Activation of nitrofurazone by azoreductases: multiple activities in one enzyme

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Azoreductases are well known for azo pro-drug activation by gut flora. We show that azoreductases have a wider role in drug metabolism than previously thought as they can also reduce and hence activate nitrofurazone. Nitrofurazone, a nitroaromatic drug, is a broad spectrum antibiotic which has until now been considered as activated in bacteria by nitroreductases. The structure of the azoreductase with nitrofurazone bound was solved at 2.08 Å and shows nitrofurazone in an active conformation. Based on the structural information, the kinetics and stoichiometry of nitrofurazone reduction by azoreductase from P. aeruginosa, we propose a mechanism of activation which accounts for the ability of azoreductases to reduce both azo and nitroaromatic drugs. This mode of activation can explain the cytotoxic side-effects of nitrofurazone through human azoreductase homologues.

Azoreductases are a group of NAD(P)H dependent flavoenzymes that have been identified in a range of bacterial species found in the gut including E. coli and P. aeruginosa. Members of this family are also found in humans where they are known as NAD(P)H quinone oxidoreductases. The importance of azoreductases in drug metabolism in the gut is well known for azo drugs used to treat inflammatory bowel disease e.g. balsalazine. Data have shown that these enzymes can reduce a number of other classes of substrate including, quinones and nitroaromatics. As a result of the diverse substrate specificity profile it is suggested that the role of azoreductases in drug metabolism may be wider than originally thought.

Nitroaromatic groups are found in a range of drugs currently on the market including the anti-inflammatory nimuselide and the parkinson’s disease treatment tolcapone. A common feature of some nitroaromatic drugs is hepatotoxicity resulting from reduction of the nitro group. Nitrofurazone was selected as an example of a nitroaromatic drug to study the mechanism of nitroreduction by azoreductases and their human homologues. Nitrofurazone is part of the 5-nitrofuran family, which are potent antibiotics, active against both Gram-positive and Gram-negative bacteria. Members of the 5-nitrofururan family are being developed as therapies for multidrug resistant bacteria e.g. M. tuberculosis. Nitrofurazone and the other members of this family have a common mode of action that requires intracellular activation by nitroreductases. As a result, mutations within two nitroreductase known as nitrofuran sensitivity genes A and B (NfsA and B) from E. coli provide nitrofurazone resistance. As humans lack nitroreductases such as these, the majority of nitroreduction will be carried out by NAD(P)H quinone oxidoreductases. Activation by nitroreductases results in four electron reduction of the nitro group to a reactive hydroxylamine metabolite that in combination with the nitroso oxidation product causes DNA damage.

Three azoreductases from P. aeruginosa, paAzoR1, paAzoR2 and paAzoR3, have been extensively characterised. A recent structural study has provided insight into the mechanism by which azoreductases activate azo drugs, and also explains their ability to reduce both azo and quinone substrates. The aforementioned ability of azoreductases to act as nitroreductases indicates they may also reduce nitrofurazone.

The structure of E. coli NfsB bound to nitrofurazone has been solved the structure showed nitrofurazone bound in what the authors described as an inactive conformation within the enzyme active site. In order for their proposed mechanism of reduction to be correct nitrofurazone has to reverse its binding orientation as a result of FMN being in the reduced state. The work described in this manuscript demonstrates that azoreductases can also activate nitrofurazone and describes the kinetics of nitrofurazone reduction by paAzoR1 as well as the structure of nitrofurazone bound to paAzoR1 in an active conformation. Based on this structure, a putative mechanism is
Nitrofurazone is reduced by pAzoR1 and other nitroaromatic drugs by bacterial azoreductases and by their human homologues.

**Results**

**Nitrofurazone is reduced by pAzoR1.** Nitrofurazone was shown to be reduced by pAzoR1, apparent $K_m$ and $V_{max}$ values were obtained via Lineweaver-Burk plots. The apparent $K_m$ for pAzoR1 was 15.7 ± 1.6 μM and the $V_{max}$ was 9.8 ± 0.6 μM s$^{-1}$ mg of enzyme$^{-1}$. Via equations (1) and (2) the rates of nitrofurazone reduction and NADPH oxidation were obtained simultaneously under the conditions described in the materials and methods. The simultaneous rates obtained during nitrofurazone reduction were 3.12 ± 0.06 μM s$^{-1}$ mg of enzyme$^{-1}$ for nitrofurazone and 5.76 ± 0.25 μM s$^{-1}$ mg of enzyme$^{-1}$ for NADPH this gives an estimate for the molar ratio of the reaction of approximately 2 moles NADPH oxidation per mole nitrofurazone reduction. Mass spectroscopy data obtained from a completed reaction carried out in ammonium bicarbonate gave a major peak at 184 Da (Supp. Fig. 1).

**Structure of pAzoR1 binding nitrofurazone.** The structure of pAzoR1 binding to nitrofurazone was solved at a resolution of 2.08 Å with final $R_{merge}$ and $R_{work}$ values of 20.4% and 16.1% respectively and with good stereochemistry (Table 1). No significant conformational changes were observed between the structures of pAzoR1 bound to methyl red and pAzoR1 bound to nitrofurazone (RMSD on carbon α positions 0.23 Å). No significant movements of the amino acid side chains within the active site were observed when the structures were compared.

Clear positive difference density was visible for the nitrofurazone bound within the active site after a single round of refinement in PHENIX (Fig. 2A). Nitrofurazone was built into the density initially in one orientation, however upon further refinement it became clear there was density for nitrofurazone in two overlapping orientations. In both orientations the nitro group of nitrofurazone is positioned above the N5 of FMN in contrast to the orientation observed in the structure of NfsB bound to nitrofurazone where the nitro is 7.7 Å from the N5 (Fig. 3F). Nitrofurazone binds in an overlapping conformation to that previously observed for balsalazide bound to pAzoR1 (Fig. 2B) and methyl red bound to the Y131F mutant of pAzoR1, as a result it makes many of the same interactions. The furan rings of both conformers overlap the salicylate ring of balsalazide in the corresponding structure and the polar tail of nitrofurazone overlaps with the position of the azo bond of balsalazide as well as partially overlapping the 4-aminobenzoyl-β-alanine ring of balsalazide (Fig. 2B).

**Discussion**

pAzoR1 binds nitrofurazone with a significantly lower $K_m$ than azo compounds while the rate of reduction is also lower. The slow rate of reduction is likely to be due to competition between nitrofurazone and NADPH for binding to the active site. Due to the very high $K_m$ of NADPH (~1.2 mM) the tight binding of nitrofurazone causes substrate inhibition. Apparent $V_{max}$ is higher than that previously observed in R. sphaeroides azoreductases (0.23 μM s$^{-1}$ mg of enzyme$^{-1}$). The rate of nitroreduction by pAzoR1 is significantly slower than in nitroreductases such as NfsB (~2.3 s$^{-1}$ for pAzoR1 compared to 225 s$^{-1}$ for NfsB) suggesting that in bacteria the majority of activation is carried out by nitroreductases.

The calculated molar ratio of approximately 2:1 for the rate of NADPH oxidation to nitrofurazone reduction is in-line with the data that were obtained from Electrospray ionisation, time of flight mass spectroscopy (ESI-TOF-MS Sup. Fig. 1B). The mass spectroscopy data agree with previously published data that show the final reaction product is consistent with the hydroxylamine reductive metabolite of nitrofurazone (MW = 184 Da).

As when determining the mechanism of reduction of azo drugs, the tautomer state of nitrofurazone bound to the enzyme is important, the tautomer that binds pAzoR1 can be determined from the structure. Nitrofurazone can exist in two tautomers (Fig. 1) that are single round of refinement in PHENIX (Fig. 2A).

**Table 1** | Processing and refinement statistics for pAzoR1 binding nitrofurazone.

|                  | pAzoR1 nitrofurazone |
|------------------|----------------------|
| Space Group      | P3;21                |
| $\alpha$, $\beta$, $\gamma$ (°) | 90, 90, 120 |
| $\alpha$, $\beta$, $c$ (Å)   | 82.20, 82.20, 108.46 |
| **Processing Statistics** | |
| Resolution Range (Å) | 29.76-2.08 |
| Unique reflections* | 25,761 [1,794] |
| $R_{merge}$*       | 0.061 [0.766] |
| $<\langle|F||F\rangle\rangle>$* | 18.6 [2.0] |
| Completeness %*    | 99.6 [95.3] |
| Multiplicity*      | 5.4 [5.2] |
| **Refinement Statistics** | |
| $R_{work}$%        | 16.07                |
| $R_{free}$%        | 20.35                |
| RMS bond angle (°) | 0.98                 |
| RMS bond length (Å) | 0.007               |

*Numbers in parentheses are for the highest resolution shell.

In both orientations the nitro group of nitrofurazone is positioned above the N5 of FMN in contrast to the orientation observed in the structure of NfsB bound to nitrofurazone where the nitro is 7.7 Å from the N5 of FMN. In this structure nitrofurazone is anchored within the active site via a network of nine

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**Figure 1 | Structure of nitrofurazone.** Figure showing the two nitrofurazone tautomers that occur in equilibrium in solution.
short range hydrogen bonds to a combination of backbone amides, the ribose chain of FMN and the side chains of Glu165 and Asn71. This is in sharp contrast to the entirely hydrophobic interactions nitrofurazone makes within the active site of paAzoR1. As the binding conformation observed in the structure with NfsB is incompatible with the proposed mechanism of reduction it was speculated that when binding to the reduced enzyme, nitrofurazone binds in the reverse orientation, which would mean it is in a similar orientation to that observed in the paAzoR1-nitrofurazone structure. High resolution structural data (1.9 Å) from a closely related nitroreductase (88% identical – all active site residues are conserved) from *E. cloacae* in both the reduced and oxidised states show that reduction of FMN has little effect on surrounding active site residues. Lower resolution (2.5 Å) structures of the reduced and oxidised forms of NfsB are also available and show there is no significant conformational changes in the active site residues as a result of FMN reduction. It is therefore unlikely that reduced NfsB would bind nitrofurazone in a different conformation. The large number of interactions which are involved with the positioning of nitrofurazone in the conformation observed in the oxidised NfsB structure would

Figure 2 | Binding of nitrofurazone to paAzoR1. A) The binding of nitrofurazone in its two alternate conformers are shown with brown carbon atoms. The FMN is shown with yellow carbon atoms, both conformations of nitrofurazone are shown with brown carbon atoms. Important side chains are labelled. Blue mesh is the refined 2F̃-F̃ map contoured at 1σ, while black mesh is unbiased F̃-F̃ map at 2.2σ. The black sphere represents a bound water molecule. (B) Comparison of balsalazide and nitrofurazone binding to paAzoR1. Colouring of nitrofurazone is as in (A) and balsalazide is in purple. This figure was produced in Pymol v1.1.31

Figure 3 | Comparison of the overall structures (A–C) and nitroaromatic binding (D–F) of paAzoR1 (A,D), hNQO2 (B,E) and NfsB (C,F). (A–C) The overall structure of the dimeric forms of each of the three proteins. In all three panels monomer A is in green and B is in turquoise. (D–F) A detailed view of nitroaromatic drug binding within the active site of the enzymes (nitrofurazone in paAzoR1 and NfsB and CB1954 in hNQO2). In all panels the flavin cofactor is in yellow (FMN for paAzoR1 and NfsB, FAD for hNQO2). The Nitroaromatic drug is in brown in panels D–F. The structure of hNQO2 bound to CB1954 is from PDB 1XI230 and the structure of NfsB bound to nitrofurazone is from PDB 1YK1.11
mean that most likely alternate conformations will be energetically less favourable.

The proposed mechanism for nitroreduction, based on the azo tautomers being reduced, is shown in Fig. 4. Reduction reactions carried out by azoreductases proceed via a Bi-Bi ping-pong mechanism\(^1\),\(^{19}\) therefore as occurs during azoreduction,\(^{19}\) substrate binding is preceded by NADPH binding. While bound to paAzoR1, NADPH transfers a hydride ion to the N5 of FMN which becomes FMNH\(^-\). NADPH is subsequently displaced by nitrofurazone which accepts the hydride from FMNH\(^-\) (Fig. 4A). The position of the nitrogen of the nitro group of each conformer of nitrofurazone within 3.6 Å of the N5 atom of FMN suggests that this is the target for hydride transfer. As the nitrogen atom of a nitro group is positively charged this also makes it a good candidate for transfer of the negatively charged hydride. To complete the reduction reaction a proton is accepted from the water molecule (Fig. 4A) which is stably bound to the protein within 3 Å of the nitro group of nitrofurazone (Fig. 2A). The reduction takes place twice according to the same mechanism, in order to get complete formation of the hydroxylamine species, therefore, a double Bi-Bi ping pong mechanism can also be proposed for nitrofurazone reduction in paAzoR1.

In addition it was noted that in the structure of paAzoR1 binding balsalazide, the oxygen of the quinoneimine tautomer of balsalazide is ~2.7 Å from the ketone of the Asn99 side chain. In the structure of paAzoR1 binding nitrofurazone one of the oxygen atoms of the nitro group is a similar distance from the carbonyl of Asn99. This positioning would allow hydrogen bond formation after reduction of either substrate which may help to stabilise the reduced form of the compound and thus may facilitate hydride transfer.

Nitrofurazone is known to be carcinogenic in mammals.\(^{19}\) Unlike bacteria, humans have no homologues of NfsA and NfsB, as a result the majority of nitrofurazone activation in humans is proposed to occur via azoreductase-like enzymes. Humans have two azoreductase homologues, NAD(P)H quinone oxidoreductase (hNQO) 1 and 2. Although bacterial azoreductases share minimal sequence similarity with hNQO1 and hNQO2, they are structurally related (Fig. 3A and B\(^{20}\),\(^{21}\)) and carry out many of the same reactions.\(^3\) Both hNQO1 and hNQO2 have been shown to activate the anti-tumour pro-drug CB1954 via nitroreduction.\(^{19}\) Activation of nitrofurazone by these enzymes may result in the carcinogenic properties in mammals and may be the cause of contact dermatitis experienced with nitrofurazone. The structure of hNQO2 binding CB1954 (Fig. 3E – PDB: 1X12\(^{20\circ}\)) shows the nitro group that is reduced, binds with the nitrogen within 3.6 Å of the N5 of FMN in a similar position to the nitro group of nitrofurazone bound to paAzoR1. As a result it is proposed that other nitroaromatics are reduced by azoreductases via the same mechanism as is described here for nitrofurazone.

### Methods

All reagents were obtained from Sigma-Aldrich (Poole, UK) unless otherwise specified.

**Enzymology.** Recombinant paAzoR1 was prepared as described previously.\(^2\) Unless otherwise stated assays were carried out in 96-well format in a reaction volume of 100 μL using a Fluostar Omega plate reader (Aylesbury, UK) typically using 10 μg paAzoR1 per well, 750 μM NADPH and varying concentrations of nitrofurazone in 20 mM Tris-HCl pH 8.0, 100 mM NaCl.

In order to estimate the stoichiometry of the enzymatic reduction of nitrofurazone by paAzoR1, the following reaction was carried out: 50 μM nitrofurazone, 400 μM NADPH, 10 μg paAzoR1. Both the oxidation of NADPH and the reduction of nitrofurazone were simultaneously followed spectrophotometrically at 340 nm and 405 nm respectively. The molar extinction coefficient of each species was determined at each λ\(_{max}\) (ε\(_{NADPH,\,340}\), 3,300 M\(^{-1}\)cm\(^{-1}\), ε\(_{nitrofurazone,\,340}\), 405 M\(^{-1}\)cm\(^{-1}\), ε\(_{nitrofurazone,\,405}\), 10,800 M\(^{-1}\)cm\(^{-1}\) and ε\(_{NADPH,\,405}\), 9,500 M\(^{-1}\)cm\(^{-1}\)). Initial velocities were estimated via the following equations:

**equation (1)** \(v_1\) (340 nm) \(\frac{d[COD_{340\,nm}]}{dt}\) = \(\frac{d[COD_{340\,nm}]}{dt}\) nitrofurazone \(\frac{d[COD_{340\,nm}]}{dt}\) nitrofurazone \(\frac{d[COD_{340\,nm}]}{dt}\) nitrofurazone \(\frac{d[COD_{340\,nm}]}{dt}\) nitrofurazone.

As \(v_1\,\text{NADPH}\) is 0 it is straightforward to determine the rate of nitrofurazone reduction \(\frac{d[COD_{340\,nm}]}{dt}\) nitrofurazone. This value is used to estimate \(\frac{d[COD_{340\,nm}]}{dt}\) nitrofurazone in equation (1). The ratio of \(\frac{d[COD_{340\,nm}]}{dt}\) NADPH to \(\frac{d[COD_{340\,nm}]}{dt}\) nitrofurazone provides an estimate for the ratio of NADPH oxidation to nitrofurazone reduction.

**Identification of the products of nitrofurazone reduction.** Identification of the final reduction products was carried out via ESI-TOF-MS. The reduced nitrofurazone was produced via the following reaction: 25 μM nitrofurazone, 0.5 mM NADPH and 100 μg paAzoR1 in a final volume of 1 ml containing 25 mM ammonium bicarbonate pH 7.9 (<0.5 mM NaCl). The reaction was monitored at OD\(_{405}\) on a Hitachi (Mannheim, Germany) U-2001 spectrophotometer and allowed to proceed to >98% completion. paAzoR1 was removed via a Sartorius (Ashtead, UK) 3 kDa molecular weight cutoff vivaspin centrifugal concentrator. The reaction mixture containing reduced nitrofurazone was lyophilised with a Lab mode (Milton Keynes, UK) ScanVac, before resuspension in 1 mL methanol. ESI-TOF-MS was carried out on a Waters (Elstree, UK) micromass mass spectrometer in negative ion mode.

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**Figure 4** | Mechanism for nitrofurazone reduction. (A) First round of nitrofurazone reduction. (B) Second round of nitrofurazone reduction resulting in hydroxylamine formation. The proton in both (A) and (B) is likely to be donated by a stably bound water molecule observed in the structure (Fig. 2A). Before (A) and between (A) and (B) NADPH binds to paAzoR1 and transfers a hydride to FMN before being replaced by nitrofurazone.
**Structure determination.** paAzoR1 was mixed with nitrofurazone to give a protein solution of 23 mg mL\(^{-1}\). paAzoR1 in 20 mM Tris-\(\text{HCl}\) \(pH\) 8.0 with 2 mM nitrofurazone and incubated for an hour before being washed with 20 mM Tris-\(\text{HCl}\) \(pH\) 8.0 to remove DMSO. Crystals grew isomorphously under the same conditions as previously described,\(^{12}\) briefly 2 \(\mu\)L protein complex was mixed with 2 \(\mu\)L 1.6 M (NH\(_4\))\(_2\)SO\(_4\) 0.1 M HEPEs \(pH\) 7.5 in sitting drop format. Data for paAzoR1-nitrofurazone were collected at beamline I02 at the Diamond Light Source (Oxon, UK) with a Quantum ADSC detector. Data were integrated via XDS,\(^{12}\) and scaled and merged with SCALA\(^{23}\) both were run as part of xia2.\(^{24}\) The structure was solved with the program PHASER,\(^{25}\) using the structure of paAzoR1 stripped of its ligands (PDB: 2V9C) as the search model. The atomic model was rebuilt and refined with Coot,\(^{26}\) Refmac 5.5\(^{27}\) and PHENIX.\(^{28}\) Initial translation, libration and screw (TLS) parameters were determined with the program TLSMD\(^{29}\) and TLS refinement was performed with PHENIX. Restraints for nitrofurazone were generated in eLBOW. Water molecules were added with PHENIX and Coot. The final model was validated via MolProbity\(^{30}\) and all residues were shown to lie within the allowed regions of the Ramachandran plot (97.8% in the preferred region and 2.2% in the allowed region). Data collection and refinement statistics are shown in table 1. The structure was deposited in the protein data bank (PDB ID: 3R6W).

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**Author contributions**
A. Ryan designed the experiments, carried out some of the experiments, carried out data analysis and wrote the manuscript. E. Kaplan carried out most of the experiments and data analysis. N. Laurieri was involved with planning of experiments as well as data analysis and contributed to writing of the manuscript. E. Lowe carried out crystallographic data collection and aided with data analysis. E. Sim coordinated the project as well as helping to write the manuscript.

**Additional information**
Supplementary Information accompanies this paper at http://www.nature.com/scientificreports

**Competing Financial Interests:** The authors declare that they hold no competing financial interests.

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