

# REVIEW ARTICLE

## Azoreductases in drug metabolism

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Azoreductases are flavoenzymes that have been characterized 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 nitrofurantoin antibiotics. The mechanism of reduction of azo compounds is presented; it requires tautomerisation of the azo compound to a quinoneimine and provides a unifying mechanism for the reduction of azo and quinone substrates by azoreductases. The importance of further work in the characterization 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 also the wider family of NAD(P)H quinone oxidoreductases, is outlined. The importance of common single nucleotide polymorphisms (SNPs) in human azoreductases is described not only in cancer prognosis but also with regard 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.

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### Abbreviations

5-ASA, 5-aminosalicylate; AcpD, acyl carrier protein phosphodiesterase; ecAzoR, *Escherichia coli* azoreductase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; hNQO1/2, human NAD(P)H quinone oxidoreductase 1 and 2; IBD, inflammatory bowel disease; NfsB, nitrofurantoin sensitive protein B; NRH, dihydronicotinamide riboside; paAzoR, *Pseudomonas aeruginosa* azoreductase; ppAzoR, *Pseudomonas putida* azoreductase; WrbA, tryptophan repressor binding protein A

## Table of Links

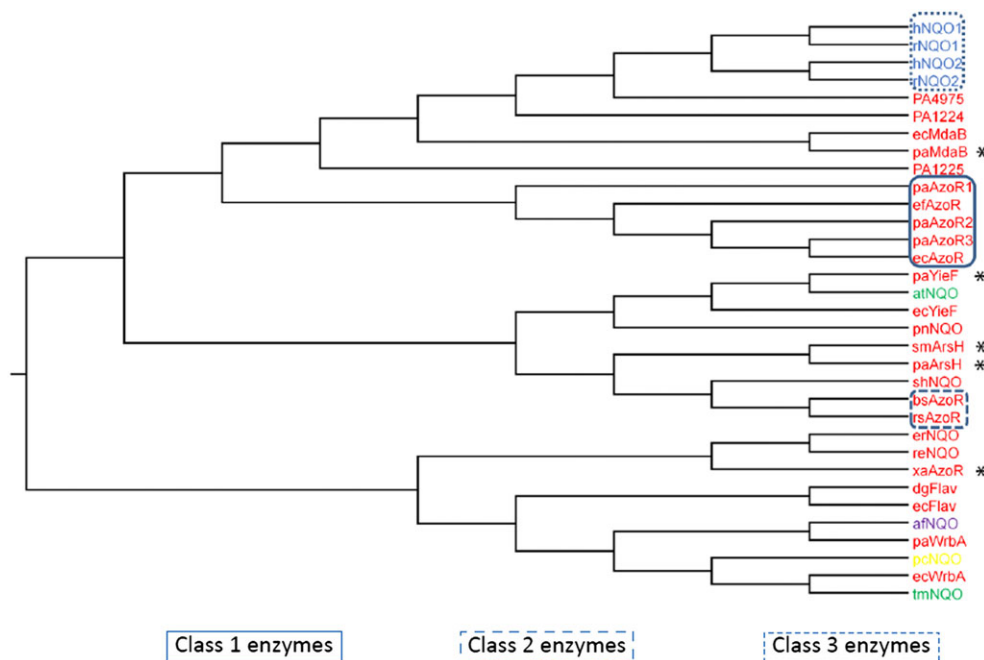
LIGANDS	
5-fluorouracil	Idarubicin
Chloroquine	Imatinib
Doxorubicin	Streptonigrin
Etoposide	Tanespimycin
Famitinib	Troglitazone
Gemcitabine	

This Table lists key ligands in this article, which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016).

## Introduction to azoreductases

Azoreductases are a group of diverse enzymes found in many bacterial and eukaryotic organisms [Figure 1 (Ryan *et al.*, 2014)]. The azoreductases discussed in this review are flavin-dependent [typically flavin mononucleotide (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 and John, 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



## 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. \* Indicates a characterized 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 *B. subtilis*, *E. faecalis* and *R. 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 uncharacterized proteins from *Staphylococcus haemolyticus*, *Ralstonia eutropha* and *Erwinia chrysanthemi*. Adapted from Ryan *et al.* (2014).

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.

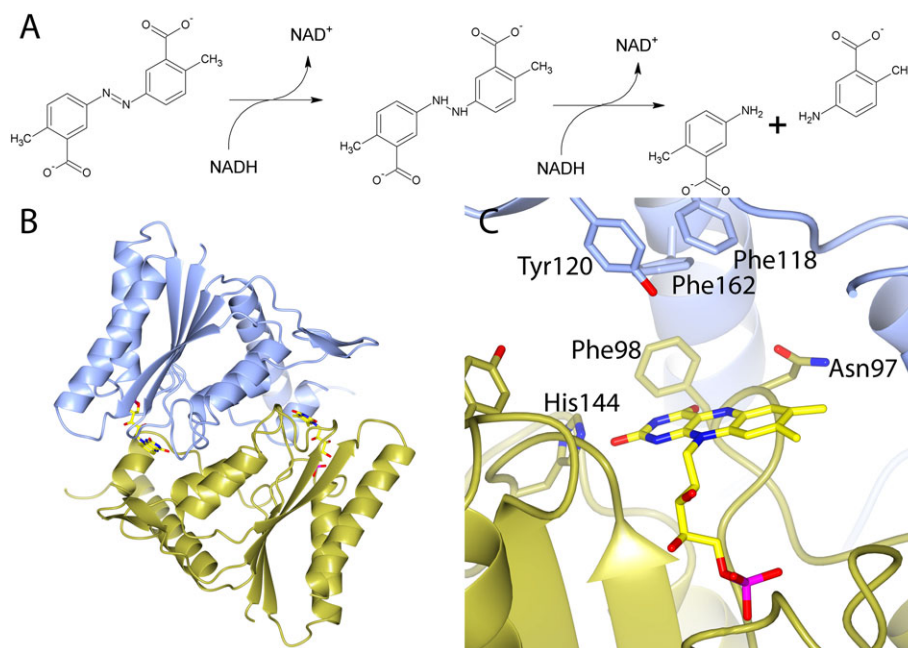
To date, the majority of studies on azoreductases have been performed on enzymes from aerobic bacteria (Nakanishi *et al.*, 2001; Crescente *et al.*, 2016); in contrast, only a single azoreductase from a strict anaerobe has been characterized (Morrison *et al.*, 2012). Several species of gut bacteria 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 is 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 (Nakanishi *et al.*, 2001; Binter *et al.*, 2009; 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. The reduction of an azo substrate requires two NAD(P)H per azo substrate (Figure 2A). As well as azo compounds, the enzymes have been shown to reduce a range of other substrates including quinones (Ryan *et al.*, 2010b; Gonçalves *et al.*, 2013; Ryan *et al.*, 2014) and nitroaromatics (Liu *et al.*, 2007a; Ryan *et al.*, 2011; Prosser *et al.*, 2013). 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 *Escherichia coli* overexpressing AzoR during treatment with menadione (Liu *et al.*,

2008) suggest that detoxification of quinones is an important physiological function.

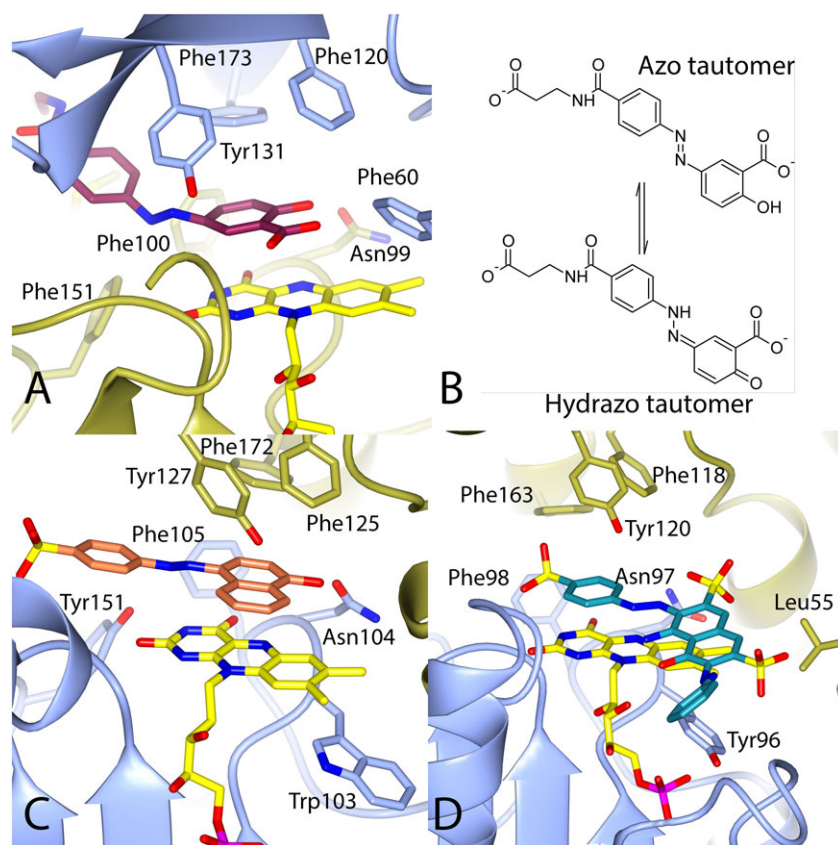
The structures of several bacterial azoreductases have been solved, and all share a characteristic homodimeric short-flavodoxin fold (Figure 2B). The active sites of these enzymes are situated at the dimer interface and are formed by residues from both monomers (Figure 2C). One molecule of flavin is bound within each active site and is required for activity. The structure of *E. coli* azoreductase (ecAzoR) depicted in Figure 2 was the first to be solved (Ito *et al.*, 2006) and is typical of the structures of those azoreductases that were subsequently solved [Figure 3 for examples (Wang *et al.*, 2007; Liu *et al.*, 2007b; Binter *et al.*, 2009; Gonçalves *et al.*, 2013; Yu *et al.*, 2014)]. Azoreductases can be encoded by a diverse range of sequences [Figure 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 (Figure 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 azo



## 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).



### Figure 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 colouring is as in 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).

compounds [Figure 3 (Wang *et al.*, 2007; Wang *et al.*, 2010; Ryan *et al.*, 2010a; Gonçalves *et al.*, 2013; 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 (Figure 3). In general, the substrates of azoreductases do not make many specific hydrophilic interactions, which would explain 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 bound to cibacron blue, a competitive NAD(P)H inhibitor, has been solved (Yu *et al.*, 2014), but this does not provide details of the 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 flavin-dependent azoreductases characterized and a number of related NAD(P)H quinone oxidoreductases [adapted in Figure 1 (Ryan

*et al.*, 2014)]. Using this tree, three distinct classes of 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 *Pseudomonas 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 the 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 and include the human NAD(P)H quinone oxidoreductase 1 and 2 (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 has been 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, tryptophan repressor binding protein A (WrbA) and YieF], which are NAD(P)H quinone oxidoreductases that have been characterized in other bacteria (Ryan *et al.*, 2014). The enzymes identified in *P. aeruginosa* were cloned, expressed and characterized, 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 characterize further members.

## Azo drugs

The use of azo compounds as drugs stretches back to the first commercially available antibiotic, prontosil (Supporting Information 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, upon reduction, release the non-steroidal anti-inflammatory 5-aminosalicylate [5-ASA (Makins and Cowan, 2001)]. In these prodrugs, 5-ASA (Supporting Information Fig. 1) is covalently linked via an azo bond to an inert carrier to prevent rapid adsorption from the digestive tract (Haagen Nielsen and Bondesen, 1983). These drugs rely upon cleavage of the azo bond by azoreductases secreted by the gut microflora in order to release 5-ASA (Peppercorn and Goldman, 1972).

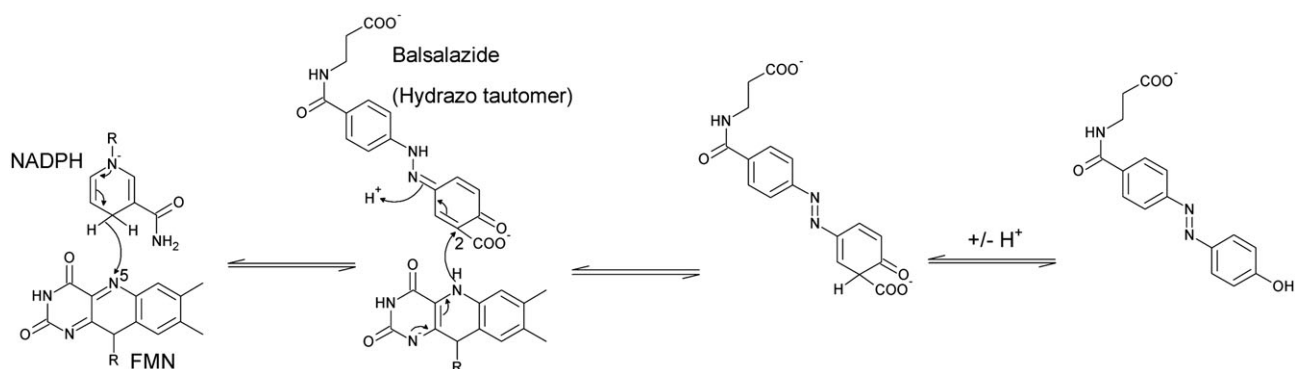
The use of azo-linked compounds for specific drug delivery to the gut remains an area of great interest. As well as anti-inflammatory compounds, azo chemistry has been used to target a range of other drugs and they include antibiotics

(Kennedy *et al.*, 2011; Deka *et al.*, 2015) and anticancer drugs (Sharma *et al.*, 2013; Plyduang *et al.*, 2014). Development continues on new azo-bonded carriers for drugs (Ruiz *et al.*, 2011b; Kim *et al.*, 2016) 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 and Karton, 2010). Other studies are investigating 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 the substrate (Figure 4). The structures of three azoreductases bound to azo substrates have been solved (Figure 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 the position of the substrate as a result of FMN reduction as a comparison of ecAzoR in the oxidized 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 [Figure 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 (Figure 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



**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.



enzymes to reduce both quinones and azo compounds (Ryan *et al.*, 2014). As a result, a novel mechanism of azoreduction was proposed based upon the reduction of the quinoneimine-containing tautomer (Figure 4). With this mechanism, the hydride is transferred to the carbon at position 2 of the quinonimine ring of balsalazide as the covalently bonded carboxylate would make the carbon  $\delta^+$ .

Subsequently, two structures of azoreductases complexed with their azo substrates (Figure 3C and D) 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 at 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

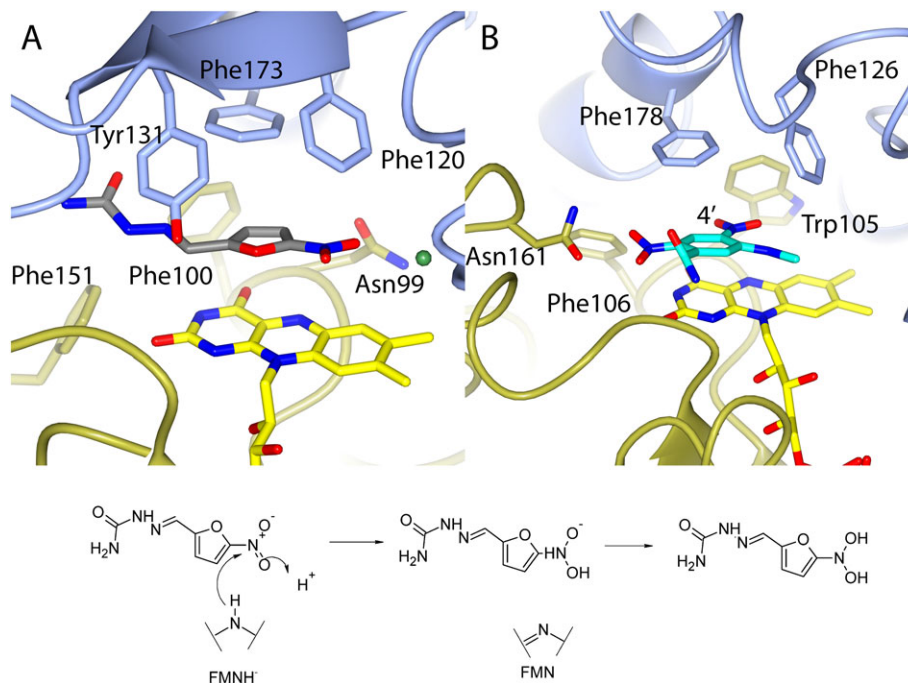
The number of drugs that incorporate a nitroaromatic group is relatively small due to their toxicity, which stems from their ability to generate ROS via redox cycling via single electron reduction. Four electron reduction of the nitro group generates a reactive hydroxylamine, which can covalently modify either proteins or DNA (Kovacic and Somanathan, 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 (Supporting Information Fig. 1), which is used for treating urinary tract infections (Garau, 2008). Although typically used as antibiotics, nitrofurans are also able to kill trypanosomatids (Patterson and Wyllie, 2014).

All nitrofurans must be activated via the 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 nitrofurazone sensitivity protein B (NfsB) in *E. coli* (Race *et al.*, 2005) or azoreductases (Ryan *et al.*, 2011). The structure of paAzoR1 was solved in complex with nitrofurazone [Figure 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, Figure 5B and C). The nitro group is within the hydrogen bonding range (3 Å) of the side chain of Asn<sup>99</sup>, which would stabilize 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 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



**Figure 5**

Reduction of nitro compounds by azoreductases. (A) The structure of nitrofurazone bound to paAzoR1; (B) the structure of CB1954 bound to hNQO2. (C) Proposed mechanism of nitrofurazone 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 nitrofurazone 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).

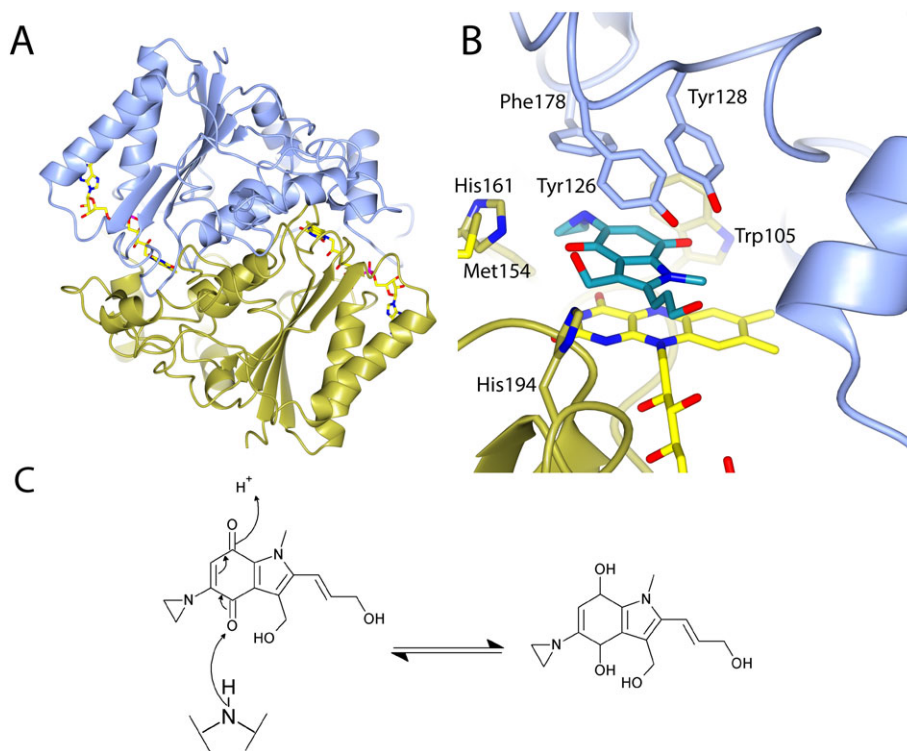
classes of substrates via the same bi-bi ping pong mechanism (Wu *et al.*, 1997). Similarly to bacterial azoreductases, hNQO1 and hNQO2 have flavodoxin-like folds and form homodimers in solution [Figure 6A (Faig *et al.*, 2000)]. Both hNQO1 and hNQO2 use flavin adenine dinucleotide (FAD) as a cofactor rather than FMN (Figure 6B). hNQO1 is an important phase II drug metabolizing enzyme that is expressed in many tissues throughout the body (Siegel and Ross, 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 the 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 tumourigenesis. 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 single nucleotide polymorphism (SNP) (P187S mutant) and cancer (Fagerholm *et al.*, 2008; Lajin and Alachkar, 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 utilizes neither NADH nor NADPH, but instead uses 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 [ $K_i$  ~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- to 100-fold greater activity against quinones than against azo substrates (Ryan *et al.*, 2014; Crescente *et al.*, 2016). 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, for example anthracyclines (Hortobagyi, 1997), or in



**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 the reduction of EO9. Protein colour coding is as in Figure 2 and in (B) EO9 has turquoise carbon atoms. (A) and (B) are based upon PDB file 1GG5 (Faig *et al.*, 2001).

clinical trials, for example  $\beta$ -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 both overexpression of NQO1 and SNPs which inactivate NQO1 are commonly found in tumours we 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 (Silvers *et al.*, 2013; Best *et al.*, 2016).

### *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 bound to bacterial (Gonçalves *et al.*, 2013; Ryan *et al.*, 2014) and mammalian azoreductases (Faig *et al.*, 2000) have been solved, as well as, more recently, the structure of *E. coli* 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 (Figure 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 (Figure 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).

**Table 1**

hNQO1 and its association with cancer chemotherapy toxicity

Drug type	Drug <sup>a</sup>	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)
			Bladder	(Matsui <i>et al.</i> , 2010)
	Epirubicin	Activation	Breast	(Fagerholm <i>et al.</i> , 2008)
			Colon	(Beall <i>et al.</i> , 1996)
	Streptonigrin	Activation	Breast	(Dehn <i>et al.</i> , 2003)
Pancreatic			(Lewis <i>et al.</i> , 2005)	
Tanespimycin	Activation	Brain	(Gaspar <i>et al.</i> , 2009)	
		Colon & Ovarian	(Kelland <i>et al.</i> , 1999)	
		Oesophageal	(Hadley and Hendricks, 2014)	
		Pancreatic	(Siegel <i>et al.</i> , 2011)	
Nucleoside analogues	Gemcitabine	Protection	Bile duct	(Buranrat <i>et al.</i> , 2010; Zeekpudsa <i>et al.</i> , 2014)
			Bile duct	(Zeekpudsa <i>et al.</i> , 2014)
	5-fluorouracil	Protection	Gastric	(Peng <i>et al.</i> , 2016)
			Liver	(Sutton <i>et al.</i> , 2015)

<sup>a</sup>The structures of all drugs are shown in Figure 1.



## 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 (Supporting Information Fig. 1). CB1954 has undergone clinical trials for the treatment of prostate cancer with virally encoded *E. coli* NfsB (Patel *et al.*, 2009). The use of virally encoded *E. coli* NfsB in combination with CB1954 is an example of gene-directed enzyme prodrug therapy, as CB1954 toxicity is reliant upon its activation by *E. coli* NfsB expressed by tumour cells (Williams *et al.*, 2015). CB1954 has also undergone clinical trials 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 [Figure 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 Asn<sup>161</sup>, which orients the molecule within the active site [Figure 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 (Figure 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 (Torreale *et al.*, 2010), nitroaromatic compounds are selectively activated by parasite-specific nitroreductases. BTZ043 is the first of a novel class of 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 characterization 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 IBD but also 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.

## Conflict of interest

The authors declare no conflicts of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.13571>

**Figure S1** Structures of compounds mentioned in the review. Numbered atoms mentioned in the text are labelled.