6 of the most problematic classes that hit all six assays to 6 classes that are not very likely to be promiscuous. Calculating the enrichment values gave a value between 41% to 625% of the problematic groups and 8% to 18% of the nonproblematic group. They concluded an enrichment of 30% would define a good filter. Although this measurement may seem arbitrary, it is a good measure of qualitative ranking between filters (16).

Lilly Research Group

The Lilly Research group has created 275 ‘rules’ for identifying promiscuity, based on around 900.000 compounds in high throughput screens against multiple families of targets (14). These rules are translated into substructural filters. The targets, for example, are kinases, G-protein coupled receptors or ion channels. The structural rules fall into 17 classes that correspond to certain mechanisms of action such as acylating, alkylating or chelating compounds. Out of these screening hits, they defined a set of 6165 promiscuous and 300877 non-promiscuous compounds, respectively. Compounds were considered promiscuous if they showed activity in five or more target families. Quinones (para-quinone filter), in this case, fall under the Michael group, as they are Michael acceptors (17). A Michael acceptor is a compound that is part of the Michael reaction (see Michael reaction), which causes the nonspecific binding. Analysis

showed that 0,746% of their promiscuous compounds contain a para-quinone Michael acceptor, and 0,157% of their non-promiscuous compounds contained the para-quinone structure. This corresponded to an enrichment value of 475% (the original was 4,75 as it did not account for the percentage).

Table 1. Information about quinone promiscuity according to different studies.

Research	Promiscuous quinones	Non-promiscuous quinones	Enrichment	Source	Definition promiscuity
Baell & Holloway	228	86	265%	370 quinone derivatives	>2 assays hits
Lilly	0,746%	0,157%	475%	6165 promiscuous, 300877 non-promiscuous compounds	>5 subfamilies hits

Next to this screening, they evaluated the Baell & Holloway PAIN substructures against a subset of their chemical library that already passed their filters. 4351 promiscuous compounds, defined as being active against five or more families of targets, and 242.266 non-promiscuous compounds, defined as being active against two or fewer families (tested >100 times) were used. They found 7 quinone_A substructures to be not promiscuous and found 5 to be promiscuous, resulting in an enrichment of 3980% (or 39,80 without accounting for the percentage).

Even though Baell & Holloway did not publish their chemical library and although they only used 6 different assays, it is still clear that quinones are a problematic substructure as their enrichment value is quite high in multiple studies (Table 1). This makes quinones true frequent hitters and it seems that the substructural filters are justified. Still, it is unclear why exactly they react with multiple assays. For that, we must delve deeper into the properties of quinones, their toxicology and their mechanisms.

Quinones and their reactivity

Quinones are cyclic compounds with two doubly bound oxygens (Figure 2). Although multiple forms exist, the 1,4-benzoquinone form is one that we will discuss here as it is the most problematic quinone among PAINS, as concluded by Baell & Holloway (12). Also, there are no approved drugs that contain 1,2-benzoquinone substructures.

There are multiple classes of drugs that contain a benzoquinone structure: 1,4-benzoquinone, 1,4-naphthoquinone and the 9,10 -anthraquinone. The PAIN substructural alert (quinone_A) is seen in multiple quinone structures.

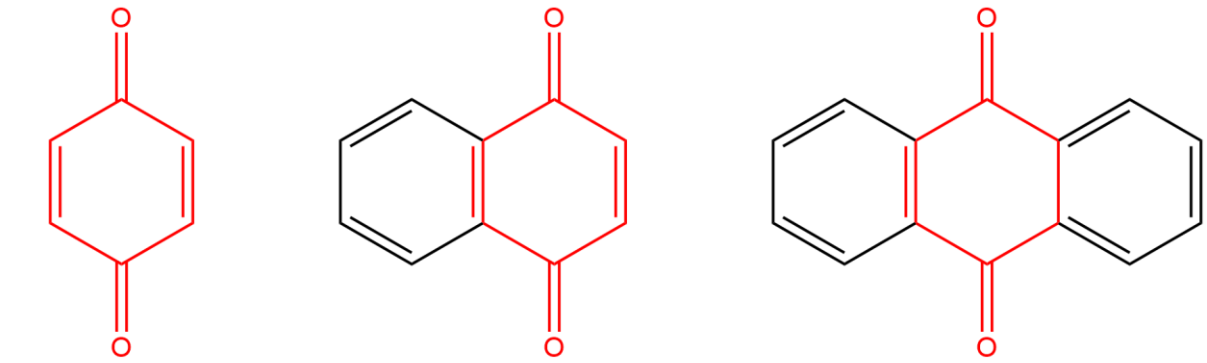

Figure 2. The chemical structure of different benzoquinones. The substructure in red indicates what is flagged by the quinone_A PAIN filter.

Quinones are very reactive. They are electrophilic on positions 2, 3, 5 and 6, which can cause them to react with nucleophiles in the body such as nucleophilic amino groups or deoxyribonucleic acid (DNA). They are Michael acceptors, as quinones are α, β-unsaturated carbonyl compounds. This can result in the alkylation of a nucleophile under the presence of a base. Thiol additions have been reported to cysteine side chains (17). This together with their redox cycling potential has been attributed to their toxicity.

Michael reaction

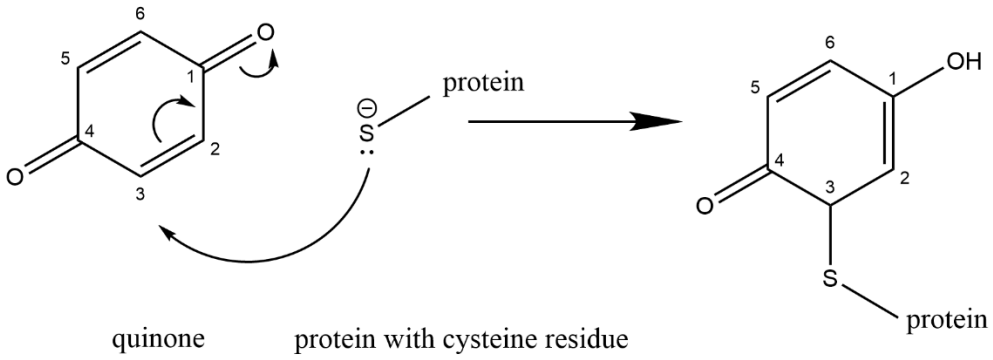

Figure 3. A Michael addition of a protein as a nucleophile to a quinone.

The Michael addition is one of the mechanisms of action (Figure 3). A nucleophile, for example, a cysteine residue, will attack the quinone, the Michael acceptor, on position 3. Electron shuffling reorganizes the quinone which gives the oxygen a lone pair, that subsequently reacts with a hydrogen source. The result is a quinone bound to the cysteine residue of a protein (17). So now, multiple quinones bind to the protein, changing the protein without a biological effect. This reaction is also partially responsible for the DNA intercalation activity of some quinone-based drugs (18).

Redox cycling

In the body, there are multiple enzymes involved in redox cycling (Figure 4). Flavoenzymes, for example, with NAD(P)H as an electron source are catalyzers for multiple redox reactions in the body (19). The catalyst helps to donate electrons from an electron source to the quinone, reducing it to a semiquinone. The semiquinone subsequently reduces oxygen to O_2^-, which is a reactive oxygen species (ROS). By reducing oxygen, semiquinone oxidizes to quinone, restarting the cycle. A semiquinone can be reduced once more, resulting in

hydroquinone. ROS is responsible for damage of cells. It is extremely reactive and will react to proteins, DNA and the cell membrane, among others. Many drugs' activity is based on this principle. For example, the cytotoxic effects of doxorubicin are partially based on the formation of ROS in the tumor cells (20).

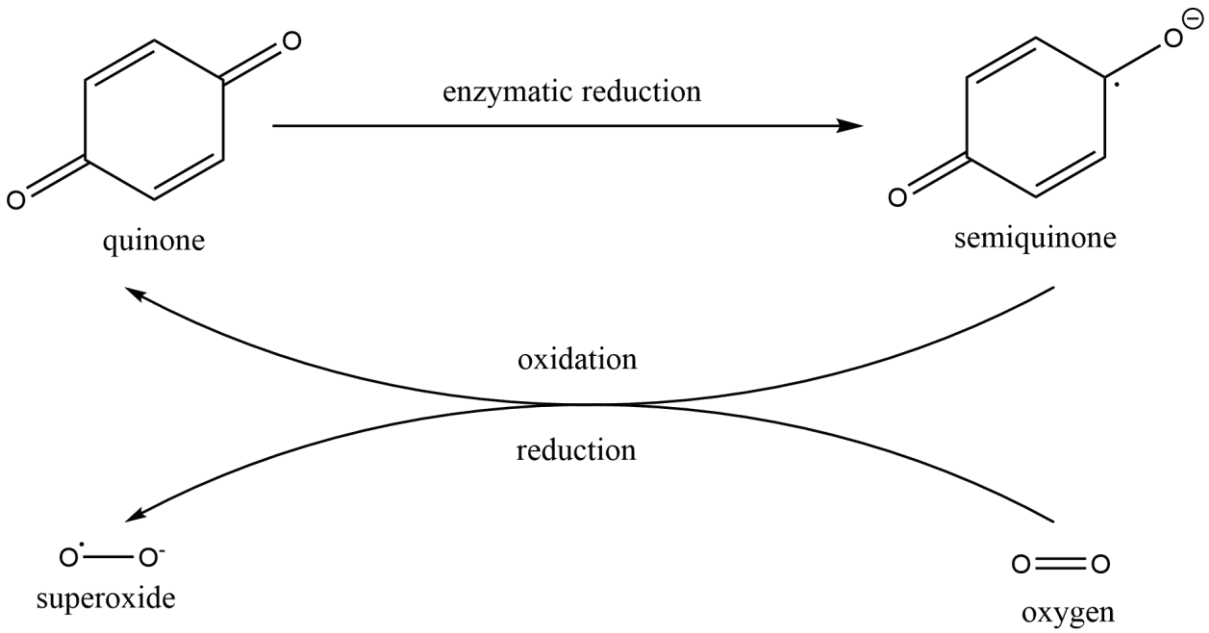

Figure 4. A schematic representation of redox cycling, with the formation of ROS. Note that the semiquinone can also be further reduced following the same cycle again.

Altering reactivity of quinones

We see that quinones are very reactive. What are the factors that contribute to this reactivity? The reactivity of a quinone derivative can be changed by substituting hydrogens on the reactive quinone core with other functional groups. Different groups will have different effects. We will mostly investigate the electron-donating and withdrawing effects of functional groups, as they affect the redox potential of quinones (21), directly changing its redox activity. There are two ways that functional groups can change electron availability. They do this by induction or by extension of its resonance structure (22).

The inductive effect is based on the electronegative properties between two σ-bonded atoms. This overlap of σ-bonds will give the electron pair more room to localize. To put this into perspective we look at the difference between oxygen and carbon atoms. An oxygen atom has an electronegativity of 3,44, while carbon has an electronegativity of 2,55 (23). This difference is more favorable for the oxygen, as it will pull the electrons more into its σ-orbital, making it more electron-dense. This results in a slight difference in charge in the molecule and more importantly will change the ability to accept or lose electrons in a reaction.

The resonance effect can be best described by the overlapping of one or multiple π-orbitals. When a molecule is π-bonded, the electrons in this shell localize over the entire π-system, making the orbitals more electron-dense. To put this into perspective, some functional groups have empty π-shells, which subsequently extend the π-system of the molecule. This withdraws the electron from the other π-orbitals onto its own. Electron donating groups have lone pairs which can localize into overlapping π-orbitals of the molecule. Depending on the functional group, the electron availability of one of these effects may be stronger than the other. A reduced quinone has restored aromaticity, which can be used by the substituent to induce this resonance effect.

Even though they are very reactive, quinones play a vital role in energy metabolism in the body as they use their electron transferring properties in multiple metabolic pathways (24)(25). Two quinones are heavily consumed in the body: coenzyme Q and vitamin K.

Below, we will go over two endogenous quinones and their pharmacological properties. After, we will look at different approved quinone drugs.

Coenzyme Q

Coenzyme Q is a 1,4- benzoquinone with a hydrophobic tail of ten isoprenyl groups (Figure 5). It is found in mitochondria of all eukaryotic cells (26). It is an essential part of the electron transport chain. There, it is responsible for multiple functions such as being an electron carrier but also functions as an antioxidant.

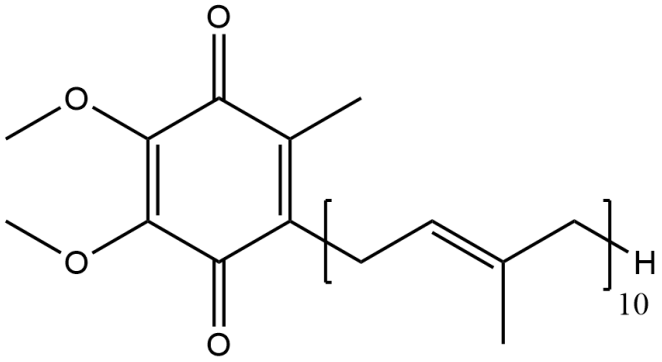

Figure 5. The chemical structure of coenzyme Q. The 10-unit isoprenyl tail has been shortened.

The mitochondrial electron transport chain is responsible for the production of adenosine triphosphate (ATP) inside cells. Broadly speaking, this chain exists of 4 protein complexes that transfer electrons from complex I to complex IV to produce ATP. Coenzyme Q plays a vital role in specifically transporting electrons from complex II to complex III.

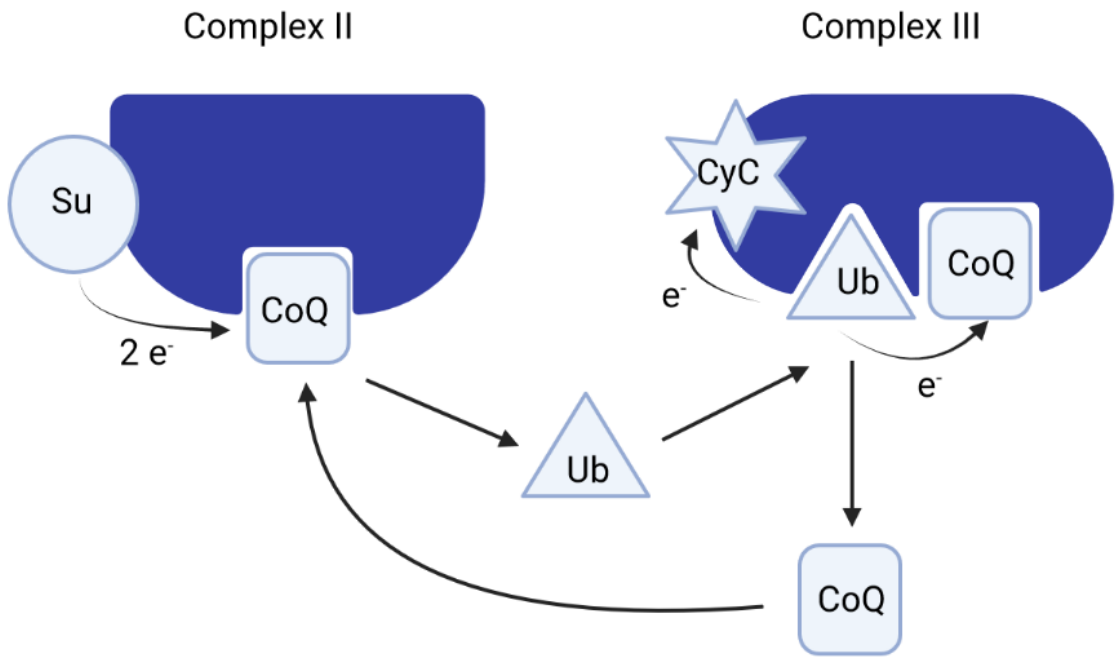

Figure 6. The electron transport chain in a mitochondrion. The thin curved arrows indicate electron transport. The full arrows indicate molecule transformation or movement. Su: succinate. CoQ: coenzyme Q. Ub: ubiquinol. CyC: cytochrome C. Created with BioRender.

Complex II transports electrons from succinate to coenzyme Q (Figure 6). Coenzyme Q is then reduced to a semiquinone and further reduced to ubiquinol by accepting another electron from complex II. Ubiquinol is the hydroquinone form of coenzyme Q. Next, it will bind together with coenzyme Q to two binding sites on complex III. Ubiquinol will undergo oxidation, moving one electron to cytochrome C and one to coenzyme Q. This oxidizes ubiquinol back to coenzyme Q while reducing coenzyme Q to its semiquinone form. Then this cycle happens again, further reducing the semiquinone to ubiquinol (27).

Coenzyme Q deficiency is associated with multiple diseases such as myopathies. These disorders are very treatable with coenzyme Q supplementation (26). These deficiencies give a possible use of benzoquinone derivatives as a function of coenzyme Q analogs. The toxicity of coenzyme Q is almost nonexistent, apart from mild gastrointestinal effects (<1%) and reduced blood pressure (28). It is deemed very safe to supplement coenzyme Q when deficient, and overdoses of coenzyme Q do not influence endogenous coenzyme Q production, nor does it accumulate in plasma (29).

It is interesting indeed that there is no severe toxicity for this quinone, which would be expected by its supposed reactivity.

Phylloquinone and menaquinone

There are multiple types of vitamin K. They are all produced by green leafy vegetables, as part of their photosynthesis (30). As humans do not produce vitamin K endogenously, it must be consumed via diet. Their common structure is a 1,4-naphthoquinone but they vary in tail length

(Figure 7). Menaquinone (vitamin K2) has a varied isoprenyl tail while phylloquinone (vitamin K1) does not have double bonds in its tail.

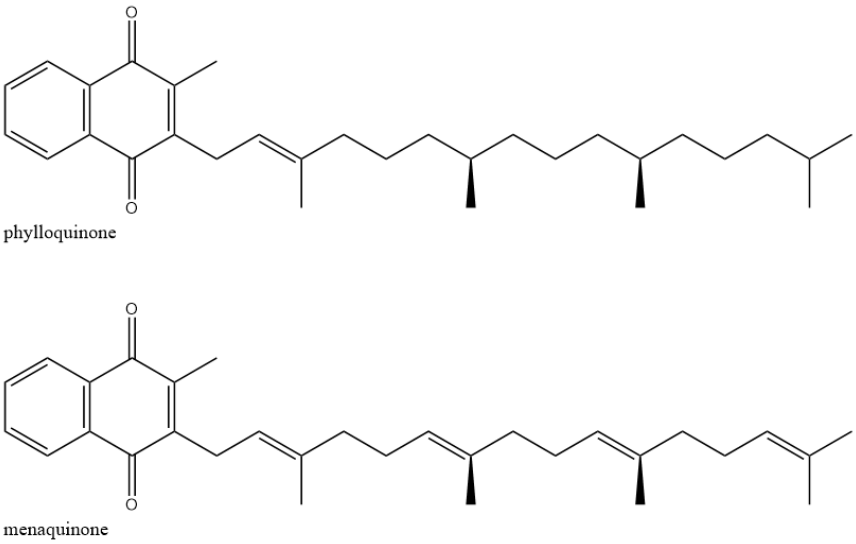

Figure 7. Chemical structures of phylloquinone and menaquinone.

Vitamin K is necessary for certain anti-coagulation mechanisms and is therefore associated with various coronary diseases (31). It also plays an important role in bone formation (32). It is a cofactor that is used during posttranslational modification of proteins where it carboxylates glutamate residues to y-carboxyglutamate (33). This happens during the last phase of protein synthesis. Vitamin K epoxide reductase reduces vitamin K to its hydroquinone state. Now in its active form, vitamin K serves as a cofactor for y-glutamylcarboxylase, which generates glutamate under the formation of a vitamin K 2,3-epoxide. Glutamate plays a role in clotting factors, as it is present on many coagulation proteins such as prothrombin (34).

Interestingly, there is no toxicity found for natural Vitamin K derivatives (35), even though they would be reactive considering their quinone substructure. Menadione is an exception, as it causes liver damage in high concentrations. Menadione is not part of the human diet, however (35).

Approved quinone derivatives, their mechanism and toxicity

9 approved drugs contain a quinone_A substructure. These were found by using the ZINC15 database (Table 2). The list consists mostly of topoisomerase II inhibitors such as mitoxantrone and the doxorubicin analogs. There is also mitomycin C, which also has anti-cancer properties. Atovaquone is antibacterial and is the only antibacterial of the quinones. Lastly, there is phylloquinone, a vitamin K1 analog that has already been discussed above.

Table 2. FDA-approved drugs containing a quinone substructure. Indications, mechanisms of action and full structures (Figure 2) are shown.

Drug	Indication	Mechanism of action	Structure
Daunorubicin Doxorubicin Valrubicin	Acute myeloid leukemia	Topoisomerase II inhibitor	Anthraquinone

Idarubicin Epirubicin			
Mitoxantrone	Acute myeloid leukemia	Topoisomerase II inhibitor	Anthraquinone
Phylloquinone	Certain bleeding disorders	vitamin K	1,4-naphthoquinone
Atovaquone	PCP, toxoplasmosis, malaria	Antimicrobial in P. falciparum and PCP	1,4-naphthoquinone
Mitomycin C	Tumors	Crosslinks DNA, bioreductive alkylation	Quinone

Below, we will go over the toxicity of these drug classes and their mechanisms.

Doxorubicin analogs

Doxorubicin (Figure 8) analogs consist of an anthraquinone backbone. Daunorubicin has its origin in the *Streptomyces pecuetius* and was discovered in the 1960s (36). From then on, multiple types of analogs have been developed but only doxorubicin, epirubicin and idarubicin have been approved (36). Below, we will discuss doxorubicin as it is the most used and effective.

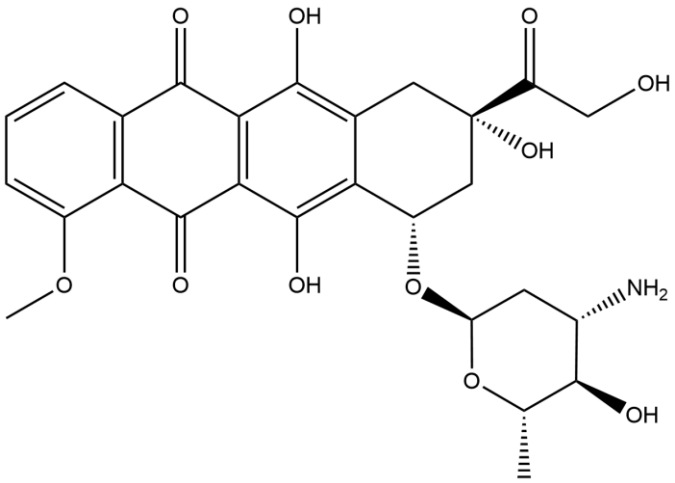

Figure 8. Chemical structure of doxorubicin.

Doxorubicin has multiple mechanisms of action in cancer cells (20)(37). One of the mechanisms of action works by inhibiting topoisomerase II. Topoisomerases are a class of enzymes that fix the problem of supercoiled DNA. When a cell replicates, its two DNA strands are separated. While one side of the DNA is separated, the other side twists in the other direction causing a lot of torsion on the DNA strand. This is called supercoiling. Topoisomerase II solves this problem by cutting both strands, which relaxes the tension and gluing it back together (20). Doxorubicin and its analogs inhibit topoisomerase II in tumor cells, by intercalating DNA. It will bind covalently to a base of one side (Figure 4) and form hydrogen bonds with the base of the other DNA strand (20). Topoisomerase II will then get trapped when

it tries to cut this part of the DNA, causing the DNA strands to break, inducing apoptosis (20). The other mechanism of action is by causing oxidative stress in the tumor cells. This happens when doxorubicin is reduced to a semiquinone by oxidizing enzymes (37)(38), which generates ROS. This happens according to the redox cycling mechanism, as discussed earlier.

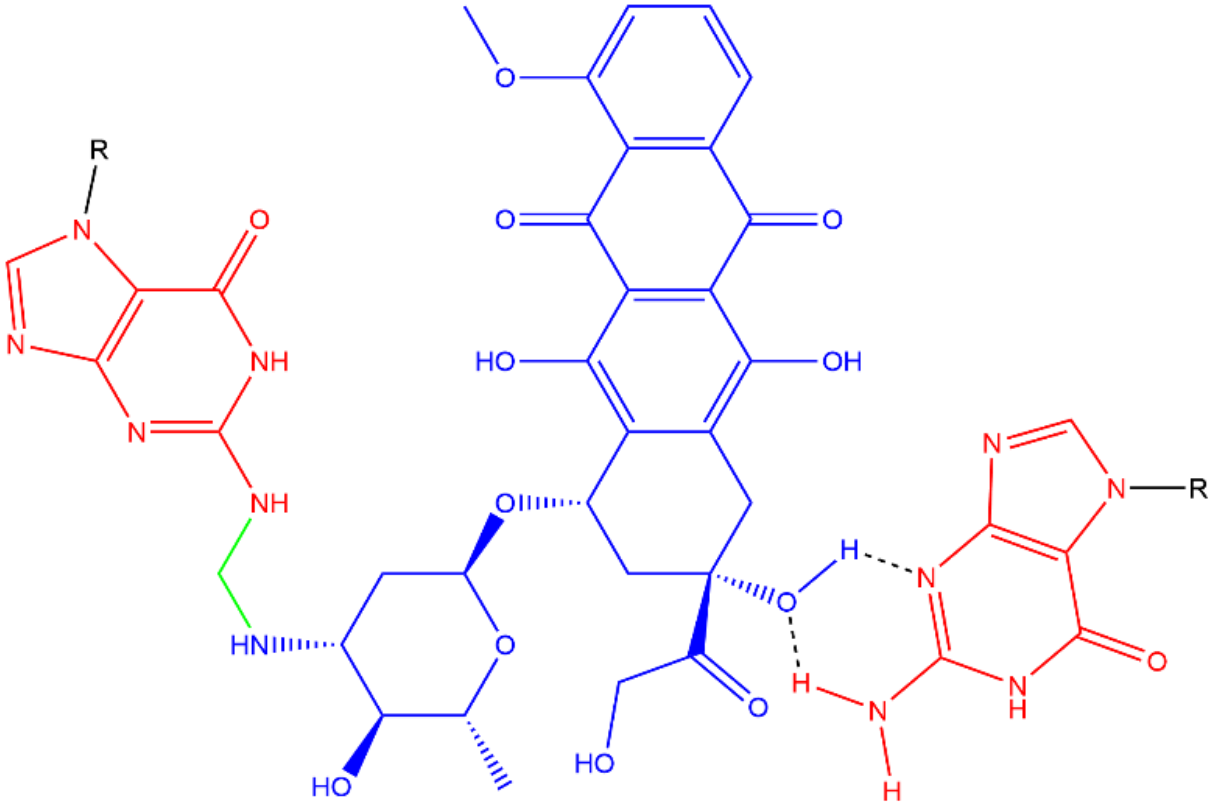

Figure 9. DNA intercalation of doxorubicin. In blue, doxorubicin is seen. Red indicates two guanine bases, both connected to a sugar backbone R. Doxorubicin is intercalated covalently with a single carbon (green). The dotted lines indicate hydrogen bonds.

Both these mechanisms have a fair share of side effects. Common side effects include cardiomyopathy in cancer patients (36), but it can also cause toxicity in the liver or kidneys (38). A pathway of cardiotoxicity of doxorubicin is by penetrating mitochondria (20). These mitochondria start to enzymatically reduce (see redox cycling) doxorubicin and start to form ROS that subsequently cause damage in the myocytes. In the liver, doxorubicin is metabolized together with the production of large quantities of ROS. This causes DNA damage, tissue damage and reduced glutathione levels, which affects the body's metabolism.

What can be learned from doxorubicin and its analogs? The quinone substructure is mostly responsible for the redox effects, which is one of the causes of its anti-tumor activity. Besides that, these properties are also the reason for its cardiotoxicity. Due to these heavy side effects, it is fair that this quinone substructure would not pass the PAIN filter.

Mitoxantrone

Mitoxantrone (Figure 10), comparable to the doxorubicin, is an anthraquinone and a topoisomerase II inhibitor. It was developed in the 1980s, to find an anticancer drug with less cardiotoxicity than doxorubicin (39). It is indicated for acute myeloid leukemia.

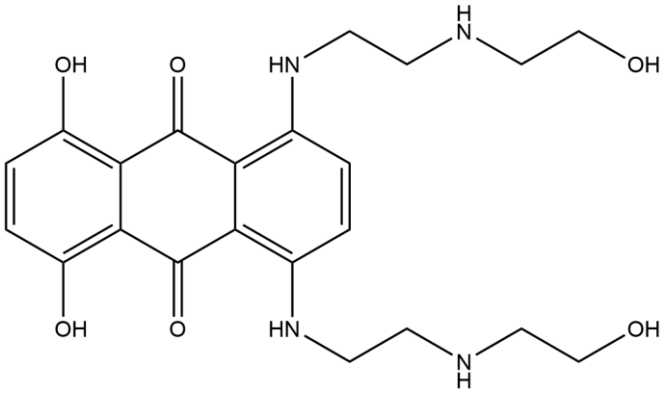

Figure 10. Chemical structure of mitoxantrone.

Its most important mode of action works by impairing the immune system by inhibiting T cell and B cell proliferation (40). There are multiple ways that mitoxantrone can induce DNA damage in these cells (39). It can induce DNA breaks by stabilizing the topoisomerase II enzyme, similar to doxorubicin; it can cause oxidative stress in the DNA strand, causing breaks; it can form covalent adducts in the DNA under the activation of myeloperoxidase; it can cause the DNA to condensate by neutralizing repulsive forces between base pairs (39).

Mitoxantrone induces toxicities that are comparable to other anthraquinones but is overall well tolerated (39). Its main toxicities are mostly hematological and gastrointestinal related as heart-related toxicities are reduced. Now the question arises: what kind of properties does mitoxantrone have, that doxorubicin does not have?

Let us look at the metabolism: mitoxantrone undergoes double oxidation, resulting in an electrophilic intermediate (Figure 11). This intermediate can then react with nucleophiles in the body such as glutathione or DNA. This is a known mechanism, comparable to the redox effect of doxorubicin but with some distinct differences.

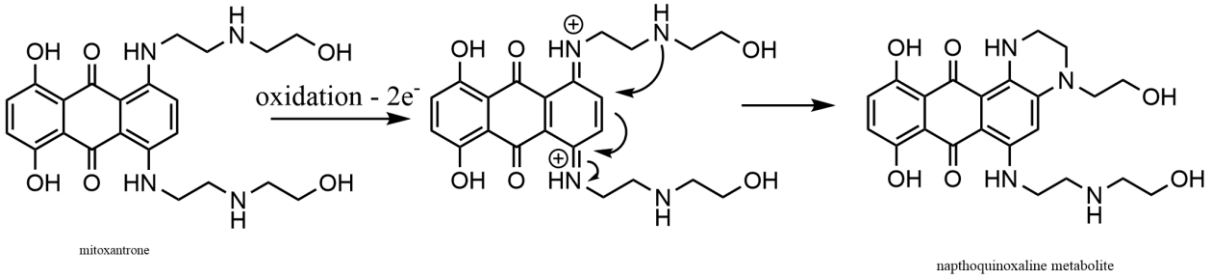

Figure 11. One of the metabolisms of mitoxantrone resulting in a reactive intermediate. The intermediate can subsequently be metabolized to a napthoquinoxaline metabolite.

One difference is that it is also susceptible to intramolecular nucleophilic attack generating a napthoquinoxaline metabolite. This metabolite is also suspected to be able to react with nucleophiles. Another big difference is that this oxidation does not occur as frequently as the redox cycling of doxorubicin (39). Also note that mitoxantrone undergoes enzymatic oxidation and not reduction like doxorubicin. This is because mitoxantrone has a relatively low reduction potential. It is therefore not as susceptible for reduction by redox enzymes. This also explains the lower ROS formation of mitoxantrone. Mitoxantrone has a reduction potential of -0.79V, while doxorubicin has a reduction potential of -0.6V (41). This decreased redox potential is seen as the foremost reason for its low cardiotoxicity as it is quite resistant to reductive metabolism in the mitochondria (42).

It is now clear to us that a quinone substructure does not have to be extremely toxic like doxorubicin. It is very important that it has a low reduction potential, so that it cannot function as a substrate for reduction enzymes. It is however innate to quinones, it seems, that it is not possible to eliminate ROS formation.

Atovaquone

Atovaquone (Figure 12) is an analog of coenzyme Q and is used for the treatment of *Pneumocystis jirovecii* pneumonia (PCP) but also has antimalarial activity against *P. falciparum*. It is the only approved antimicrobial of the quinones, although mitoxantrone also showed weak antibacterial activity (39). High quinine shortages in the 1950s pushed the development of atovaquone, resulting in the creation of an oral antimalarial without major side effects (43). Together with vitamin K analogs, atovaquone is the only naphthoquinone that is approved for use.

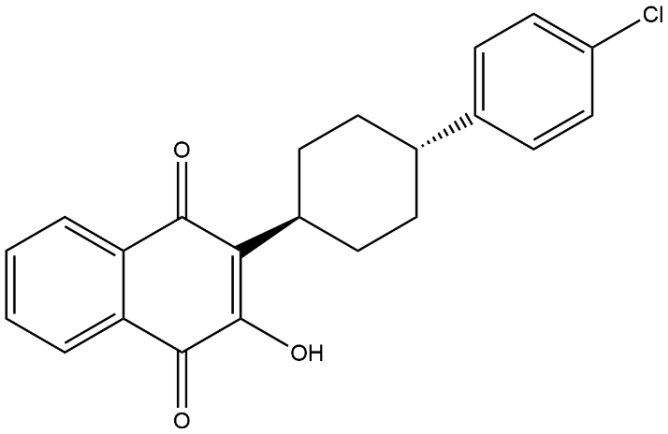

Figure 12. Chemical structure of atovaquone.

Atovaquone is a competitor of coenzyme Q. In parasitic malarial cells, this subsequently inhibits the mitochondrial electron transport chain (43). It does this by inhibiting the cytochrome bc_1 complex (complex III) at its quinone binding site (44). This complex usually binds to endogenous coenzyme Q, which functions as an electron donor that is used to produce ATP. Atovaquone binds to this binding site, disrupting the membrane potential, halting ATP production and eventually killing the cell within minutes (44) (Figure 6). Research has shown that atovaquone can also act as an anti-tumor agent (45). It does this the same way as in the *P. falciparum*, but by inhibiting the cytochrome bc_1 complex in tumor cells, slowing down oxidative phosphorylation, eventually leading them to apoptosis.

There has been little known toxicity for atovaquone, as there have been cases of overdoses of 31500mg atovaquone with few side effects (43). Even though there has been reported a link to the use of atovaquone and hepatoxicity, atovaquone is found to be inert in liver microsomes (43). This side effect is very rare and could also have been a side effect of proguanil use (46). This makes atovaquone quite tolerable in humans. The foremost reason for atovaquones' low toxicity is that it does increase ROS formation in human mitochondria, but the overall mitochondrial respiration is not increased significantly (45).

Mitomycin C

Mitomycin C is used as an anti-cancer drug for various cancers such as adenocarcinomas in the stomach and pancreas (47). It is originally isolated from the *Streptomyces caespitosus* (48). It has quite a complex chemical containing a quinone structure and also contains an aziridine group (Figure 13).

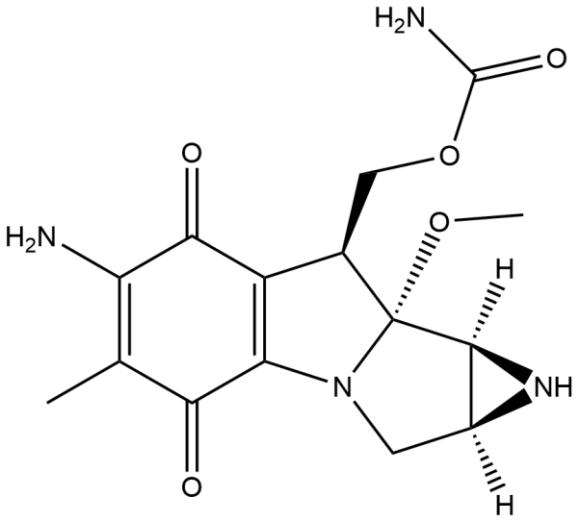

Figure 13. Chemical structure of mitomycin C.

It is an alkylating agent which crosslinks DNA, causing apoptosis of tumor cells. For mitomycin C to be active, the molecule needs to be either enzymatically or chemically reduced into its semiquinone form. Subsequently, this will activate its aziridine ring, which causes most of the alkylation (49).

Let us discuss the mechanism. First, a double enzymatic reduction takes place, which converts the quinone to a semiquinone followed by hydroquinone. Then, under the loss of methanol, the nitrogen in the indole group gets a positive charge. Hydrogen is then lost which is then used to convert the hydroquinone back to a semiquinone, opening the aziridine ring. The now open aziridine ring is then susceptible to a nucleophilic attack according to the Michael reaction (see Michael reaction), using the hydrogen to stabilize back to hydroquinone. After the leaving of $OCONH_2$, another nucleophilic attack on the DNA happens, which doubly crosslinks the DNA to mitomycin C (18) (Figure 14).

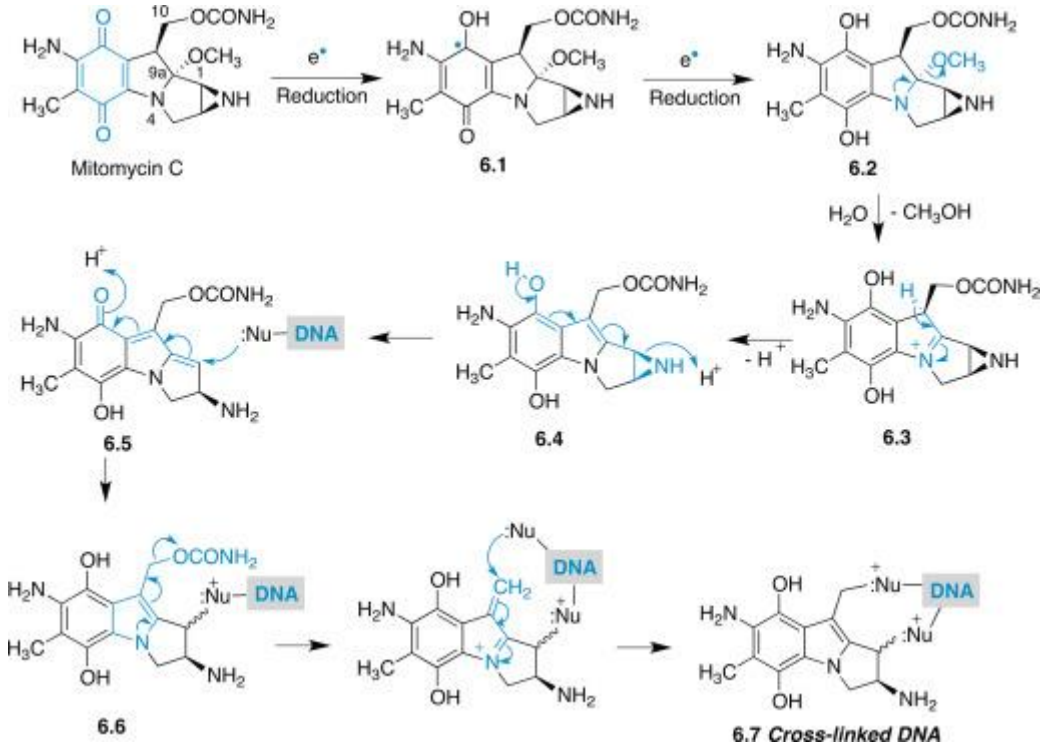

Figure 14. the mechanism of action of mitomycin C (19).

The reduction to a semiquinone is responsible for its mode of action, but can we assume this mechanism is also responsible for its toxicity? As stated earlier, semiquinones are very reactive and generate ROS, which causes all kinds of cell damage. Next to that, mitomycin C is not completely selective for tumor cells, so cell damage of other cells is to be expected. Main adverse effects include thrombocytopenia and leukopenia (49), but pulmonary events are also quite common such as interstitial pneumonia (50). These are all common side effects of non-selective anti-tumor drugs, as they do not distinguish tumor cells from non-tumor cells (51).

What can we learn from mitomycin C? For starters, it seems that quinones are often used as an antitumor agent. These come with various side effects, which are explained by their innate cytostatic function. Next to that, the quinone substructure is used as part of its reactivity, as it first undergoes enzymatic reduction and performs a Michael addition. These two mechanisms are both nonspecific mechanisms that cause promiscuity in PAINS.

Reduction potentials of quinones

We found that the reduction potential of the quinones plays a vital role in their ability to redox cycle. The low toxicity quinones have the following redox potentials: -0,163V for coenzyme Q, -0,260V for menaquinone and phylloquinone in water versus a normal hydrogen electrode (NHE) (52). Doxorubicin was found to have a lower redox potential of -0,32V (53) with NHE. It becomes a challenge to put the redox potentials of the other drugs into perspective because different studies use different voltammetric techniques and electrodes, and none were found that are based on NHE. We can estimate where it should be compared to the others.

Atovaquone also has a relatively low redox potential (-0,51V) compared to menadione (vitamin K3, -0,14V), and is attributed to its relatively low activity towards the cytochrome complex (54). Unfortunately, is not possible to put atovaquone in perspective because no reduction potential versus HSE was found of menadione.

Another good example is that we have found that mitoxantrone has a reduction potential of -0,79V and doxorubicin 0,60V measured in a cyclic voltammogram using an Ag/AgCl reference (41). Additionally, this research suggests that the cytochrome bc1 complex has more affinity towards quinones that have a redox potential within a certain range, mitoxantrone being outside this range while doxorubicin is in this range. This is a very important distinction to be made, as it would give a stricter definition of the probable reactivity of quinones.

If we look at the differences between non substituted benzoquinone, 1,4-naphthoquinone and 9,10-anthraquinone we find that benzoquinone has a redox potential of 711mV, 1,4-naphthoquinone has 493mV and 9,10-anthraquinone has a reduction potential of 155mV (55). These are measured in alcohol and are not comparable with the reduction potentials of the approved quinone-based drugs. This could suggest that substituted anthraquinones and naphthoquinones will also have a lower redox potential than quinones, which could be the reason that drugs such as doxorubicin and mitoxantrone have relatively low potentials compared to coenzyme Q.

Online databases and definition of a new repository

Multiple online chemical databases exist, such as the ZINC15 and the ChEMBL database. These databases are public databases that contain chemical data about lots of compounds. They will also tell you if the compound flags a PAIN filter. The ZINC15 database only contains the original PAIN filters by Baell & Holloway and has created their own alerts for reactivity, but the ChEMBL database has implemented multiple different filters (Lilly, PAIN, Dundee, Glaxo). Another PAIN database is BADAPPLE, which is not based on substructures but on analysis of molecular scaffolds (56). It gives a certain score that indicates a predicted likeliness of nonspecific binding. The scores go from 0 to 300+, with higher scores the likelihood of promiscuity increases. The question remains: how would a new online repository for PAINS be defined?

Firstly, it would need to contain the PAIN structural filters by Baell and other publicly known filters. These are the foundation of the repository, which is similar to the ChEMBL database. A combination of known filters should be defined, as this would make the repository overarching of all known databases. Also, the BADAPPLE filters should be included as their scoring system is useful. Secondly, a summary of how these PAIN filters are derived should be included in the database (Table 1). For example, the Baell filters are based on 6 AlphaScreen^{TM} HTS campaigns, but their exact data is not available to the public. This could give medicinal chemists information to take their PAIN filters with a grain of salt, or not depending on their interpretation. Also, the enrichment factor should be included because it gives a solid basis for the validity of the PAIN alert. Thirdly, it should contain available structure-activity relation (SAR) data of the compound. It would also be useful to show SAR data of structurally similar compounds. Structural similarity can be calculated by the Tanimoto coefficient. Baell & Holloway used this coefficient to exclude compounds with more than 85% similarity (12) from their chemical library. This seems a good threshold for structural similarity. Both these things will increase information to the medicinal chemist and can aid them in making their decision to optimize the compound. This SAR data can be pulled from the PubChem BioAssay database using PubPy or similar extensions (57). The input of a compound can be in SMILES notation. This notation represents compounds as strings of characters and makes the computable comparison between compounds easy. See Figure 15 for an overview.

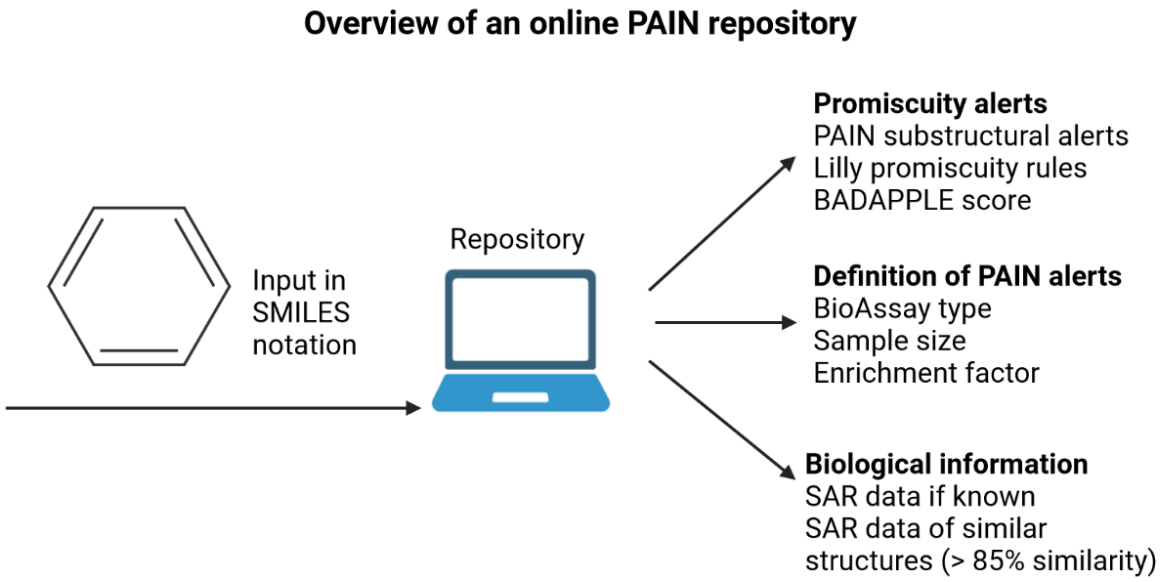

Figure 15: an overview of a new online repository for PAINS, created with BioRender.

Analysis

In this part of the report, we will analyze our findings and answer the research question. To answer the question: 'what are the prospects of quinone derivatives in drug discovery?' we must first go back to our findings. We will first justify the quinone_A filter.

Justification of the quinone_A filter

We found that multiple PAIN filters see quinones as problematic. These filters are justified, as they are based on the measurements across multiple assays. Also, the quinone filters have very high enrichment factors. This means that if a positive screening hit contains a quinone substructure, you should always tread very carefully if you want to pursue this hit further. It is of utmost importance to perform counter-screens against unrelated biological targets to validate this hit as being biologically active and not being active by nonspecific binding. The hit should be further validated by SAR measurement against the target. One should realize that this is the next step in drug discovery anyways, after a positive hit. This validation would root out false positives, but it would be avoided entirely if the PAIN filters were more accurate. Also, it is not favorable to always do this due to monetary and time restraints. The question remains: is this worth it for quinone derivatives?

Analysis of the low toxicity quinones

We have found that vitamin K analogs and coenzyme Q have very low toxicity. This, compared to the other approved quinone-containing drugs, is quite interesting. It would seem at first, that naturally occurring quinones are not as reactive as synthetic quinones. That point of view is not correct as mitomycin C and daunorubicin also come from natural sources (*Streptomyces)*, contain a quinone structure but are extremely reactive and toxic. One could argue that endogenous human and plant-based quinones are relatively safe, but this would most likely not be true as only one species of plant-based quinones was examined. Also, the opposite is

observed, as atovaquone is deemed to be relatively safe and is completely synthetic. The tolerability could be attributed to the fact that it is a coenzyme Q analog and is, therefore, less toxic by nature although there are no findings that confirm nor dispute this argument. Vitamin K analogs and coenzyme Q have relatively low redox potentials compared to doxorubicin, which might be the reason for their low toxicity by ROS formation.

Looking at the chemical structures of phylloquinone and coenzyme Q, multiple things immediately catch the eye. Firstly, both molecules have a large hydrophobic tail. Secondly, both quinones have a methyl group at position 2 of the ring. Thirdly, phylloquinone has a 1,4-substituted phenyl on positions 5 and 6, whereas coenzyme Q has two methoxy groups. These functional groups are responsible for changes in the reactivity of a quinone. Looking at atovaquone, we see it is also a 1,4-naphthoquinone like phylloquinone. It has a hydroxyl at position 2 and a relatively large tail. The methoxy groups on coenzyme Q are electron-donating by resonance, and withdrawing by induction, resulting in a weak electron-donating effect. The 1,4-naphthoquinone phylloquinone also receives electrons by its neighboring benzene through resonance. The methyl group is also weakly electron-donating by induction. Comparing the toxic mitoxantrone to coenzyme Q, the immediate difference that stands out is that mitoxantrone is an anthraquinone. The quinone structure contains two substituted benzene groups. Also, the anthraquinone structure is substituted with nitrogen, which is electron-withdrawing. This strong withdrawing effect by induction and resonance might pull electrons away from the quinone reaction core affecting its reactivity.

Although it seems that these quinones with low reduction potential and low toxicity contain mostly electron-donating groups, it is futile to analyze this by hand as there are too many factors to consider that are not discussed here. Although we anticipated that there would be a clear pattern that showed if we compared functional groups of approved drugs, this was not the case. It is a challenge to specifically characterize each functional groups' electron influence by hand because the exact extent is based on two different effects. The electron effect on the σ-bond is not well comparable to π-effects.

Further research on substituent effects

For further research, the effect of functional groups on quinones should be studied. It might be useful to derive Hammett parameters of functional groups on substituted quinones using molecular electrostatic potential analysis, for example (58). This would give each functional group a score based on their electrostatic potential and allows additional statistical analysis to be performed. This could allow a correlation to be found between reactivity and this score. This could also be extended to other properties like reduction potential.

Another similar direction could be to study the differences between quinones that are active in bioassays versus quinones that are inactive. Statistical analysis should be performed that looks at the differences in functional groups between a large number of different quinones. This data could be retrieved from PubChem's Bioassay database. Perhaps a trend can be found between the presence of certain functional groups or a combination of multiple and assay activity. Should this generate significant results, certain rules can be established that would characterize quinones to be more likely promiscuous or non-promiscuous. The rules would place more value on intermolecular variability. Also, this kind of research can be performed on not just the quinone_A filter but on any substructure that can have substituents.

Both these approaches would be able to extend the existing PAIN rules by taking functional groups into account, be it via electrostatic potential or statistical analysis of substituents.

Perhaps with these methods, a more accurate promiscuity filter can be created to filter out false positives.

Analysis of the approved drugs

As quinones show high reactivity using multiple mechanisms, their propensity for nonspecific binding is very high. When we look at approved drugs that do contain quinones, we see that their toxicity is also quite high and is based on these mechanisms. For example, doxorubicin's cardiotoxicity is mostly a side product of its capacity to redox cycle thereby generating ROS.

Furthermore, the drugs that are approved fall in only a couple of classes with very specific activities. With the anti-tumor agents, their entire mechanism of action works by generating ROS and by DNA intercalation, which are the two mechanisms that make quinones problematic in HTS screening. The antimalarial agent atovaquone, however, works by targeting quinone-specific binding places on the parasitic enzyme. The vitamin K analogs are active in posttranslational protein modification. The last two drugs influence cell functions that endogenously require quinones. It is, therefore, only logical that if an HTS campaign is performed against these kinds of targets, quinones are the drugs you want to look for.

Reduction potentials of quinones

An important factor is the reduction potential of discovered quinones. A lower redox potential corresponds to lower binding to oxidoreductases that contribute to redox cycling, as we see in mitoxantrone. It might therefore be useful to be wary of this when finding a quinone hit. As this property corresponds directly to its ability to redox cycle, binding to the screening target might be a true positive depending on the value of the redox potential. This does create challenges though because redox potential is measured and there has yet to be a completely accurate way to compute this. Successful research has been performed that tries to appropriate these potentials by computation, although only relatively small organic molecules were studied (59). It would be of great value if reasonably accurate reduction potentials could be approached using this method, as knowing this would decrease potential toxicity. One could hypothesize that decreasing the reduction potential of a quinone-based drug will decrease redox cycling and its' additional toxicity and the drug will therefore rely on its' ability to intercalate as its main pathway of toxicity.

As seen in mitoxantrone and atovaquone, there is relatively low ROS formation in human mitochondria. Mitoxantrone has reasonable side effects, however, while atovaquone has no severe side effects. Also, it seems that ROS formation is responsible for the heaviest toxicities when comparing doxorubicin with mitoxantrone. Chemically, this makes sense because redox cycling continues until the oxidoreductase has exhausted oxygen. Decreasing the reduction potential would most likely decrease the efficacy of the drugs if its mechanism is based on redox cycling. Also, anthraquinones have the lowest reduction potential of the three discussed quinone forms, benzoquinone having the highest. This suggests loosely that anthraquinones reduce less quickly than benzoquinones and 1,4-naphthoquinones. This seems counter-intuitive as doxorubicin has high redox capacity with ROS formation compared to coenzyme Q, for example. The truth of the matter is that reduced coenzyme Q (ubiquinol) is immediately quenched by antioxidants in the mitochondria or is oxidized back to coenzyme Q as part of its mechanism which stops the usual formation of ROS. Additionally, it becomes a challenge to compare the two because they are chemically so different since they both have different substitutions.

Furthermore, no good comparison of the drugs could be made regarding redox potential since there were no studies that used the same voltammetric technique.

Prospects of quinone derivatives

In the future, it could be likely that there will be more quinone-based anti-tumor agents, as they are very effective, and their entire efficacy relies on their quinone structure. Medicinal chemists must, however, be very wary about its redox potential as seems to be a reasonable measure of the amount of ROS formation.

The nontoxicity of vitamin K and coenzyme Q suggests that analogs can be developed rather safely. But, as they are well tolerated and very effective treatments for deficiencies, it seems that there is no big incentive for the development of new analogs. Also, we did not investigate vitamin K analogs that did not pass clinical trials. Atovaquone is also a potent antimalarial with few side effects so quinone-based antimalarials would likely not have added value.

Although it seems that there are very specific exceptions as discussed above, benzoquinones are still very promiscuous as observed in the Baell and Lilly research. They should therefore mostly be disregarded unless clear SAR validation has been performed.

Shortcomings and limitations

For the justification of the quinone_A filter, we looked at two different filters and their foundation. We only went briefly over the assay type that these studies used, and we could have looked at the difference in assays and the emergence of false positives of quinones. We know that there are lots of factors accounting for reactivity, such as the assay type but also the molecular environment the assay contained. Perhaps an assay could be developed that is relatively innate to the quinones reactivity. Such an assay would have to contain no nucleophiles and would not have a capacity for redox reactions. If such an assay can be created, a much more accurate screen can be performed which only accounts for immediate reactivity with a biological target.

To extend the quinone_A filter, we mostly looked at the redox capacities of quinones as the basis of their reactivity. We did not look concretely at functional groups that could affect their ability to react as Michael acceptors, although it is very likely that functional groups do influence this as they can make the quinone more electrophilic. Also, we did not look at functional groups that add steric hindrance to molecules. This could very well change the reactivity of a quinone as well as change its biological activity with certain enzymes. We did not account for this when researching the approved drugs. Also, the comparison of functional groups was quite limited, as the researcher had only limited organic chemistry knowledge. Besides, we only looked at the currently approved drugs, instead of comparing true positives and false positive hits of an HTS campaign. This would generate more accurate results as the sample size is much bigger and would also more effectively define rules to mitigate false positives. More advanced research into the influence of functional groups needs more knowledge about molecular orbital theory and analysis techniques of these groups. Likewise, it would need a solid bioinformatics background.

Reviewing the prospects of quinone-based drugs, we looked at the mechanisms and toxicology of currently approved drugs. We did not look at quinone-based drugs that are in the development pipeline and the ones that have not gotten approved, however. If this has been done, perhaps other conclusions could be made about the future of the drug class.

Conclusions

We found that the quinone_A filter is justified. Researchers should be very careful optimizing quinones and it is recommended to perform counter screens and validation with SAR research. The definition of a PAIN filter might be extended by accounting for functional groups, and further statistical research should be performed that determines differences between true and false-positive hits. Further research should also be done to statistically analyze the effects of functional groups on the reduction potential of quinones. Moreover, benzoquinones have strong anticancer properties, based on their nonspecific binding mechanism, that might make them useful for progression although their toxicity is troublesome. Electron chain active quinones and vitamin K analogs have low toxicity and high efficacy and could therefore be pursued further if incentive allows it. It can be concluded with reasonable confidence that most benzoquinone HTS hits should be disregarded unless validated with counter-screens.

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