

PEARLS

Nitrofurans: Revival of an “old” drug class in the fight against antibiotic resistance

Vuong Van Hung Le^{1,2*}, Jasna Rakonjac^{1,2*}**1** School of Fundamental Sciences, Massey University, Palmerston North, New Zealand, **2** Maurice Wilkins Centre, University of Auckland, Auckland, New Zealand* v.le@massey.ac.nz (VVHL); J.Rakonjac@massey.ac.nz (JR)

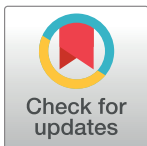
Introduction

Antibiotic resistance is one of the greatest contemporary threats to the human health, which has increasingly been undermining the effectiveness of existing antimicrobial therapies. Development of novel antibiotics is inarguably the key to combat this threat, yet this is a lengthy process (10 to 15 years) at a hefty price tag of approximately US\$1.3 billion for development of an approved drug [1,2]. A new drug, once introduced into the market, also faces the risk of drug resistance emergence. It is, therefore, essential to diversify therapeutic strategies, including revival and reintroduction of “old” antibacterials for treating multidrug-resistant pathogens [3]. Nitrofurans class of synthetic molecules, introduced in the 1940s and 1950s, belongs to this category [4]. Several nitrofurans are currently on the market: nitrofurazone for topical infections and urinary catheter coating, nitrofurantoin for urinary tract infections, and furazolidone for bacterial diarrhea and *Helicobacter pylori* infections. Here, we highlight aspects of this drug class that have recently been unraveled, laying foundation for future improvements and judicious uses of nitrofurans against ever-expanding antibiotic-resistant bacteria.

Nitrofuran-activating enzymes in *Escherichia coli*

Nitrofuran compounds are prodrugs. In *E. coli*, they are activated via reduction by 2 type I oxygen-insensitive nitroreductases, NfsA and NfsB. These enzymes catalyze a stepwise 2-electron reduction of the nitro moiety into reactive nitroso and hydroxylamino derivatives, one of which is considered responsible for the antibacterial activity of nitrofurans (Fig 1A) [5–7]. Peterson and colleagues reported the existence of type II nitroreductase activity in *E. coli* that reduces nitrofuran by 1-electron transfer mechanism and is sensitive to molecular oxygen (Fig 1A) [8]. The identity of enzymes catalyzing the oxygen-sensitive nitrofuran reduction has not been revealed until 2019, when we identified a novel nitrofuran-activating enzyme by selecting for furazolidone-resistant mutants in an *nfsA nfsB E. coli* double knock-out strain. A total of 15 independently isolated mutants resistant to bactericidal concentration of furazolidone contained mutations in the *ahpF* gene, which encodes a component of the antioxidant alkyl hydroperoxide reductase [9]. Subsequent enzymatic assays of purified AhpF protein determined that this enzyme is a type II oxygen-sensitive nitroreductase [9].

The antibacterial mechanism of the nitrofuran derivatives, once activated by nitroreductases, is ill defined. Multiple effects have been observed, including DNA lesions and oxidative stress and inhibition of the RNA and protein biosynthesis [10–12]. However, it remains to be clarified which of these affected targets (DNA, RNA, and protein) are directly attacked by the nitroreductase-activated nitrofuran derivatives and which are simply downstream effects of the interaction between these derivatives and bacterial essential targets. We hypothesize that the nitro moiety at the C₅ of the furan ring, once reduced by nitroreductases, acts as a warhead



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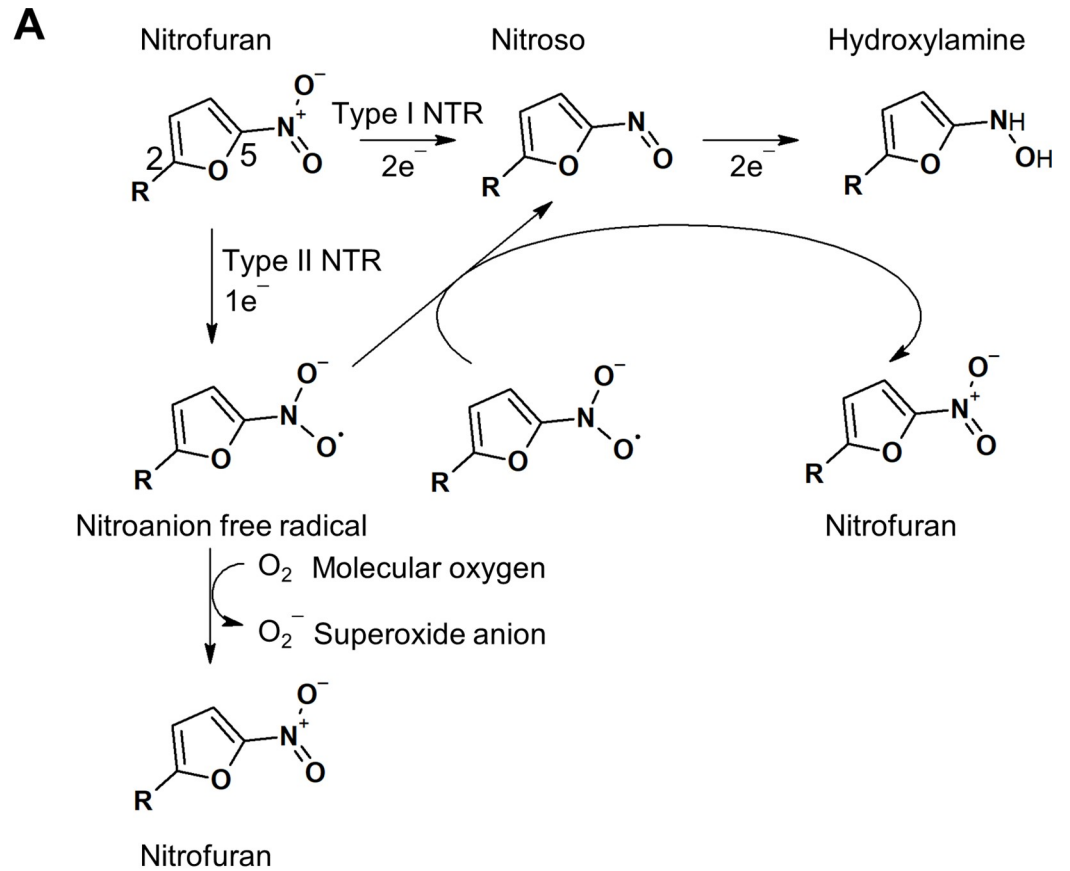
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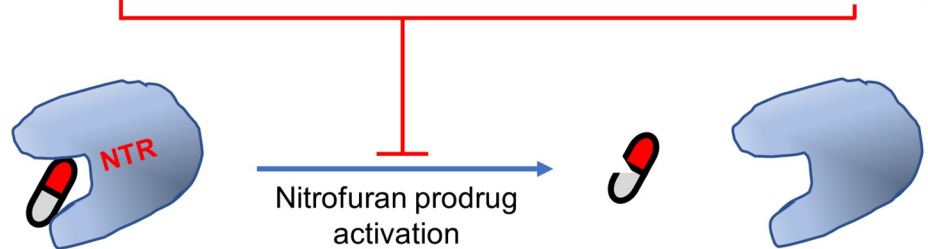
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B

Mutations in *nfsA*, *nfsB*, *ahpF*
[direct disruption of nitroreductase structure and function]

Mutations in *ribE*
[short supply of the nitroreductase cofactor]



C

Mutations in *bla_{CTX-M-14}*
[gain ability to recognize nitrofurans as its substrates]

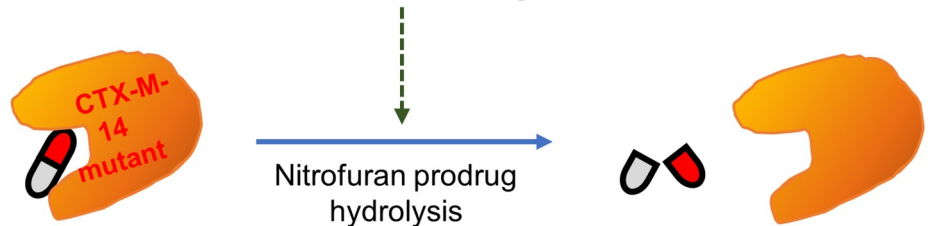


Fig 1. Mechanism of nitrofurantoin activation and resistance. (A) Schematic pathway of nitrofurantoin reduction by NTR enzymes. C₅ and C₂ of the furan ring are numbered. (B) Nitrofurantoin resistance by mutations that disrupt prodrug activation. (C) Nitrofurantoin resistance mechanism by a mutant of β -lactamase CTX-M-14 (*bla*_{CTX-M-14}) that catalyzes nitrofurantoin hydrolysis. NTR, nitroreductase.

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to covalently modify the targets, while the side chain at the C₂ of the furan ring defines its selectivity, depending on how well it fits into the ligand-binding pocket of a target. Future experiments looking into the interaction between nitrofurantoin and individual *E. coli* essential proteins (approximately 300 in total) in the presence and absence of a nitroreductase may shed light on the nitrofurantoin cognate targets.

Existence of potential unknown nitrofurantoin-activating enzymes in *E. coli* presents another interesting question for this antibiotic class. The observations that nitrofurantoin still retain antibacterial activity against the triple *nfsA nfsB ahpF* mutant, either individually or in a synergistic manner with sodium deoxycholate, an antimicrobial bile salt [9,13], point to the existence of these enzymes, although contribution of these enzymes to the overall antibacterial activity of the nitrofurantoin (nitrofurantoin and furazolidone) assayed in these studies may not be remarkable. The modest effect on nitrofurantoin minimum inhibitory concentration (MIC) of these unknown enzymes could be due to low protein expression or low affinity for the nitrofurantoin tested. Identification of these nitrofurantoin-activating enzymes in *E. coli* and understanding their biology (physiological functions, regulation of protein expression/action, and enzyme–nitrofurantoin molecular interaction) would provide useful knowledge to facilitate development of new nitrofurantoin-based antibacterial therapies. For example, in the case of potential low expression of the activating enzyme, structure-based virtual screens with an ultra-large library [14] targeting the expression regulator of the candidate enzyme or targeting the enzyme allosteric site can be employed to seek the adjuvant molecules that up-regulate the enzyme expression or enhance the enzyme activity, respectively; these adjuvant molecules can be used with nitrofurantoin antibiotics for improved antibacterial potency. Alternatively, rational design of nitrofurantoin analogs such that they favorably bind to the redox active site of the activation enzyme through *in silico* enzyme–drug docking simulation, followed by experimental validation, may generate novel nitrofurantoin candidates that are different from clinically used nitrofurantoin drugs in the terms of prodrug activation and resistance mechanisms. A similar strategy can be used to identify novel nitrofurantoin that are effective against bacteria outside of the spectrum of action of current nitrofurantoin, e.g., *Pseudomonas aeruginosa*.

Nitrofurantoin resistance mechanisms in *E. coli*

Mutations of *nfsA* and *nfsB* are the major bacterial mechanism for gaining resistance to nitrofurantoin, in both laboratory and clinical strains of *E. coli*. There is one exception in which a de novo–selected nitrofurantoin-resistant *E. coli* strain had the wild-type *nfsA* and *nfsB* alleles but contained an in-frame deletion in the *ribE* gene that encodes an enzyme in the biosynthesis of flavin mononucleotide, an essential NfsA/NfsB cofactor (Fig 1B) [15]. Perhaps due to the reduced bacterial growth rate caused by the *ribE* mutation, mutations in this gene have not been reported in *E. coli* clinical isolates and are unlikely to pose a significant threat to the efficacy of nitrofurantoin treatment.

The inability to prepare for the unknown often leads to disastrous consequences. In this spirit, a possible novel nitrofurantoin resistance mechanism(s), besides well-known *nfsA/nfsB* mutations, must receive due attention and resources in order to sustain the utility of this drug class. While the prevalence of nitrofurantoin resistance among *E. coli* clinical isolates in recent epidemiology surveys around the world is still low [9], nitrofurantoin-hyperresistant isolates with the

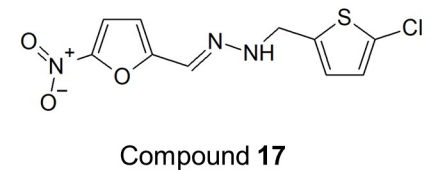
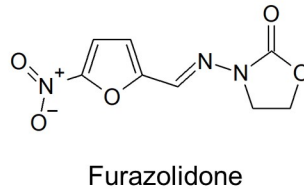
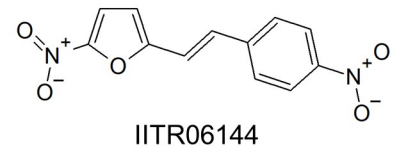
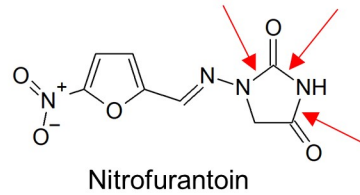
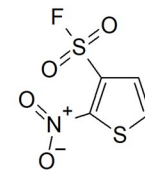
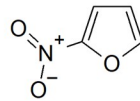
A**B**

Fig 2. New nitrofuran molecules IITR06144 and compound 17 (A) and a nitroaromatic pharmacophore 2-nitrothiophene-3-sulfonyl fluoride (B). Red arrows indicate the amide bonds of nitrofurantoin that are hypothetically hydrolyzed by the CTX-M-14 triple mutant variant.

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MIC higher than 128 $\mu\text{g}/\text{mL}$ have been encountered [15,16]. This high level of resistance cannot solely be explained by mutations in the *nfsA*, *nfsB*, and *ahpF* genes and points to unknown resistance determinants that are already circulating in pathogenic strains.

Of great concern is the reported nitrofurantoin resistance in *E. coli* uropathogenic clinical isolates in one hospital in North Wales (United Kingdom) in 2020 [17]. These isolates were found to have a mutated version of the extended spectrum β -lactamase CTX-M-14, which differs from the wild-type protein by 3 nonsynonymous changes (T55A, A273P, and R277C). When recombinantly overexpressed in an *E. coli* laboratory strain, the mutated CTX-M-14 enzyme caused hyperresistance to nitrofurantoin while retaining the ability to render β -lactam resistance [17]. The purified enzyme was able to hydrolyze nitrofurantoin in the *in vitro* enzymatic assay (Fig 1C). Although the exact hydrolytic products remain to be determined, we speculate that the triple CTX-M-14 mutant, by analogy to β -lactam drugs, may cut at the amide bonds in the hydantoin ring (Fig 2A, red arrows) such that the hydrolytic products have lower affinity to the activation enzymes or to the essential targets of nitrofurantoin. While the work is preliminary, this finding is very worrisome. The recent switch from trimethoprim/sulfamethoxazole combination to nitrofurantoin as the first-line therapy for urinary tract infections has majorly increased the exposure of *E. coli* to nitrofurantoin and will very likely select for this triple mutant of CTX-M-14. Threat of the CTX-M-14-mediated nitrofuran-resistant mutant is high, given that this is one of the 2 most predominant types of the extended spectrum β -lactamases globally [18]. Further investigation is urgently needed to understand the prevalence and expression of this CTX-M-14 variant that causes resistance to nitrofurantoin, the mechanism of nitrofurantoin hydrolysis by this variant and probably other β -lactamases,

the potential of known β -lactamase inhibitors to block the hydrolyzing activity against nitrofurans. Additionally, *in vitro* evolution experiments with clinically important β -lactamase enzymes, directed toward nitrofurantoin hydrolysis and resistance, would predict what mutations may be selected under nitrofurantoin exposure in clinical settings. These efforts will help maintain the effectiveness of nitrofurantoin against the β -lactamase-positive *E. coli*.

New nitrofurans for the future of infectious disease therapies

There have been very few attempts to develop next-generation nitrofuran antibacterial drugs in several decades. Over the past few years, however, work on nitrofurans has been revived, in parallel with their resurgence as an effective treatment option in the context of widespread resistance to other antibiotics. One prominent trend is to search for nitrofuran analogs that have a broadened spectrum and increased potency in comparison to the existing clinically used nitrofurans, such as IITR06114, a novel nitrofuran recently discovered in a small molecule screen [19], and compound 17 identified in a hit-to-lead optimization effort (Fig 2A) [20]. Extensive medicinal chemistry efforts have been undertaken to design novel antimycobacterial agents from the nitrofuran scaffold, culminating in a number of candidates with the submicromolar to nanomolar *in vitro* MICs [21,22]. It remains to be seen whether these nitrofuran candidates will be successfully brought to clinical trials in the years to come. Development of a new nitroaromatic pharmacophore, 2-nitrothiophene-3-sulfonyl fluoride, is also worth noting (Fig 2B). This new molecule has a similar molecular structure to nitrofurans and shares the mechanism of nitroreductase-mediated activation, providing a promising starting point from which novel potent drugs can be developed, including those that may be effective against formidable Gram-negative pathogens, such as *Acinetobacter baumannii* and *P. aeruginosa*, which naturally have high nitrofuran MICs [23].

Given that development of new drugs takes years, it is also important to conserve and enhance the effectiveness of already available nitrofurans. This can be achieved by combinations with other antimicrobial agents, capitalizing on interactions between them, either via synergistic interactions or collateral sensitivity. A synergistic interaction means that the antibacterial potency of 2 antibiotics, when used in combination, is stronger than the combined effect of the individual antibiotic when used alone. Such an effect was, for example, reported in the 2-way combinations of nitrofurans with the secondary bile salt deoxycholate [13], and vancomycin [24], and further enhanced in the triple combination of nitrofurans, deoxycholate, and vancomycin [25]. Meanwhile, another beneficial interaction that can be exploited is collateral sensitivity, where a resistance mechanism to one antibiotic confers increased sensitivity to another. For example, loss-of-function mutation of the protease-encoding *lon* gene causing tigecycline resistance results in hypersensitivity to nitrofurantoin [26]. Collateral sensitivity leads to a strategy of switching treatment from tigecycline to nitrofurantoin in sequential therapies to eliminate the strains resistant to the former, while increasing the chance of a successful bacterial clearance with the latter [26].

In contrast to high potency against *E. coli*, nitrofurans have very limited inhibitory effect, if any, on growth of *P. aeruginosa*, an opportunistic Gram-negative pathogen that causes respiratory system infections and urinary tract and soft tissue infections. In a screen for quorum sensing inhibitors from a library of Food and Drug Administration (FDA)-approved drugs, nitrofurazone was found to inhibit PqsE, a regulator of *P. aeruginosa* quorum sensing system. Nitrofurazone suppressed pathways regulated by this protein, including biofilm formation and production of virulence factor pyocyanin [27]. This finding opens an avenue for the repurposing of nitrofurans as antivirulence drugs to attenuate the severity of *P. aeruginosa* infections [28].

Conclusions

The spread of antimicrobial resistance has led to a resurgence of nitrofurans, an old class of antibiotics. Recent studies have shed light on the mechanism of nitrofuran activation and raised urgent questions about emerging resistance mechanisms. Current efforts, such as search for improved nitrofuran derivatives, development of new pharmacophores, drug combinations, and repurposed uses of nitrofurans, hold a promise to make this drug class an important weapon in the combat against multidrug-resistant bacterial pathogens in the future.

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