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A minireview on oxygen-insensitive nitroreductases: reaction mechanisms and their biomedical impact

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In this paper, we try to summarise some historically identified structural and catalytic properties of an important super-family of flavoenzymes, the oxygen-insensitive bacterial nitroreductases (NRs). We also review how these mechanistic properties have underpinned diverse roles in medicine and biomedical research, with an emphasis on the last decade. NRs perform the NAD(P)H-dependent two/four-electron reduction of nitroaromatic compounds ($ArNO_2$) and the two-electron reduction of quinones (Q) without the formation of free radicals of these compounds. This has significant implications for their behaviour in living systems. We reviewed the structural, catalytic and potentiometric properties of NRs and their oxidant substrate specificity, as well as the structural factors influencing it. Biomedical aspects of the function and application of NRs were also reviewed, including the importance of NRs in antibiotic resistance, their applications in cancer gene therapy, and NR-mediated cellular ablation technologies.

Keywords: nitroreductases, enzymatic catalysis, nitroaromatic compounds, hydride transfer, GDEPT

INTRODUCTION

For a long time, nitroaromatic compounds ($ArNO_2$) have maintained their importance in relation to industrial processes, environmental pollution, and pharmaceutical application. Because of contamination of groundwater and soil at military and industrial sites by $ArNO_2$ that exhibit various harmful activities, there has been a significant interest in understanding and applying biological processes for their degradation. On the other hand, the redox activity of the nitro group has contributed to its importance in medicinal chemistry. Importantly, both the biodegradation of environmental pollutants such as explosives and the manifestation of toxicity/therapeutic action of nitroaromatic drugs may involve similar initial steps, the single- or two-electron reduction of $ArNO_2$ performed by numerous flavoenzymes and/or their physiological redox partners, metalloproteins.

Bacterial type I oxygen-insensitive nitroreductases (NRs) are one of the most important groups of enzymes responsible for the biodegradation or therapeutic/toxic effects of $ArNO_2$, the formulae of the most relevant representatives of which are presented in Fig. 1. NRs catalyse reduced nicotinamide adenine dinucleotide or its phosphate (NAD(P)H)-dependent multistep reductions in $ArNO_2$ with the formation of nitroso (ArNO), hydroxylamine (ArNHOH) and potentially amino ($ArNH_2$) products. These NRs are called oxygen-insensitive because, unlike the products of the single-electron $ArNO_2$ reduction, free radicals, their reaction products, are resistant to oxygen or are reoxidised quite slowly. Most type I NRs are 2 × 24–27 kDa dimers, containing one FMN per subunit. They

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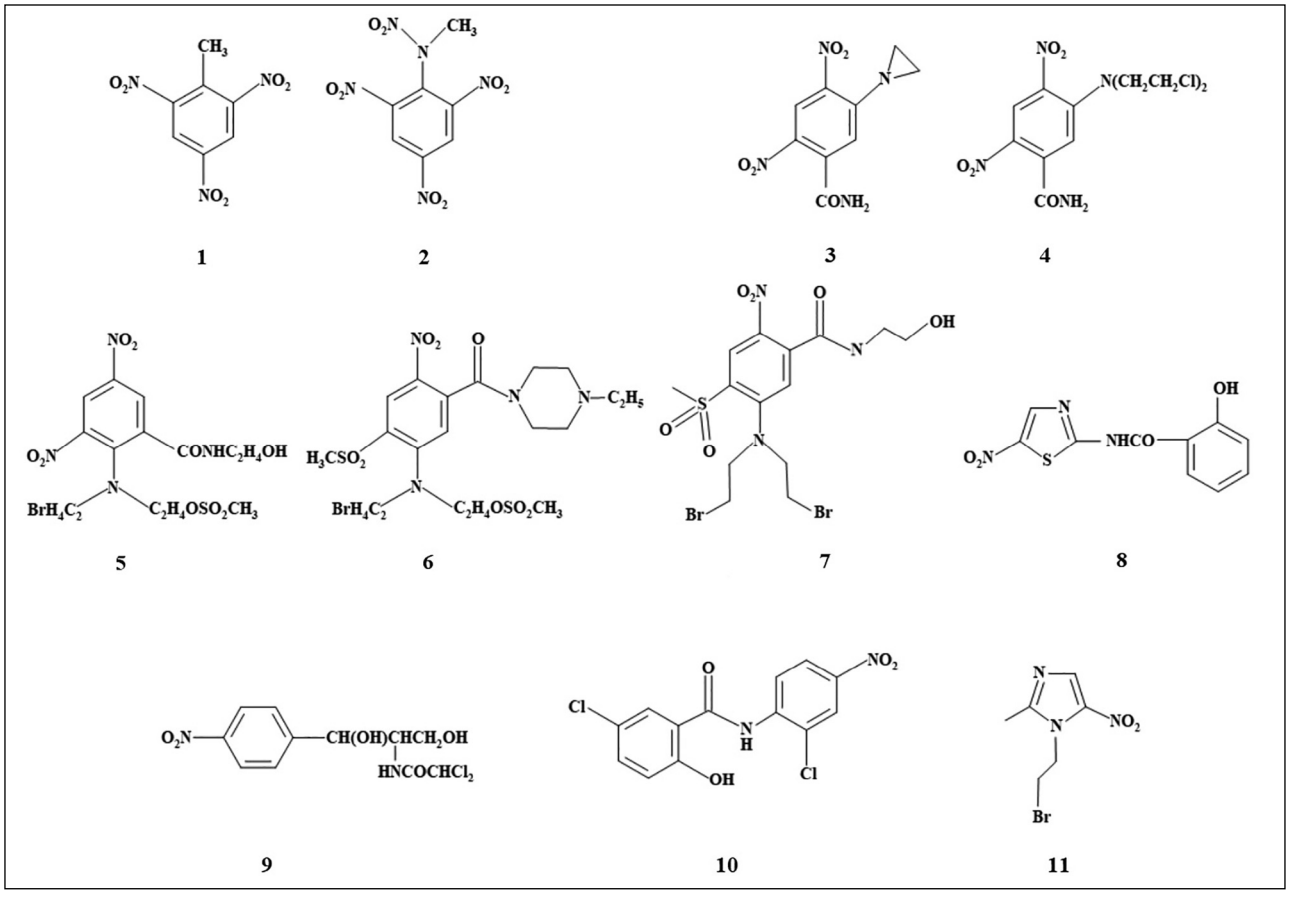


Fig. 1. The structures of nitroaromatic compounds discussed in this work: TNT (1), tetryl (2), CB-1954 (3), SN-23862 (4), PR-104A (5), SN-36506 (6), SN29176 (7), tizoxanide (8), chloramphenicol (9), niclosamide (10) and metronidazole (11)

may reduce a wide spectrum of oxidants, including nitroaromatics, quinones, riboflavin derivatives, and inorganic complexes [1–10]. The physiological functions and physiological electron acceptors for NRs are not clearly characterised. It is suggested that they participate in the antioxidant defense of microorganisms as part of the *soxRS* regulon, whose genes are upregulated in response to oxidative stress [11]. Despite of the large NR superfamily (>24,000 known sequences), only a small fraction has been experimentally characterised [12]. Typically, among the numerous groups of type I NRs, two major subfamilies are distinguished according to their primary sequence similarity with *Escherichia coli* nitroreductases A or B (NfsA and NfsB). Usually, group A nitroreductases can use only NADPH as reducing substrate [12], whereas group B nitroreductases catalyse the oxidation of NADH and NADPH. In this paper, we try to summarise some historically identified structural and catalytic properties of NRs, as well as their application areas, with an emphasis on the last decade.

STRUCTURAL, CATALYTIC AND THERMODYNAMIC PROPERTIES OF OXYGEN-INSENSITIVE NRs

The oxygen-insensitive NRs are dimeric enzymes with FMN cofactor localised in the intersubunit space [12]. They were discovered more than 40 years ago [1]; however, their more systematic studies started in 1990–2000s, including the crystallographic characterisation of *E. coli* NfsB and NfsA [7, 13–15]. These more recent studies revealed that each subunit of NfsA contains two domains, a core domain of four β-strands surrounded by α-helices and an excursion domain, with residues 165–210 containing two helices, G and H, and long loops. The *N*-terminal helix, A, of one subunit interacts with the core domain of the other subunit, as does helix H, and the excursion domain crosses over the dimer interface. The major dimer interface is at helix E [16]. Similar structural motifs are characteristic of other NRs (Fig. 2). Typically, the *re*-plane of the isoalloxazine ring of FMN

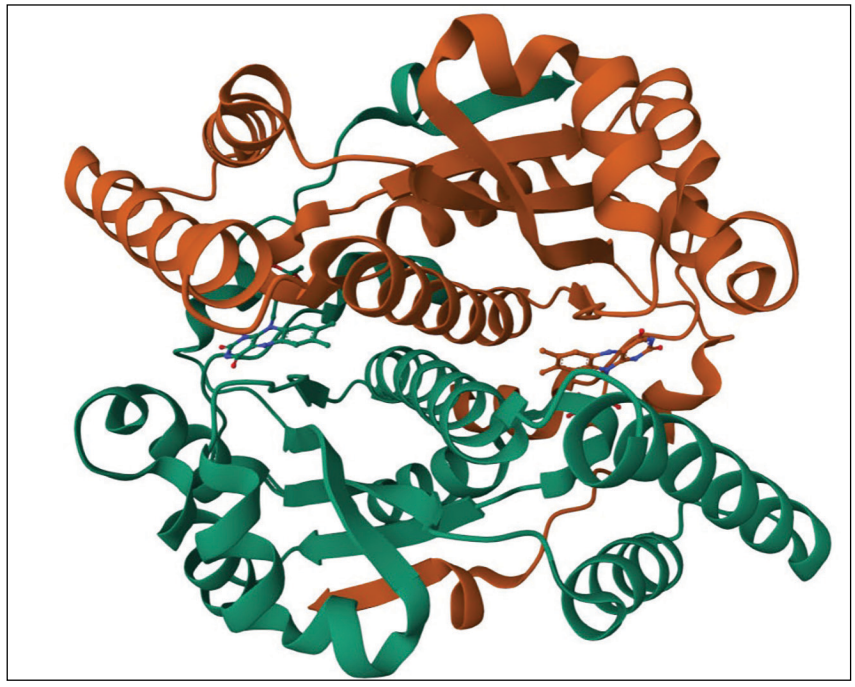
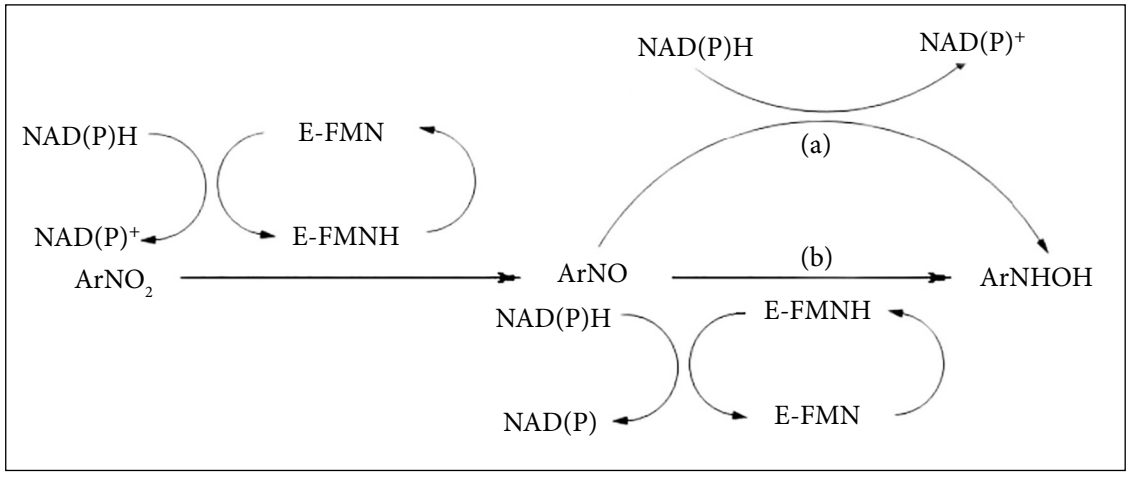


Fig. 2. Structure of *Escherichia coli* nitroreductase B (NfsB) with FMN molecule in the active centre (PDB: 1DS7)

is accessible to a solvent and to substrates. In group B nitroreductase from *Enterobacter cloacae*, the polar groups that interact with the pyrimidine ring of isoalloxazine and its N5 include Lys14, and Asn71 and Lys74. On the dimethylbenzene side, Ile164, Tyr144 and Leu145 also follow the bend of the flavin. Ser39 and Ser40 from the other monomer, which are located above the central isoalloxazine ring, move very little upon reduction of the flavin [17]. Another well-examined group B enzyme is *Vibrio fischeri* 26 kDa NAD(P)H:FMN oxidoreductase (FRase I). Even though the homology between *E. coli* NfsB and FRase I is not high (~30%), several core amino acids are still conserved in each active site (Phe124, Ser40(42), Asn71(73), Gly166). The main biological role of FRase I is the reduction of free FMN into $FMNH_2$, which is essential for bioluminescence of bacteria [5].

In this context, it should be noted that attempts to perform X-ray structural analysis of NRs complexes with $NAD(P)^+$ were often unsuccessful. The available data show that in NfsB, the nicotinamide ring of bound NAD(P)H is stacked between Phe124' from another subunit and isoalloxazine. In *E. coli* NfsA, the 2'-phosphate group of NADP(H) interacts electrostatically with Arg203 and Arg208, which determines the specificity of group A NRs for NADP(H) [16]. Phe42Asn/Ala mutations have been observed to decrease the k_{cat}/K_m of NADPH to 3–5% of the original value [10]. On the other hand, a Phe42Tyr mutation lowered the k_{cat}/K_m of NADPH by 40–60%. It is suggested that the substitution of Phe by short-chain Ala may result in considerable conformational changes, whereas Tyr keeps a similar conformation. Therefore, this may indicate that Phe42 does not act as a spacial obstacle, but contributes to the stabilisation of the active site. Another specific amino acid, which interacts with nicotinamide and isoalloxazine rings, is Ser39(40) [14].

Typically, the catalysis of oxygen-insensitive NRs follows a 'ping-pong' mechanism, which means that the reductant (NAD(P)H) and the oxidant bind at the same (or sufficiently overlapping) site at different redox forms of the enzyme. Sometimes reactions are complicated by inhibition by oxidants [18, 19]. NRs perform the initial two-electron reduction of $ArNO_2$ with the formation of nitroso (ArNO) products. However, since ArNO are better oxidants than the corresponding $ArNO_2$ [20], they are further easily reduced into hydroxylamines (ArNHOH) (Scheme). The mechanism of this reduction step is not sufficiently clear, but it has been shown that the main role in it may be played by the direct (nonenzymatic) reduction of ArNO by NAD(P)H (Scheme, pathway (a)) [21].



Scheme. Pathways of the nonenzymatic (a) and enzymatic (b) formation of ArNHOH from ArNO

On the other hand, studies of nitrobenzene and nitrosobenzene reduction by *E. coli* NfsB show that the latter is reduced 10^4 times faster than nitrobenzene [15].

Because both Ar-NO and Ar-NHOH alkylate DNA and other cellular nucleophiles, these reactions may be responsible for the cytotoxic/therapeutic action of $ArNO_2$. Another important group of oxidants, quinones, are reduced in a two-electron manner into hydroquinones [18]. Determination of the rate-limiting catalysis step is complicated because some NRs, such as *Enterobacter cloacae* NR, have a maximal turnover rate under steady-state conditions exceeding 1000 s^{-1}, at 25°C, which is beyond the capabilities of stopped-flow technique [22]. At pH 7.0 and 25°C, the maximal reduction rate of FMN of *E. coli* NfsB exceeds 500 s^{-1}, i.e. it is approaching stopped-flow limits [15]. The maximal rate of nitrofurazone reduction, acquired by extrapolating to infinite NADH concentration, is equal to 255 s^{-1} [15]. This indicates that the reaction is limited by the oxidative half-reaction.

The stopped-flow studies of *E. cloacae* NR at 4°C and pH 7.0 revealed that the enzyme was reduced by NADH with a maximal rate of 700 s^{-1} and that *p*-nitrobenzoic acid reoxidised it with a maximal rate of 1.9 s^{-1}, i.e. 400 times more slowly. This shows that the rate-limiting step of catalysis also could be the oxidative half-reaction [23, 24]. A similar conclusion would hold for other NRs, where the maximal reaction rate depends on the structure of the oxidant [21, 22, 25–30]. In this context, one may note that the kinetic isotope effect was observed in both reductive and oxidative half-reactions of *E. cloacae* NR using deuterated NADH [24]. It shows that the H atom transferred from NADH to the N-5 position of isalloxazine is subsequently transferred to the nitroaromatic oxidant. On the other hand, it is important to understand that the analysis of the reoxidative half-reaction is quite complex, since during the reduction of $ArNO_2$, ArNO is formed which is a much better oxidant. Another hypothesis is that both NR subunits do not function simultaneously, i.e. they function one after another [16]. Unfortunately, there are insufficient kinetic data to reject or confirm this hypothesis. Our recent study of the relatively slow-acting TdsD1 nitroreductase showed that complete FMN reduction could be described equally well as a one- or two-step process [31]. Another important aspect is the formation of $ArNH_2$ as the final reduction product, which is only observed in some cases, although this problem is relevant in biomedicine. NfrA from *Bacillus LMA* exhibits 40% homology with NfsB and reduces 3,5-dinitrotrifluoromethyl-benzene to a diamine product with k_{cat} = 18 s^{-1} [32], i.e. much faster than nitrofurazone. *S. typhimurium* NR quantitatively reduces nitrobenzene into aniline [33]. A recent study shows that *Haemophilus influenzae* NR-B reduces chloroamphenicol into a corresponding amine with k_{cat} = 10.2 s^{-1} [34]. This NR possesses an undisclosed substrate specificity because it reduces the more powerful oxidant metronidazole with a lower rate, k_{cat} = 0.34 s^{-1} with the formation of its hydroxylamine metabolite. It remains unclear whether the tendency for amine formation is determined by the properties of $ArNO_2$ or nitroreductase

or by both factors. It has been suggested that the possibility of amine formation increases with the reduction potential of $ArNO_2$ and the size of their aromatic system [29]. However, it should be noted that the nature of these reactions remains poorly understood, e.g. the energetics of the ArNHOH/$ArNH_2$ transition is poorly characterised, except for quantum mechanical calculations performed in vacuo, but not in aqueous media [35].

The values of the standard midpoint redox potential (two-electron reduction of the FMN cofactor at pH 7.0, $E°_7$) of oxygen-insensitive NRs may provide some information on their catalysis. These data as well as some data at pH 7.5–8.0 are presented in Table 1. They show that the $E°_7$ of most NRs are close to –0.200 V, i.e. that the reoxidation of their reduced forms by $NAD(P)^+$ ($E°_7$ = –0.320 V) may not be taken into account, with the possible exception of the *Vibrio harveyi* NADPH:FMN oxidoreductase (Table 1). At the other extreme is the suggested $E°_7$ of the peroxiredoxin-nitroreductase hybrid enzyme of *Thermotoga maritima* (Prx-NR), ≥ –0.155 V (Table 1). However, a distinctive feature of oxygen-insensitive NRs is the instability of their FMN semiquinone, 0.01% at equilibrium for *E. cloacae* NR [36]. No appreciable FMN semiquinone formation was observed during the photosensitised reduction of NfsA as well [40]. In this case, the high redox potential of the FMN semiquinone/reduced FMN pair makes the initial single-electron transfer thermodynamically unfavourable, and likely determines the mode of the two-electron (hydride) reduction. Probably, this is associated with the hampered transition from the bent structure of the isoalloxazine ring of reduced FMN (the N5-N10 axis diverts from coplanarity by 25°) to a putatively planar semiquinone form [17].

OXIDANT SUBSTRATE SPECIFICITY OF NRs

The first studies of the oxidant specificity of NfsA, NfsB and *Vibrio fischeri* NAD(P)H:FMN reductase showed that the log of the reduction rate of compound at its fixed concentration, or its bimolecular reduction rate constant (k_{cat}/K_m), increase with its single-electron reduction potential (E^1_7) [1]. Those trends were scattered, but observed in both group A and group B NRs [4, 5]. When studying a limited number of compounds from various groups, those trends described the reactivity of $ArNO_2$, quinones, and flavin derivatives. Although two/four-electron $ArNO_2$ reduction takes place, the E^1_7 was used as the correlation parameter because the energetics of two-electron $ArNO_2$ reduction in aqueous media has not yet been characterised [35]. This led to an initial assumption that the active centres of NRs are mobile and plastic, capable of binding oxidants of various sizes and shapes. Later, crystallographic studies of NRs complexes with

Table 1. **Potentiomentric data of nitroreductases**

Enzyme	Standard redox potential, V	Conditions
E. cloacae NR (B) [36]	–0.190	Equilibration with redox mediator, PIPES, KCl, pH 7.0
Vibrio fischeri NAD(P)H:FMN oxidoreductase (B) [9]	–0.215	Equilibration with redox mediator, phosphate, pH 7.0
E. coli NfsB [37]	–0.199	Equilibration with redox mediator, Tris, NaCl, pH 7.0
E. coli NfsB [38]	–0.218	Direct electrochemical reduction, phosphate, KCl, 10% glycerol, pH 7.5
Vibrio harveyi NADPH:FMN oxidoreductase (A) [9]	–0.255	Equilibration with redox mediator, phosphate, pH 7.0
E. coli NfsA [39]	–0.264	Direct electrochemical reduction, phosphate, KCl, 10% glycerol, pH 7.5
E. coli NfsA [40]	–0.215	Equilibration with NADP(H) analogue, phosphate, pH 7.0
B. tequilensis NR (B) [30]	–0.236	Equilibration with redox mediator, phosphate, pH 7.0
T. maritima peroxiredoxin-nitroreductase hybrid enzyme [41]	–0.185	Equilibration with redox mediators, Tris, pH 7.6.
M. smegmatis PnBA [29]	–0.190	Equilibration with redox mediator, pH 7.0

oxidants were performed, as well as their computer modelling. The available crystallographic data demonstrate the different modes of the oxidants binding and their interaction with isoalloxazine ring of FMN. Initially, two binding sites of CB1954 (Fig. 1) were found in different subunits of *E. coli* NfsB, including the residues of Lys14, Lys74, Ser12 (A), and Phe124, Asn71, Gly166 (B) [14]. The parallel computer modelling data indicate that site B can provide a more efficent π-π interaction of CB-1954 with an isoalloxazine ring, evidently, due its stacking with Phe124 [7]. On the other hand, nitrofurazone binds identically in both subunits of NfsB; however, its conformation may be nonproductive, because only an amide group and not a nitrofuran ring interacts with isoalloxazine [15]. It also appears that Phe124, which shields isoalloxazine from a solvent, retards the reduction of bulky oxidants such as riboflavin, because a Phe124Ser substitution in NfsB significantly increases the riboflavin reduction rate, whereas the rates of reduction of quinones and nitroaromatics are affected insignificantly [5]. Similarly, the structural specificity of NfsB was demonstrated by the fact that analogues of the cancer prodrug SN-23862 (Fig. 1) with positively charged alkylamine substituents at the 1-position were poor or inactive substrates despite their favourable redox potential [28]. To our knowledge, systematic studies of NfsB to obtain quantitative structure-activity relationships (QSARs) have not been performed, as the focus has been on therapeutically relevant compounds. However, studies on group B *Enterobacter cloacae* NR showed that the reactivity of $ArNO_2$ was higher than that of quinones with the same E^1_7 [18, 22]. Furthermore, the reactivity of quinones depends weakly on their E^1_7, while 2-hydroxy-1,4-naphthoquinone derivatives have an anomalously high reactivity [18].

Regarding group A NRs, computer modelling data suggest that Ser40, Ser41 and Phe42 are essential for the binding of CB1954 (Fig. 1) to NfsA [10]. Phe42 substitution into Tyr, Asn or Ala resulted in 52, 96 and 99% decreases in the nitrofurazone reaction rate [10]. On the other hand, the following X-ray studies of the complexes of oxidised NfsA showed that nitrofurantoin binds to Arg15, Ser41, Lys67 and Asp225, but in an unproductive conformation [39]. However, most importantly, the use of the reduced form of FMN in computer modelling enabled a structure to be obtained with the productive orientation of the NO_2 group of nitrofurantoin towards the N5 position of isoalloxazine. Following this approach, a comprehensive study of *E. coli* NfsA kinetics and computer modelling was performed [40]. It showed that the enhanced reactivity of 2-hydroxy-1,4-naphthoquinones, and the abnormally low activity of nitracrine (*N*',*N*'-dimethyl-*N*-(1-nitroacridin-9-yl)-propane-1,3-diamine), SN-36506 (also known as CP-506; Fig. 1), thionine and safranin T could be explained by H-bond formation with Arg15, Arg133 and Ser40, whereas their π–π interactions with the isoalloxazine ring are not important. It is interesting to note that the calculated distance between the *N*5 of the isoalloxazine and the oxygen of the nitro or quinone carbonyl groups varied between 3.0–5.0 Å for efficient oxidizers and 6.0–9.0 Å for poor ones. It is noteworthy that the poorly reactive oxidants had more negative reaction activation entropies than the well-reactive ones, which is consistent with the current understanding of the activation of single-step hydride transfer.

The specificity of oxidant binding to NRs is also related to another problem of their functioning, inhibition by dicoumarol. Dicoumarol efficiently inhibits NfsB, acting as a competitive inhibitor to NADH (K_i = 2 μM (0.1 M phosphate, pH 7.0 [25]) or 10 μM (0.01 M Tris-HCl, pH 7.0 [15])) and as an uncompetitive inhibitor to nitrofurazone or menadione. It also acts as a competitive vs NADPH inhibitor (K_i ~ 10 μM) in NfsA-catalysed reactions [21]. This indicates that dicoumarol binds more effectively to an oxidised enzyme form. According to the X-ray data, dicoumarol binds to *Vibrio fischeri* NAD(P)H:FMN reductase in the bent conformation, one of its rings interacting with an isoalloxazine ring, and another one with Phe124 [42]. Since Phe124 is conserved in other group B nitroreductases, it is safe to suggest that this is a universal common dicoumarol binding mechanism. On the other hand, Ile43 of *V. fischeri* FRase I also participates in dicoumarol binding, and it corresponds to Phe42 of NfsA and to Thr41 of NfsB.

To our knowledge, despite the discovery and characterisation of new nitroreductases, QSAR studies of their oxidants have not been intensive. However, we would like to compare the QSAR data of the two NRs that we conducted. The first

is our recently studied TdsD1 nitroreductase [31]. Despite its very low homology to NfsA and NfsB, ~20%, it has a very similar substrate specificity, most notably expressed as a significantly increased activity of 2-hydroxy-1,4-naphthoquinones. Although the tertiary structure of this enzyme has not been determined, sequence comparison and computer modelling have shown that its Arg27 and Ser52(53) residues may be in the FMN environment and match the X-ray structural data of NfsA (Arg15 and Ser40), as well as NfsB with analogous amino acids (Table 2). This may determine the enzyme specificity for 2-hydroxy-1,4-naphthoquinones. The second object of our studies was the peroxiredoxin-nitroreductase (Prx-NR) hybrid enzyme of *Thermotoga maritima*. This hybrid enzyme consists of 321 amino acids including an FMN-containing NR domain at the C-terminus [43]. Its sequence analysis (residues 142–321, GenBank accession number NP_228196) revealed a modest homology, 20–24%, with the sequences of *E. coli* NR-B and *E. cloacae* NR [19]. The residues analogous to Phe124, Phe70, Ser40, Lys14 and Lys74, which participate in the binding of nitroaromatic compounds or the inhibitor dicoumarol in *E. coli* NR-B or *E. cloacae* NR, are absent in Prx–NR. On the other hand, this shows a possible conservation of several residues participating in the binding of the isoalloxazine ring of FMN in NfsA, Arg15 (Arg154 in Prx–NR) and Gly131 (Gly235 in Prx–NR) (Table 2). Due to those structural features, the enzyme was still inhibited by micromolar concentrations of dicoumarol. However, due to the lack of structural equivalents at Ser39-41, it did not exhibit an enhanced reactivity towards 2-hydroxy-1,4-naphthoquinones, and also its reactivity towards $ArNO_2$ was lower than that towards quinones of the same redox potential.

THE IMPORTANCE OF NRs IN ANTIBIOTIC RESISTANCE

NRs have been implicated in both resistance and sensitivity to several important antibiotics. Perhaps the most important example is metronidazole (Fig. 1), a nitroaromatic prodrug that is generally converted to its active state under anaerobic conditions via nitroreductase activity by bacterial one-electron ferredoxin reductases and flavin reductases, although its complete activation and resistance pathways are not well understood [45]. The prominent pathogen *Helicobacter pylori* provides an exception to this, as mutations to the nitroreductase encoding *rdxA* gene are solely responsible for resistance in a large portion of clinical and laboratory isolates [45–47]. Nonsense and missense mutations are generally attributed to the loss of RdxA function [47]; however, single amino acid changes in RdxA may also provide

Table 2. **Conservation or similarity of amino acids in the NR sequences with the active centre residues of NRs involved in the binding of oxidants or dicoumarol**

E. coli **NfsB**	***E. cloacae*** **NR**	***E. coli*** **NfsA**	***E. faecium*** **NrmA**[f]	***B. tequilensis*** **NR**[g]	**TdsD1**[h]	***T. maritima*** **Prx-NR**[i]
Lys14[a]	Lys14	Arg15[b]	Arg18	Lys19	Arg27	Arg154[f]
Ser39[a]	Ser39	Ser39	Ser43	Ser44	Ser52	–
Ser40[a, c]	Ser40	Ser40[b]	Ser44	Ser45	Ser53	–
Thr41[a, c]	Thr41[d]	Ser41	–	–	Tyr54	–
Phe124[a]	Phe124[d]	–	Tyr123	–	–	–
–	–	Tyr128	–	–	–	Phe260
Gly166	Gly166	Gly131[e]	Gly161	Gly170	Gly140	Gly235
Asp168	Asp168	Arg133[b]	Glu163		Asp142	–
Lys205	Lys205	Lys167[e]	–	Lys209	Arg186	–

[a] Binding of nitroaromatic oxidants and dicoumarol, X-ray crystallography [15]; [b] binding of nitroaromatic and quinone oxidants, computer modelling [40]; [c] binding of nitroaromatic oxidants, X-ray crystallography [15]; [d] binding of aromatic ligands, X-ray crystallography [17]; [e] binding of nitroaromatic oxidants, X-ray crystallography and computer modelling [39]; [f] sequence comparison [44]; [g] binding of nitroaromatic oxidants, computer modelling [30]; [h] computer modelling [31]; [i] sequence comparison [19].

resistance. It is unclear whether these amino acid changes affect the biological function of RdxA or its function on metronidazole alone [48]. In some *rdxA* mutant strains, mutations in another nitroreductase gene, *frxA*, have been observed to further enhance resistance to metronidazole [45].

The anthelmintic niclosamide (Fig. 1) has recently shown promise as a topical antibiotic in Phase II clinical trials [49], and its nitro substituent is important in the protonophore (cytotoxic oxidative phosphorylation uncoupling) activity of the drug [50, 51]. Deletion of nitroreductase genes in niclosamide-sensitised *E. coli* has been shown to greatly increase susceptibility to niclosamide with a 32-fold decrease in MIC. Individual overexpression of *E. coli* nitroreductases NfsA, NfsB and AzoR reestablished resistance in NR knockout *E. coli*, with increases in the half-maximal inhibitory concentration (IC_{50}) between 10- and 15-fold. Variants of *E. coli* NfsA conferring increased niclosamide resistance, generated via multisite saturation mutagenesis around the active site, displayed a strong collateral sensitivity to the nitroaromatic prodrugs metronidazole and nitrofurantoin [51]. This suggests that if niclosamide resistance mediated by evolved nitroreductase enzymes were to arise in the clinic, it could potentially be treated by nitroaromatic antibiotics.

More recently, independent teams have reported that chloramphenicol resistance in *E. coli* can be conferred by overexpression of NfsB from *Haemophilus influenzae* or NfsA from *E. coli* [34, 52]. In the mass-mutagenesis study investigating active site residues of *E. coli* NfsA, it was found that abolition of native quinone reductase activity by substitution of R225 greatly improved resistance to chloramphenicol [52]. However, nitroreductase-mediated resistance to chloramphenicol in clinical isolates has not yet been reported.

APPLICATIONS OF NRs IN CANCER GENE THERAPY

NRs have long offered promise for gene-directed enzyme prodrug therapy (GDEPT), a precision medicine strategy that seeks to deliver genetically-encoded prodrug-converting enzymes to the tumour environment, and thereby sensitise cancerous cells to a systemically delivered prodrug therapy [53].

The first significant application of NRs in GDEPT occurred in the 1990s, when researchers identified *E. coli* NfsB as a potent enzyme capable of activating prodrugs such as CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) (Fig. 1). CB1954 itself was initially developed as a chemotherapeutic that showed a potent single-agent activity in rat Walker 256 carcinoma models [54], but was subsequently found to be relatively inert in human tumours [55]. When combined with NfsB in GDEPT, however, CB1954 was effectively converted into a cytotoxic DNA cross-linking agent, showing promise in both *in vitro* and *in vivo* models [56]. The first human trials using the NfsB/CB1954 system began in the early 2000s, using adenoviral vectors to deliver the *nfsB* gene directly into tumors [57, 58]. Although the trials demonstrated proof-of-concept in terms of partial responses and safety at the administered dose of CB1954, that dose was 10–100 fold lower than the K_M of NfsB, and it seems likely that a consequent lack of prodrug activation contributed to decisions to not further progress the therapy in that form [59].

Recent breakthroughs in NR-based GDEPT (gene–directed enzyme–prodrug therapy) have advanced both the enzymatic and prodrug components. Medicinal chemistry innovations have yielded second- and third-generation nitroaromatic prodrugs – notably, a series of mustard-class compounds including PR–104A, SN29176 (Fig. 1) and CP-506 – that deliver an enhanced potency, improved local bystander effects and superior pharmacological profiles [60–62]. This has in turn required the discovery and/or engineering of new NRs that can efficiently activate each prodrug, as well as offer other advantages. For example, Copp et al. (2017) used directed evolution to engineer variants of the *E. coli* NR NfsA that were not only enhanced in their abilities to convert PR-104A into a potent cytotoxin with a substantial bystander effect, but also had the ability to convert positron emission tomography probes to cell-entrapped forms [63]. This offers prospects for clinicians to assess the efficacy and safety of NR tissue distribution during GDEPT regimens, prior to administering the prodrug. Overall, these complementary developments in medicinal chemistry and enzymology have expanded the therapeutic promise and practicality of nitroreductase-based GDEPT strategies.

NR-MEDIATED CELLULAR ABLATION TECHNOLOGIES

Conceptually similar to GDEPT, but offering a far greater control to scientists, NRs have become an important tool for targeted cell ablation to study tissue development, disease and regeneration. In zebrafish (*Danio rerio*), the NR ablation system has been used to model degenerative conditions and interrogate regenerative responses in organs such as the pancreas, retina and heart [64, 65]. This is achieved by creating a fully transgenic model organism that contains a prodrug-converting NR gene in all cells, but with NR expression confined to defined cell types by use of tissue-specific promoters. The NR then converts an otherwise innocuous prodrug such as metronidazole into a potent cytotoxin. As the toxic metabolite for metronidazole remains entrapped within NR-expressing cells [66, 67], ablation is confined to the targeted cell population and can be modulated by adjusting the timing and dose of prodrug exposure [68].

NR-mediated cell ablation was first demonstrated in zebrafish using *Escherichia coli* NfsB, enabling the targeted ablation of neuronal, endocrine and immune cell types with a greater control than earlier laser or optogenetic methods [64, 65, 69]. While widely used, *E. coli* NfsB and subsequent engineered variants were limited by a relatively poor catalytic efficiency with metronidazole, requiring high prodrug concentrations that caused systemic toxicity and prevented the complete ablation of some cell types. A major advance came with the development of NTR2.0, a rationally engineered *Vibrio vulnificus* NfsB variant with a substantially improved metronidazole reductase activity [70]. NTR2.0 enables robust ablation at much lower metronidazole concentrations and is now considered the benchmark enzyme for NR-mediated ablation in zebrafish and related systems. It has facilitated studies of diverse cell types, including rod photoreceptors [70], retinal ganglion cells [71], neutrophils [72] and pancreatic β-cells [73], many of which could not be effectively ablated by first-generation NR systems.

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DEGUONIUI NEJAUTRIŲ NITROREDUKTAZIŲ APŽVALGA: REAKCIJŲ MECHANIZMAI IR JŲ BIOMEDICININIS POVEIKIS

S a n t r a u k a

Straipsnyje siekiama apibendrinti svarbios flavininių fermentų superšeimos – deguoniui nejautrių bakterijų nitroreduktazių (NR) – kai kurias istoriškai nustatytas struktūrines ir katalitines savybes bei aptarti, kaip šios savybės nulėmė įvairius šių fermentų vaidmenis medicinoje ir biomedicininiuose tyrimuose, ypatingą dėmesį skiriant pastarojo dešimtmečio rezultatams. NR atlieka NAD(P)H-priklausomą nitroaromatinių junginių ($ArNO_2$) dvi- keturelektroninę redukciją ir dvielektroninę chinonų (Q) redukciją, nesusidarant šių junginių laisviesiems radikalams. Tai turi didelę įtaką NR vaidmeniui gyvosiose sistemose. Apžvelgiamos NR struktūrinės, katalitinės ir potenciometrinės savybės, jų oksidatorių substrato specifiškumas bei struktūriniai veiksniai, darantys įtaką šiam specifiškumui. Be to, aptariami biomedicininiai NR funkcijos ir taikymo aspektai, apimantys jų reikšmę atsparumui antibiotikams, taikymą vėžio genų terapijoje ir NR-medijuojamose ląstelių abliacijos technologijose.