



## Azoreductases in drug metabolism

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## Azoreductases in drug metabolism

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### Abstract

Azoreductases are flavoenzymes that have been characterised in a range of prokaryotes and eukaryotes. Bacterial azoreductases are associated with the activation of two classes of drug, azo drugs for the treatment of inflammatory bowel disease and nitrofurans antibiotics. The mechanism of reduction of azo compounds is presented that requires tautomerisation of the azo compound to a quinoneimine and provides a unifying mechanism for the reduction of azo and quinone substrates by azoreductase. The importance of further work in characterisation of azoreductases from enteric bacteria is highlighted to aid in the development of novel drugs for the treatment of colon related disorders.

Human azoreductases are known to play a crucial role in the metabolism of a number of quinone containing cancer chemotherapeutic drugs. The mechanism of hydride transfer to quinones, which is shared not only between eukaryotic and prokaryotic azoreductases but the wider family of NAD(P)H quinone oxidoreductases, is outlined. The importance of common SNPs in human azoreductases is described not only in cancer prognosis but also due to their effects on the efficacy of quinone drug based cancer chemotherapeutic regimens. This highlights the need to screen patients for azoreductase SNPs ahead of treatment with these regimens.

### Non-approved abbreviations

5-ASA: 5-aminosalicylate, AcpD: acyl carrier protein phosphodiesterase, ecAzoR: *E. coli* azoreductase, FAD: flavin adenine dinucleotide, FMN: Flavin mononucleotide, hNQO1/2: human NAD(P)H quinone oxidoreductase 1 and 2, IBD: inflammatory bowel disease, MdaB: Modulator of drug activity B, NAD(P)H: Nicotinamide adenine dinucleotide (phosphate) reduced, NfsB: Nitrofurazone sensitivity protein B, NRH:(Wu *et al.*, 1997) dihydronicotinamide riboside, paAzoR: *P. aeruginosa* azoreductase, ppAzoR: *Pseudomonas putida* azoreductase, WrbA: Tryptophan repressor binding protein A

## Introduction to azoreductases

Azoreductases are a group of diverse enzymes found in many bacterial and eukaryotic organisms (Fig 1 (Ryan *et al.*, 2014)). The azoreductases discussed in this review are flavin-dependent (typically FMN) enzymes that are able to reductively cleave compounds containing an azo bond. Azo compounds are defined as those that contain an  $R_1-N=N-R_2$  group, where  $R_1$  and  $R_2$  are typically aromatic groups. Azoreductases are primarily cytosolic enzymes, however they have been shown to be secreted during exposure of bacteria to azo dyes (Morrison *et al.*, 2015). The physiological role of these enzymes in the bacteria remains unclear, however they are constitutively expressed during growth *in vitro* (Chen *et al.*, 2005; Wang *et al.*, 2007) which suggests a role in homeostasis. The majority of azoreductase research is focused on their use in bioremediation where they can be used for the treatment of waste water contaminated with azo dyes from the textile and cosmetics industries (Singh *et al.*, 2015). This review is unique in focusing on their role in the activation of several classes of drug.

To date the majority of studies on azoreductases have been performed on enzymes from aerobic bacteria (Crescente *et al.*, 2016; Nakanishi *et al.*, 2001), in contrast only a single azoreductase has been characterised from a strict anaerobe (Morrison *et al.*, 2012). Several species of gut bacteria have been identified to have azoreductase activities (Wang *et al.*, 2004) however studies on individual azoreductases from enteric bacteria are very limited. Due to the anaerobic environment of the gut it will be important to improve our understanding of the azoreductases which predominate in these bacteria in order to help design novel drugs.

Reduction of substrates by azoreductases requires the use of either NADH or NADPH as an electron donor in a bi-bi ping pong mechanism (Binter *et al.*, 2009; Nakanishi *et al.*, 2001; Wang *et al.*, 2010). Reduction is an obligate two electron process where a hydride is transferred from NAD(P)H to FMN and then on to the second substrate. Reduction of an azo substrate requires two NAD(P)H per azo substrate (Fig 2A). As well as azo compounds, the enzymes have been shown to reduce a range of other substrates including quinones (Gonçalves *et al.*, 2013; Ryan *et al.*, 2014; Ryan *et al.*, 2010b) and nitroaromatics (Liu *et al.*, 2007a; Prosser *et al.*, 2013; Ryan *et al.*, 2011). The physiological substrate of azoreductases remains unclear, however the high specific activity of azoreductases when reducing quinones (Ryan *et al.*, 2014) and increased survival of *E. coli* overexpressing AzoR during treatment with menadione (Liu *et al.*, 2008), suggest that detoxification of quinones is an important physiological function.

The structures of several bacterial azoreductases have been solved and all share a characteristic homodimeric short-flavodoxin fold (Fig 2B). The active sites of these enzymes are situated at the dimer interface and are formed by residues from both monomers (Fig 2C). One molecule of flavin is bound within each active site and is required for activity. The structure of *Escherichia coli* azoreductase (ecAzor) depicted in Figure 2 was the first to be solved (Ito *et al.*, 2006) and is typical of those whose structures were subsequently solved (figure 3 for examples (Binter *et al.*, 2009; Gonçalves *et al.*, 2013; Liu *et al.*, 2007b; Wang *et al.*, 2007; Yu *et al.*, 2014)). Azoreductases can be encoded by a diverse range of sequences (Fig 1 (Ryan *et al.*, 2014)) as a result there are very few conserved residues in the protein and the ones that are present are thought to provide structural stability (Ryan *et al.*, 2010a). The substrate binding pocket is typically lined by hydrophobic and aromatic residues (Fig 2C). Several of these aromatic residues are part of the  $\beta$ -hairpin that forms

the "lid" of the active site and these have been shown, via mutagenesis, to play an important role in determining the substrate specificity of the enzyme (Wang *et al.*, 2010). The FMN is anchored by a series of sequence independent hydrogen bonds to a structural motif referred to as the FMN binding cradle (Ryan *et al.*, 2010a) which is conserved in both bacterial and eukaryotic azoreductases.

The structures of several azoreductases with bound substrates have been solved and these include with azo compounds (Fig 3 (Gonçalves *et al.*, 2013; Ryan *et al.*, 2010a; Wang *et al.*, 2007; Wang *et al.*, 2010; Yu *et al.*, 2014)), nitroaromatics (Ryan *et al.*, 2011) and quinones (Gonçalves *et al.*, 2013; Ryan *et al.*, 2014). These substrates all lie sandwiched between the isoalloxazine rings of the flavin, with which they form  $\pi$ - $\pi$  stacking interactions, and the aromatic residues of the  $\beta$ -hairpin (Fig 3). In general the substrates of azoreductases do not make many specific hydrophilic interactions explaining the ability of the active site to accommodate a range of hydrophobic substrates. So far no structure has been solved with a nicotinamide cofactor bound to an azoreductase. The structure of azoreductase with bound cibacron blue, a competitive NAD(P)H inhibitor, has been solved (Yu *et al.*, 2014) but this does not give details of binding due to the structural differences between it and NAD(P)H.

### Phylogeny of azoreductases

Defining which enzymes have azoreductase activity is a complex matter. In 2014 a phylogenetic tree was published that brought together all of the characterised flavin-dependent azoreductases and a number of related NAD(P)H quinone oxidoreductases (adapted in Fig. 1 (Ryan *et al.*, 2014)). Using this tree three distinct classes of characterised azoreductases can be defined. Class 1 is the most widespread and studied class of bacterial azoreductases. These enzymes have, since the sequencing of the first bacterial genomes been misidentified as acyl carrier protein phosphodiesterases (AcpD). Although several of these enzymes have been tested none have been shown to have AcpD activity (Nakanishi *et al.*, 2001; Wang *et al.*, 2007). In figure 1, class 1 azoreductases include *P. aeruginosa* azoreductases 1-3 (paAzoR1-3 (Ryan *et al.*, 2010a; Wang *et al.*, 2007)), *Enterococcus faecalis* azoreductase (Chen *et al.*, 2004) and ecAzoR (Nakanishi *et al.*, 2001). All members of this class share at least 30-40% sequence identity and are readily identifiable in most bacterial genomes. Class 2 bacterial azoreductases are much less common in bacterial genomes and share no significant sequence identity with class 1 enzymes. Class 2 enzymes do however share the same enzymatic activities and overall fold as enzymes from class 1 (Binter *et al.*, 2009). In figure 1, class 2 are represented by azoreductases from *Bacillus subtilis* (Binter *et al.*, 2009) and *Rhodobacter sphaeroides* (Bin *et al.*, 2004). Class 3 enzymes are the mammalian azoreductases including hNQO1 and hNQO2 that will be discussed in subsequent sections.

The reason for inclusion of the NAD(P)H quinone oxidoreductases in figure 1 was that a novel mechanism for azoreduction was proposed (see below (Ryan *et al.*, 2010a)) which suggested that the quinone and azo reduction shared the same mechanism in these enzymes (Ryan *et al.*, 2010b). To support this enzymes were identified in *P. aeruginosa* from four families (ArsH, modulator of drug activity B, WrbA and YieF) which are characterised NAD(P)H quinone oxidoreductases in other bacteria (Ryan *et al.*, 2014). The enzymes identified in *P. aeruginosa* were cloned expressed and characterised and all except WrbA (the most distantly related homologue) showed azoreductase activity (Crescente *et al.*, 2016). This indicates that the azoreductase family is larger than originally thought and more work is needed to characterise further members.

## Azo drugs

The use of azo compounds as drugs stretches back to the first commercially available antibiotic, prontosil (Sup Fig 1) a sulphonamide prodrug, which was identified in the 1930s (Colebrook *et al.*, 1936) and was later used to identify the first azoreductase (Fouts *et al.*, 1957). Prontosil was among the first drugs to be used to illustrate the importance of the mammalian gut microflora in drug metabolism (Gingell *et al.*, 1971). In modern clinics azo drugs, such as olsalazine, are used to treat inflammatory bowel disease (IBD) and ulcerative colitis (Lautenschlager *et al.*, 2014). Azo drugs for the treatment of IBD are pro-drugs that release the non-steroidal anti-inflammatory 5-aminosalicylate (5-ASA (Makins *et al.*, 2001)) upon reduction. In these prodrugs 5-ASA (Sup Fig 1) is covalently linked via an azo bond to an inert carrier to prevent rapid adsorption from the digestive tract (Haagen Nielsen *et al.*, 1983). These drugs rely upon cleavage of the azo bond by azoreductases secreted by the gut microflora in order to release 5-ASA (Peppercorn *et al.*, 1972).

The use of azo-linked compounds for specific drug delivery to the gut remains an area of great interest. As well as anti-inflammatory compounds a range of other drugs are being targeted using azo chemistry and they include antibiotics (Deka *et al.*, 2015; Kennedy *et al.*, 2011) and anticancer drugs (Plyduang *et al.*, 2014; Sharma *et al.*, 2013). Development continues on new azo bonded carriers for drugs (Kim *et al.*, 2016; Ruiz *et al.*, 2011b) as well as linking pairs of drugs together (Ruiz *et al.*, 2011a). Studies are making use of the azo linkage in new systems such as using azo polymers as a coating material which is degraded to release the drug (Saphier *et al.*, 2010). Other studies investigate the use azo linked nanoparticles to release drugs into the colon (Naeem *et al.*, 2014) or azo containing hydrogels to release olsalazine upon reduction (Li *et al.*, 2010).

## The mechanism of azoreduction

The basic mechanism for azoreduction by all FMN dependent azoreductases is described in figure 2A. In this mechanism N5 of FMN accepts a hydride during oxidation of NAD(P)H and donates it upon reduction of substrate (Fig 4). The structures of three azoreductases with azo substrates bound have been solved (Fig 3) and in each the azo bond is not in an optimal position for hydride transfer (distance from N5 of FMN varies 4.8–6.3 Å, transfer distance should be ~3.5 Å). There is unlikely to be a significant shift in position of the substrate as a result of FMN reduction as comparison of ecAzoR in the oxidised and reduced states showed little conformational change to the residues surrounding the active site (Ito *et al.*, 2008). This makes direct transfer of the hydride to the substrate azo bond unlikely and leaves the question of what is the site of electron transfer.

The answer was provided when the structure of balsalazide bound to paAzoR1 was solved (Fig. 3A (Ryan *et al.*, 2010a)). The clue was that although balsalazide should be planar due to its conjugated system the electron density clearly indicated a 50° bend in the molecule at the azo bond. In order to account for this anomaly an alternative explanation was put forward in which the azo compound was in fact in the hydrazo tautomer (Fig. 3B). Tautomerisation introduces an sp<sup>3</sup> hybridized nitrogen into the azo bond which would account for the bend in the electron density. Tautomerisation also means the salicylate ring forms a quinoneimine structure that is in a more optimal position to be reduced and would account for the ability of these enzymes to reduce both quinones and azo compounds (Ryan *et al.*, 2014). As a result a novel mechanism of azoreduction was proposed based

upon reduction of the quinoneimine containing tautomer (Fig. 4). In this mechanism the hydride is transferred to the carbon at position 2 of quinonimine ring of balsalazide as the covalently bonded carboxylate would make the carbon  $\delta^+$ .

Subsequently two structures of azoreductases complexed with their azo substrates (Fig. 3C and 3D) have been published (Gonçalves *et al.*, 2013; Yu *et al.*, 2014) which show the azo compounds in a more planar conformation. Both azo drugs should be able to tautomerise to form a hydrazo tautomer. In both structures a carbon atom from the quinoneimine ring formed via tautomerisation of the azo substrate is in a similar position to carbon 2 of balsalazide and in an optimal distance for hydride transfer (3.4 Å and 3.7 Å) consistent with the mechanism in figure 4.

### Nitrofurans and other nitroaromatic drug activation by azoreductases

The number of drugs that incorporate a nitroaromatic group is relatively small due to their toxicity which stems from their ability to generate ROS via redox cycling via single electron reduction. Four electron reduction of the nitro-group generates a reactive hydroxylamine which can covalently modify either proteins or DNA (Kovacic *et al.*, 2014). Among the most heavily studied nitroaromatic azoreductase substrates are the nitrofurans antibiotics. The most commonly studied nitrofurans are, nitrofurazone which is a topical antibiotic for treating burns (Ungureanu, 2014), and nitrofurantoin (Sup Fig 1) used for treating urinary tract infections (Garau, 2008). Although typically used as antibiotics nitrofurans are also able to kill trypanosomatids (Patterson *et al.*, 2014).

All nitrofurans must be activated via reduction of their nitro group to a reactive hydroxylamine (Whiteway *et al.*, 1998). In bacteria this can be achieved either via dedicated nitroreductases such as NfsB in *E. coli* (Race *et al.*, 2005) or azoreductases (Ryan *et al.*, 2011). The structure of paAzoR1 was solved in complex with nitrofurazone (Fig 5A (Ryan *et al.*, 2011)). The nitro group of nitrofurazone is positioned over the N5 of FMN to allow optimal hydride transfer (3.6 Å – Fig. 5B and C). The nitro group is within hydrogen bonding range (3 Å) of the side chain of Asn99 which would stabilise the reduced form and also a water molecule which could donate a proton required for reduction.

### Mammalian azoreductases

Like bacteria, eukarya including mammals have azoreductases. In humans the azoreductases are referred to as NAD(P)H quinone oxidoreductase 1 and 2 (hNQO1 and hNQO2 (Wu *et al.*, 1997)). Although they share limited sequence identity (<10%) to either class 1 or 2 azoreductases they are able to reduce many of the same classes of substrates via the same bi-bi ping pong mechanism (Wu *et al.*, 1997). Similarly to bacterial azoreductases, hNQO1 and hNQO2 have flavodoxin-like folds and form homodimers in solution (Fig 6A (Faig *et al.*, 2000)). Both hNQO1 and hNQO2 use FAD as a cofactor rather than FMN (Fig 6B). hNQO1, is an important phase II drug metabolising enzyme that is expressed in many tissues throughout the body (Siegel *et al.*, 2000). hNQO1 is overexpressed in many cancers including, lung (Li *et al.*, 2015), breast (Yang *et al.*, 2014) and pancreatic tumours (Lewis *et al.*, 2005). hNQO1 is known to control degradation of a range of proteins by the proteasome including the tumour suppressors p53 (Asher *et al.*, 2001) and p73 (Asher *et al.*, 2005) which is likely to contribute to its role in tumorigenesis. There is also an association between the common (allelic frequency 0.22 in Caucasians and 0.45 in Asian populations (Kelsey *et al.*, 1997)) C609T NQO1 SNP (P187S mutant) and cancer (Fagerholm *et al.*, 2008; Lajin *et al.*, 2013). The P187S

mutation results in a reduced affinity of hNQO1 for FAD and thus reduced enzymic activity and an increased susceptibility to proteolysis (Lienhart *et al.*, 2014).

The role of hNQO2 in cells is less clear than for hNQO1. hNQO2 expression is less widespread than hNQO1 and is mainly found in muscle and kidney (Jaiswal, 1994). hNQO2 is unusual in that it utilises both NADH and NADPH instead using dihydronicotinamide riboside (NRH (Wu *et al.*, 1997)). Like hNQO1, hNQO2 is involved with the regulation of proteasomal degradation of some proteins such as cyclin D1 (Hsieh *et al.*, 2012). hNQO2 has a common SNP (C659T or L47F) that does not affect enzymic activity but makes hNQO2 more susceptible to proteolysis (Megarity *et al.*, 2014). hNQO2 SNPs have been linked to prostate cancer (Mandal *et al.*, 2012), colorectal cancer (Chen *et al.*, 2016) and prognosis in breast cancer (Hubackova *et al.*, 2012). hNQO2 has been associated with the activation of chemotherapeutics (Celli *et al.*, 2006; Jamieson *et al.*, 2011) and hNQO2 SNPs are associated with the cardiotoxicity of the anthraquinone idarubicin (Megias *et al.*, 2015). Both imatinib ( $K_i \sim 40$  nM (Bantscheff *et al.*, 2007)) and chloroquine ( $K_i = 0.6$   $\mu$ M (Kwiek *et al.*, 2004)) are known inhibitors of NQO2 and hNQO2 is likely to be a secondary target for both drugs in the cell.

### Mammalian azoreductases and chemotherapeutic drugs

Azoreductases in bacteria have been shown to have 10-100 fold greater activity against quinones than against azo substrates (Crescente *et al.*, 2016; Ryan *et al.*, 2014). Quinones are highly cytotoxic due to their ability to undergo redox cycling via one or two electron reduction, but can also cause alkylation of cellular protein and DNA (Bolton *et al.*, 2000). As a result quinones are not used as antibiotics however several quinones are either in use as cancer chemotherapeutics e.g. anthracyclines (Hortobagyi, 1997), or in clinical trials e.g.  $\beta$ -lapachone (currently in phase I/II trials for a range of cancers (Li *et al.*, 2014)). Quinones such as Atovaquone are also used for the treatment of malaria (Looareesuwan *et al.*, 1996). Metabolites of several drugs, including etoposide (Smith *et al.*, 2014), famitinib (Xie *et al.*, 2013) and troglitazone (Yamamoto *et al.*, 2002) have been identified as having either quinone or related quinoneimine functional groups that have been linked to their hepatotoxicity. As a result it is important to understand the role of human azoreductases in the cytotoxicity of quinone drugs.

The cytotoxicity of a range of quinone based cancer chemotherapeutics are altered by hNQO1 activity (Table 1). Many of these drugs are cytotoxic as they undergo redox cycling via reduction by NQO1 to their quinol form before oxidation back to the quinone and release of ROS (Docampo *et al.*, 1979). In contrast the reduction of tanespimycin to its quinol makes it a more potent inhibitor of its target Hsp90 (Guo *et al.*, 2005). Although hNQO1 is primarily linked with the toxicity of quinone based chemotherapeutics it has been linked to some nucleoside analogues (Table 1). The reason for the change in resistance to nucleoside analogues is also thought to be linked to their ability to generate ROS (Aresvik *et al.*, 2010) and the antioxidant role played by hNQO1 (Bauer *et al.*, 2012). As a result of the occurrence of both overexpression of hNQO1 and inactivating mutants of the enzyme as causative agents of cancer, this data would suggest that treatment of cancer with chemotherapeutics activated by hNQO1 is best undertaken after genotyping the patient. Alternatively fluorogenic substrates are under development that could circumvent the need for genotyping, significantly speeding up the process (Best *et al.*, 2016; Silvers *et al.*, 2013).

### The mechanism of quinone reduction by hNQO1 and other azoreductases

In order to better understand the mechanism of quinone reduction by flavoproteins the structures of a number of quinones have been solved bound to bacterial (Gonçalves *et al.*, 2013; Ryan *et al.*, 2014) and mammalian azoreductases (Faig *et al.*, 2000) as well as a recent structure *E. coli* tryptophan repressor binding protein A (WrbA (Degtjarik *et al.*, 2016)). There are also structures of several quinones under development as chemotherapeutics complexed with hNQO1 (Faig *et al.*, 2001; Pidugu *et al.*, 2016). EO9 or Apaziquone is a good example of an NQO1 substrate (Walton *et al.*, 1991), it has entered phase 3 clinical trials for the treatment of bladder cancer (Phillips *et al.*, 2013). The structure of EO9 bound to hNQO1 is typical of other quinones bound to bacterial and mammalian azoreductases. In the structure of EO9 bound to hNQO1 the quinone oxygen is 3.6 Å from the N5 of FMN in an ideal location for electron transfer (Fig 6A). A quinone oxygen is similarly positioned in structures of paAzoR1 (3.7 Å (Ryan *et al.*, 2014)) and ppAzoR (3.6 Å (Gonçalves *et al.*, 2013)) bound to anthraquinone-2-sulphonate. Transfer of the hydride to the carbonyl oxygen of the quinone as the mechanism of quinone reduction (Fig 6C) is supported by recent quantum mechanical calculations which were performed on a high resolution structure of WrbA bound to benzoquinone (Degtjarik *et al.*, 2016).

### Mammalian azoreductases and nitroaromatic drugs

As discussed above nitroaromatics are not commonly used in treatment due to their cytotoxic side effects however like quinones there are ongoing efforts to use them for the treatment of intractable diseases a good example of this is the prodrug CB1954 (Sup Fig 1). CB1954 has undergone clinical trials for the treatment of prostate cancer with virally encoded *E. coli* NfsB (Patel *et al.*, 2009) and in a range of tumours with NRH (Middleton *et al.*, 2010). As with nitrofurazone one or other of the nitro groups must be reduced to a hydroxylamine in order to activate the compound. hNQO2 selectively reduces the 4' nitro of CB1954 (Fig 5B (Fu *et al.*, 2005)). Bacterial azoreductases also reduce the CB1954 (Liu *et al.*, 2007a; Prosser *et al.*, 2013) but at both nitro groups equally. The reason for the nitro-group selectivity in hNQO2 is believed to be Asn161 which orients the molecule within the active site (Fig. 5B (AbuKhader *et al.*, 2005)). The 4' nitro group of CB1954 is positioned in close contact with the N5 of FMN (3.5 Å) suggesting the mechanism of reduction is as described for nitrofurazone (Fig 5C).

There is a lot of research targeting difficult to treat infections using nitroaromatic drugs. In neglected tropical diseases such as malaria (Cakmak *et al.*, 2011) and sleeping sickness (Torreele *et al.*, 2010) nitroaromatic compounds are selectively activated by parasite specific nitroreductases. BTZ043 is the first of a novel class of novel nitroaromatic drugs (Lechartier *et al.*, 2012) that are showing promise for the treatment of extensively drug resistant *Mycobacterium tuberculosis* (Pasca *et al.*, 2010) and is now entering phase I clinical trials. As a result it is important to improve our understanding of nitroreduction by the human enzymes to avoid side effects resulting from host specific interactions with these novel classes of drugs.

### Conclusions

Azoreductases are a diverse and adaptable protein family that play an important role in the metabolism of drugs by the gut microflora. In the area of bacterial azoreductases one of the key

challenges remains the characterisation of azoreductases encoded by bacteria found in the natural gut microflora in order to improve the design of novel azo prodrugs for the treatment of not only inflammatory bowel disease but other diseases of the colon.

In recent years the importance of the human azoreductases in the area of cancer chemotherapy is coming to the fore. As increasing numbers of chemotherapy drugs rely on hNQO1 and hNQO2 for toxicity the need to genotype patients for the presence of inactivating mutations must now be considered not only for treatment with quinone based drugs but also nucleoside analogues.

### Competing interests' statement

None

### Figures

#### Figure 1: Phylogenetic tree showing relationships between azoreductases.

Those enzymes in red text are from bacteria, those in blue are from mammals and those in green from plants. Class 1 enzymes are boxed with a solid line, Class 2 with a dashed line and Class 3 in a dotted line. \* indicates a characterised azoreductase outside the three defined classes. paYieF, paArsH and paMdaB are azoreductases from *P. aeruginosa*. paWrbA, PA1224, PA1225, and PA4975 are NAD(P)H quinone oxidoreductases from *P. aeruginosa*. bsAzoR, efAzoR and rsAzoR are azoreductases from *Bacillus subtilis*, *Enterococcus faecalis* and *Rhodobacter sphaeroides*. rNQO1 and rNQO2 are rat azoreductases. xaAzoR is a flavin-independent azoreductase from *X. azovorans*. ecMdaB, ecYieF and ecWrbA are NAD(P)H quinone oxidoreductases from *E. coli*. afNQO, pnNQO, tmNQO, pcNQO and atNQO are NAD(P)H quinone oxidoreductases from *Archaeoglobus fulgidus*, *Paracoccus denitrificans*, *Triticum monococcum*, *Phanerochaete chrysosporium* and *Arabidopsis thaliana* respectively. smArsH is an azoreductase from *Sinorhizobium meliloti*. dgFlav and ecFlav are flavodoxins from *Desulfovibrio gigas* and *E. coli* respectively. shNQO, reNQO and erNQO are uncharacterised proteins from *Staphylococcus haemolyticus*, *Ralstonia eutropha* and *Erwinia chrysanthemi*. Adapted from (Ryan *et al.*, 2014).

#### Figure 2: the structure of a typical bacterial azoreductase and its mechanism of azoreduction.

(A) Mechanism for the reduction of an azo drug (olsalazine) by flavin-dependent azoreductases. (B) Characteristic homodimeric flavodoxin fold of ecAzoR, a class 1 bacterial azoreductase, (C) detailed view of the active site of ecAzoR. In (B) and (C) monomers are coloured blue and gold. The FMN with sticks and yellow carbon atoms. In (C) residues surrounding the active site are shown as sticks and labelled. (B) and (C) are based upon the structure of ecAzoR PDB 1V4B (Ito *et al.*, 2006) and were generated in CCP4MG (McNicholas *et al.*, 2011).

#### Fig 3: the binding of azo substrates to azoreductases.

(A) The structure of balsalazide bound to paAzoR1. (B) The tautomeric forms of balsalazide that occur in solution. (C) The structure of Orange I bound to AzrC from *Bacillus* sp 29. (D) The structure of reactive black 5 bound to *Pseudomonas putida* azoreductase (ppAzoR). The colouring is as per figure 2. The structures are based upon PDBs (A) 3LT5 (Ryan *et al.*, 2010a), (C) 3W79 (Yu *et al.*, 2014) and (D) 4C14 (Gonçalves *et al.*, 2013).

**Figure 4: Proposed mechanism for the reduction of balsalazide by paAzoR1.**

For simplicity only the isoalloxazine ring of FMN and the nicotinamide group of NADPH are shown.

**Figure 5: Reduction of nitro compounds by azoreductases.**

(A) The structure of nitrofurzone bound to paAzoR1, (B) the structure of CB1954 bound to hNQO2. (C) Proposed mechanism of nitrofurzone reduction in paAzoR1. In (A) and (B) protein colour coding is as in figure 2. For ease of interpretation only one of the two possible binding orientations for nitrofurzone is shown in (A) with grey carbon atoms, while water is shown as a green ball. In (B) CB1954 is shown with turquoise carbon atoms and the 4' nitrate is labelled. These images are based upon PDB files (A) 3R6W (Ryan *et al.*, 2011) and (B) 1X12 (Fu *et al.*, 2005).

**Figure 6: Binding of the chemotherapeutic EO9 to hNQO1**

(A) Overall structure of human hNQO1. (B) Binding of EO9 to hNQO1. (C) Mechanism of hydride transfer during reduction of EO9. Protein colour coding is as per figure 2 and in (B) EO9 has turquoise carbon atoms. (A) and (B) are based upon PDB file 1GG5 (Faig *et al.*, 2001).

**Sup Fig 1: Structures of compounds mentioned in the review.**

Numbered atoms mentioned in the text are labelled.

Drug type	Drug*	Role of NQO1	Tumour type	Citation
Quinone	$\beta$ -lapachone	Activation	Breast	(Glorieux <i>et al.</i> , 2016)
			Lung	(Bey <i>et al.</i> , 2007)
			Skin	(Li <i>et al.</i> , 2014)
			Liver	(Li <i>et al.</i> , 2014; Park <i>et al.</i> , 2014)
			Pancreatic	(Ough <i>et al.</i> , 2005)
	Doxorubicin	Protection	Bile duct	(Zeekpudsa <i>et al.</i> , 2014)
	Epirubicin	Activation	Bladder	(Matsui <i>et al.</i> , 2010)
	Streptonigrin	Activation	Breast	(Fagerholm <i>et al.</i> , 2008)
			Colon	(Beall <i>et al.</i> , 1996)
			Breast	(Dehn <i>et al.</i> , 2003)
	Tanespimycin	Activation	Pancreatic	(Lewis <i>et al.</i> , 2005)
			Brain	(Gaspar <i>et al.</i> , 2009)
			Colon & Ovarian	(Kelland <i>et al.</i> , 1999)
Oesophageal			(Hadley <i>et al.</i> , 2014)	
Nucleoside analogues	Gemcitabine	Protection	Pancreatic	(Siegel <i>et al.</i> , 2011)
			Bile duct	(Buranrat <i>et al.</i> , 2010; Zeekpudsa <i>et al.</i> , 2014)
	5-fluorouracil	Protection	Bile duct	(Zeekpudsa <i>et al.</i> , 2014)
			Gastric	(Peng <i>et al.</i> , 2016)
			Liver	(Sutton <i>et al.</i> , 2015)

**Table 1: hNQO1 and its association with cancer chemotherapy toxicity.**

\*The structures of all drugs are shown in Supplementary Figure 1.

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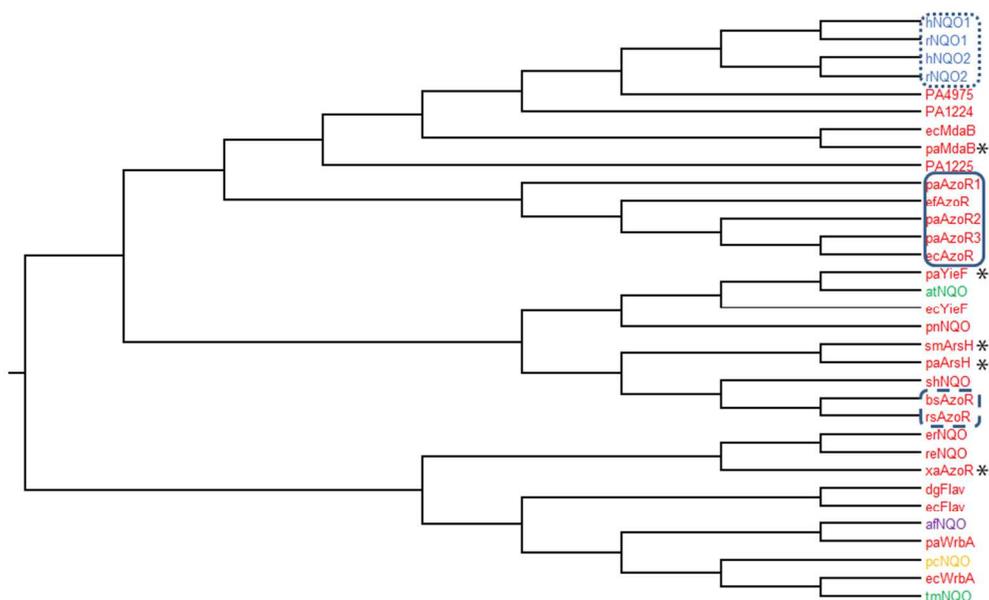


Figure 1: Phylogenetic tree showing relationships between azoreductases.

Those enzymes in red text are from bacteria, those in blue are from mammals and those in green from plants. Class 1 enzymes are boxed with a solid line, Class 2 with a dashed line and Class 3 in a dotted line. \* indicates a characterised azoreductase outside the three defined classes. paYieF, paArSH and paMdaB are azoreductases from *P. aeruginosa*. paWrbA, PA1224, PA1225, and PA4975 are NAD(P)H quinone oxidoreductases from *P. aeruginosa*. bsAzoR, efAzoR and rsAzoR are azoreductases from *Bacillus subtilis*, *Enterococcus faecalis* and *Rhodobacter sphaeroides*. rNQO1 and rNQO2 are rat azoreductases. xaAzoR is a flavin-independent azoreductase from *X. azovorans*. ecMdaB, ecYieF and ecWrbA are NAD(P)H quinone oxidoreductases from *E. coli*. afNQO, pnNQO, tmNQO, pcNQO and atNQO are NAD(P)H quinone oxidoreductases from *Archaeoglobus fulgidus*, *Paracoccus denitrificans*, *Triticum monococcum*, *Phanerochaete chrysosporium* and *Arabidopsis thaliana* respectively. smArSH is an azoreductase from *Sinorhizobium meliloti*. dgFlav and ecFlav are flavodoxins from *Desulfovibrio gigas* and *E. coli* respectively. shNQO, reNQO and erNQO are uncharacterised proteins from *Staphylococcus haemolyticus*, *Ralstonia eutropha* and *Erwinia chrysanthemi*. Adapted from (Ryan et al., 2014).

334x206mm (72 x 72 DPI)

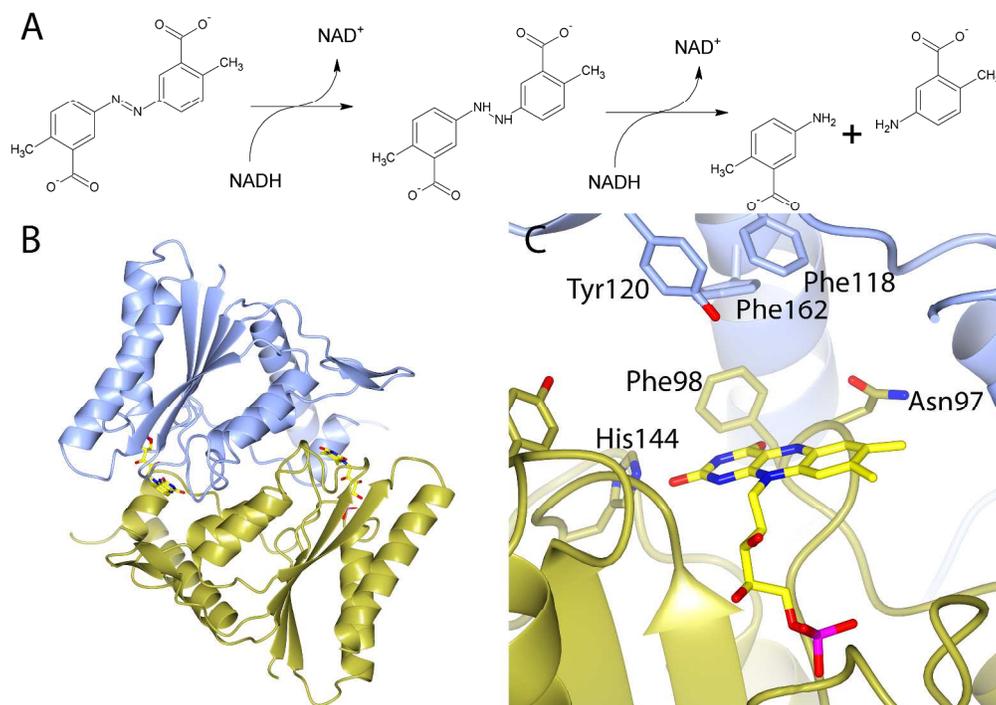


Figure 2: the structure of a typical bacterial azoreductase and its mechanism of azoreduction. (A) Mechanism for the reduction of an azo drug (olsalazine) by flavin-dependent azoreductases. (B) Characteristic homodimeric flavodoxin fold of ecAzoR, a class 1 bacterial azoreductase, (C) detailed view of the active site of ecAzoR. In (B) and (C) monomers are coloured blue and gold. The FMN with sticks and yellow carbon atoms. In (C) residues surrounding the active site are shown as sticks and labelled. (B) and (C) are based upon the structure of ecAzoR PDB 1V4B (Ito et al., 2006) and were generated in CCP4MG (McNicholas et al., 2011).

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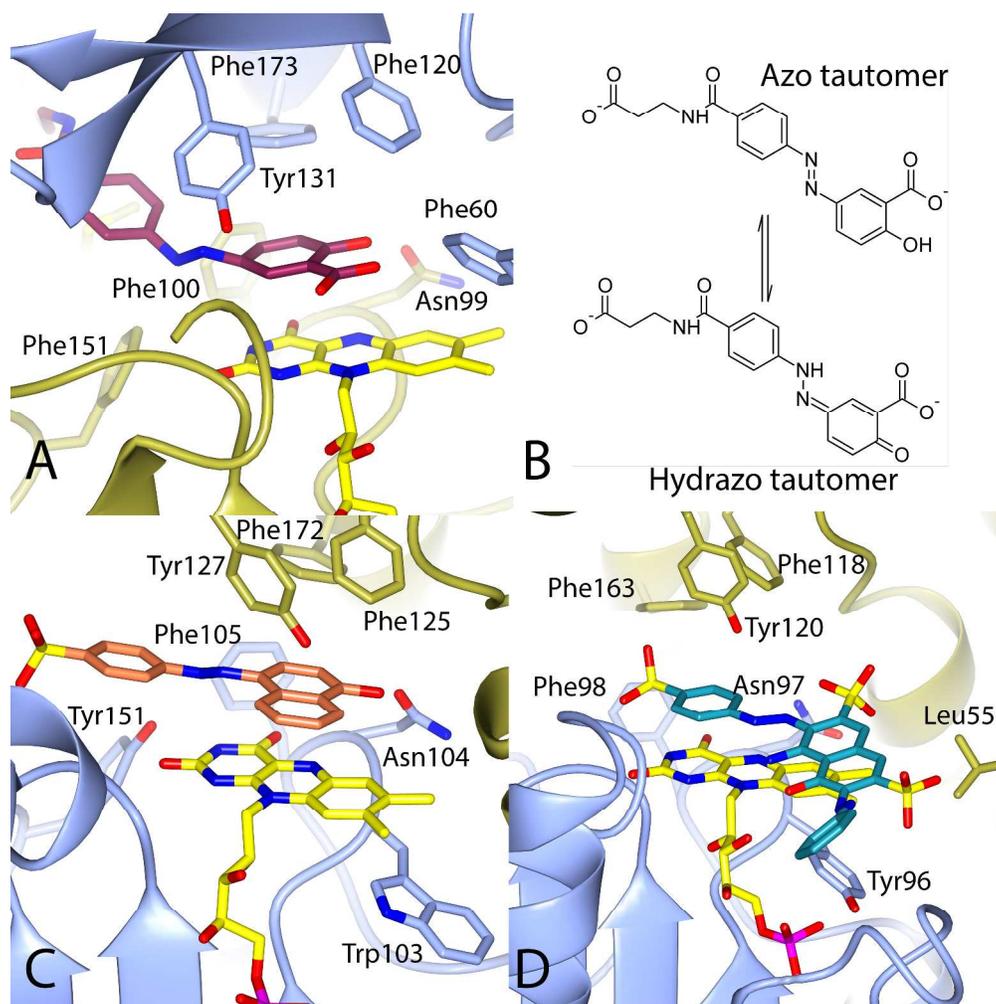


Fig 3: the binding of azo substrates to azoreductases. (A) The structure of balsalazide bound to paAzoR1. (B) The tautomeric forms of balsalazide that occur in solution. (C) The structure of Orange I bound to AzrC from *Bacillus* sp 29. (D) The structure of reactive black 5 bound to ppAzoR. The side chains contributed by each monomer of the azoreductases are coloured a different colour, FMN is in yellow sticks. The structures are based upon PDB codes (A) 3LT5 (Ryan et al., 2010a), (C) 3W79 (Yu et al., 2014) and (D) 4C14 (Gonçalves et al., 2013).

372x372mm (300 x 300 DPI)

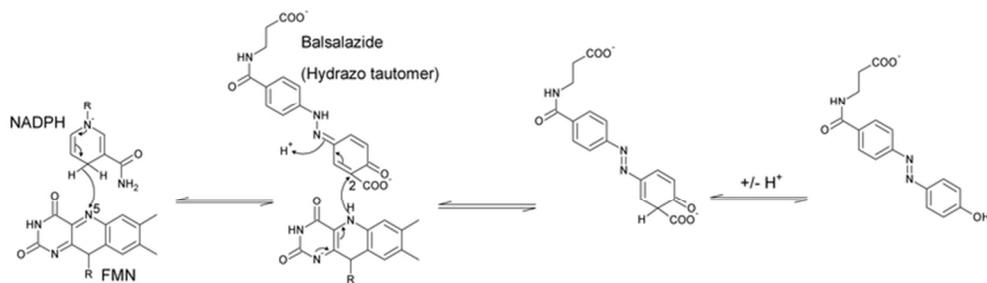


Figure 4: Proposed mechanism for the reduction of balsalazide by paAzoR1. For simplicity only the isoalloxazine ring of FMN and the nicotinamide group of NADPH are shown.

64x18mm (300 x 300 DPI)

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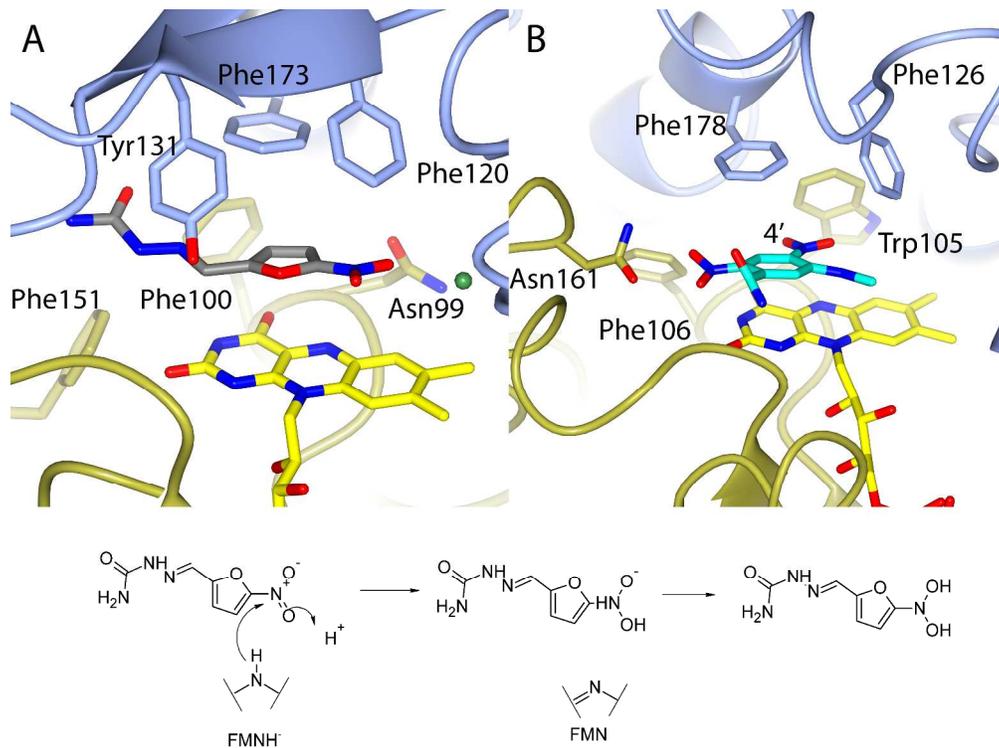


Figure 5: Reduction of nitro compounds by azoreductases.

(A) The structure of nitrofurzone bound to *paAzoR1*, (B) the structure of CB1954 bound to *hNQO2*. (C) Proposed mechanism of nitrofurzone reduction in *paAzoR1*. In (A) and (B) protein colour coding is as in figure 2. For ease of interpretation only one of the two possible binding orientations for nitrofurzone is shown in (A) with grey carbon atoms, while water is shown as a green ball. In (B) CB1954 is shown with turquoise carbon atoms and the 4' nitrate is labelled. These images are based upon PDB files (A) 3R6W (Ryan et al., 2011) and (B) 1X12 (Fu et al., 2005).

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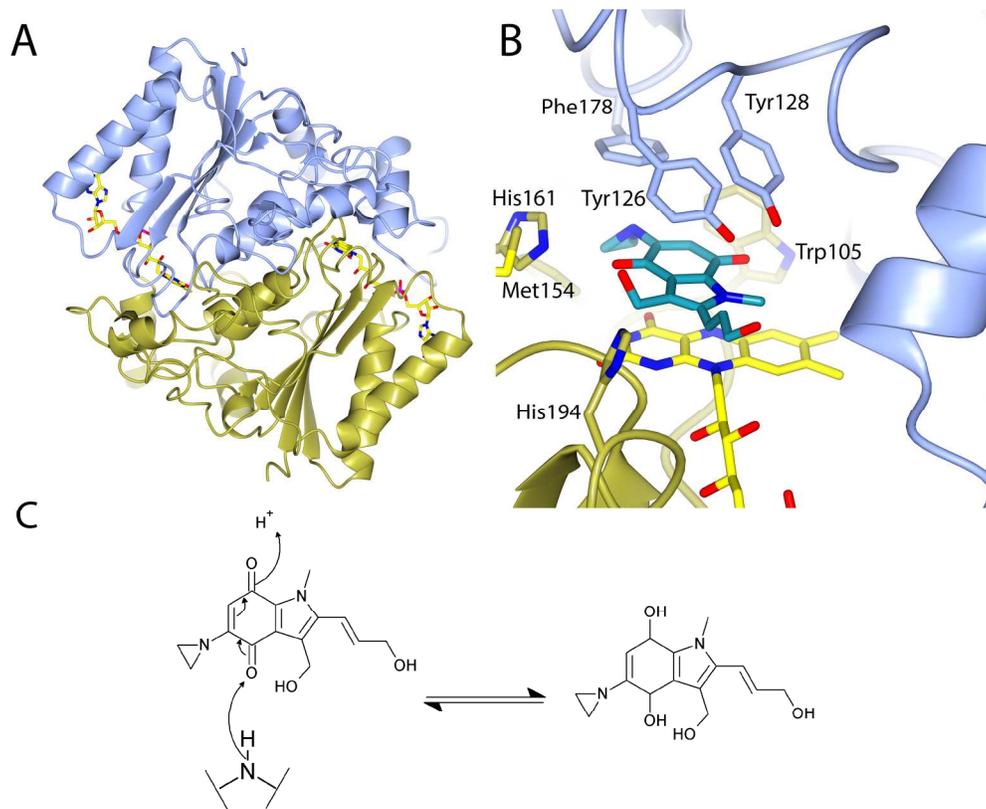
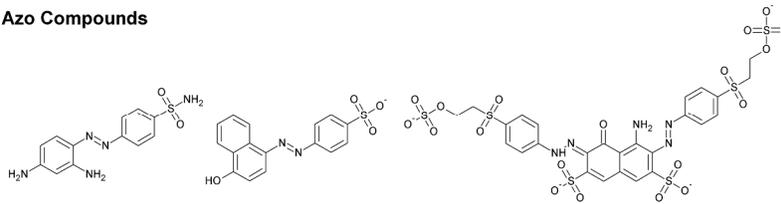


Figure 6: Binding of the chemotherapeutic EO9 to hNQO1  
 (A) Overall structure of human hNQO1. (B) Binding of EO9 to hNQO1. (C) Mechanism of hydride transfer during reduction of EO9. Protein colour coding is as per figure 2 and in (B) EO9 has turquoise carbon atoms. (A) and (B) are based upon PDB file 1GG5 (Faig et al., 2001).

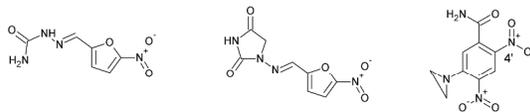
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**Azo Compounds**

Prontosil

Orange I

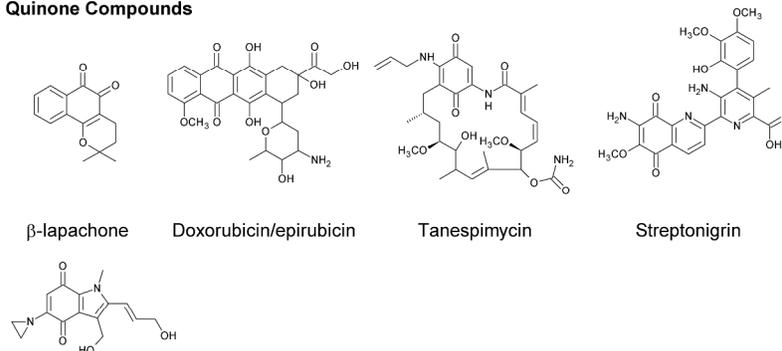
Reactive black 5

**Nitroaromatic Compounds**

Nitrofurazone

Nitrofurantoin

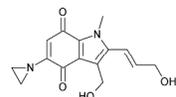
CB1954

**Quinone Compounds** $\beta$ -lapachone

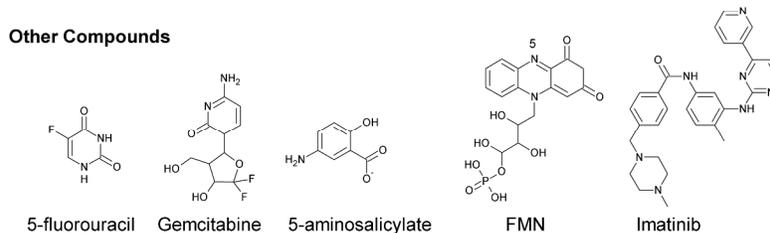
Doxorubicin/epirubicin

Tanespimycin

Streptonigrin



EO9 (Apaziquone)

**Other Compounds**

5-fluorouracil

Gemcitabine

5-aminosalicylate

FMN

Imatinib

274x404mm (300 x 300 DPI)