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Current and Future Prospects of Nitro-compounds as Drugs for Trypanosomiasis and Leishmaniasis

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Abstract: Interest in nitroheterocyclic drugs for the treatment of infectious diseases has undergone a resurgence in recent years. Here we review the current status of monocyclic and bicyclic nitroheterocyclic compounds as existing or potential new treatments for visceral leishmaniasis, Chagas' disease and human African trypanosomiasis. Both monocyclic (nifurtimox, benznidazole and fexinidazole) and bicyclic (pretomanid (PA-824) and delamanid (OPC-67683)) nitro-compounds are prodrugs, requiring enzymatic activation to exert their parasite toxicity. Current understanding of the nitroreductases involved in activation and possible mechanisms by which parasites develop resistance is discussed along with a description of the pharmacokinetic / pharmacodynamic behaviour and chemical structure-activity relationships of drugs and experimental compounds.

Keywords: Human African trypanosomiasis; Chagas' disease; Visceral leishmaniasis; Nifurtimox; Benznidazole, Fexinidazole, Pretomanid; Delamanid; Nitro-drugs.

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1. INTRODUCTION

Unicellular flagellated parasites belonging to the order Kinetoplastida are the causative agents of Chagas' disease (*Trypanosoma cruzi*), human African trypanosomiasis (HAT) (*T. brucei gambiense* and *T. b. rhodesiense*) and visceral leishmaniasis (VL) (*Leishmania. donovani* and *L. infantum*) [1]. Collectively, these insect-transmitted diseases represent a considerable health burden to millions of people, with current total deaths estimated at 74,000 per annum and an annual health burden of 5 million disability adjusted life years (DALYs) (Global Health Estimates 2014 Summary Tables available at http://www.who.int/healthinfo/global_burden_disease/en/). There are no approved vaccines available and, although vector control measures such as insect traps, indoor residual spraying of insecticides and long-lasting insecticide-treated bed-nets are valuable in reducing disease transmission, they are inadequate on their own for disease elimination. Unfortunately, the current drugs used to treat these life-threatening diseases are inadequate due to a variety of reasons including poor efficacy, drug resistance, toxicity, the need for hospitalisation and cost of treatment. Safer, affordable broad-spectrum treatments are required.

Interest in nitro-compounds as anti-trypanosomal agents originated with the nitrofuran, nitrofurazone, (Figure 1) discovered in 1944 by Dodd and Stillman and used as a topical antibiotic during World War II [2]. Clinical trials in the early 1960s revealed the potential therapeutic value of nitrofurazone in the treatment of Chagas' disease and HAT (see review [3]). However, toxicity due to polyneuropathy and haemolytic anaemia were serious limitations. Nonetheless, nitrofurazone can be seen as the forerunner of nifurtimox, introduced in the late 1960s and still in use today for Chagas' disease and HAT. It is noteworthy in this respect that only clinical and experimental nitro-compounds and oxaboroles have broad spectrum activity across the trypanosomiases and leishmaniases – other drugs such as the arsenicals, antimonials, amphotericin formulations and aminoglycosides are restricted to clinical use for a specific disease. A review of the broad spectrum activity of nitroimidazoles against parasites, mycobacteria and anaerobic Gram positive and Gram negative bacteria has recently been published [4].

2. CURRENT NITRO-DRUGS

Studies as early as the 1950's established that compounds containing a nitrofuran moiety possess activity against experimental models of *T. cruzi* infection (reviewed in [5]). Subsequent experiments established that the nitrofuran derivative nitrofurazone (Figure 1) could cure mice infected with *T. cruzi*, provided that the dosing regimen was lengthy and uninterrupted. Shortly afterwards nitroimidazoles, such as metronidazole (Figure 1) were also found to be active against *T. cruzi in vitro*. Further work on these two compound classes led to the development of nifurtimox (Lampit™, Bayer 2502, Figure 1) and benznidazole (Rochagan™, Radanil™, Ro 7-1051, Figure 1) as anti-trypanosomal chemotherapies (reviewed in [5,6]).

2.1. Benznidazole and Nifurtimox

Despite the fact that Chagas' disease and *T. cruzi* were discovered more than 100 years ago, there are only 2 available treatments for the disease, both of which are nitro-drugs; the nitrofuran nifurtimox, developed by Bayer, and the nitroimidazole benznidazole, developed by Roche. Both drugs are effective at treating acute-Chagas' disease, although there are geographical variations in efficacy (reviewed in [6]). Of the two, benznidazole is better tolerated, and is therefore the first-line treatment for acute-Chagas' disease and recent chronic infections. However, both drugs have a number of side effects that can reduce patient compliance [7] and have been shown to be mutagenic in the Ames test [8].

The effectiveness of benznidazole treatment in patients with chronic-Chagas' disease is not clear. Several studies have shown that benznidazole can reduce parasite levels in chronic-Chagas' patients; however, there is conflicting evidence as to whether or not reduced parasitaemia leads to improved clinical outcomes (reviewed in [6]). Given the adverse drug reactions associated with benznidazole, it has been necessary to carefully assess potential treatment benefits in a number of separate trials (see below).

The effect of continued *T. cruzi* infection upon patients suffering from chronic chagasic cardiomyopathy (CCC) is not definitively understood (reviewed in [6,9]). In order to assess if benznidazole treatment had an effect

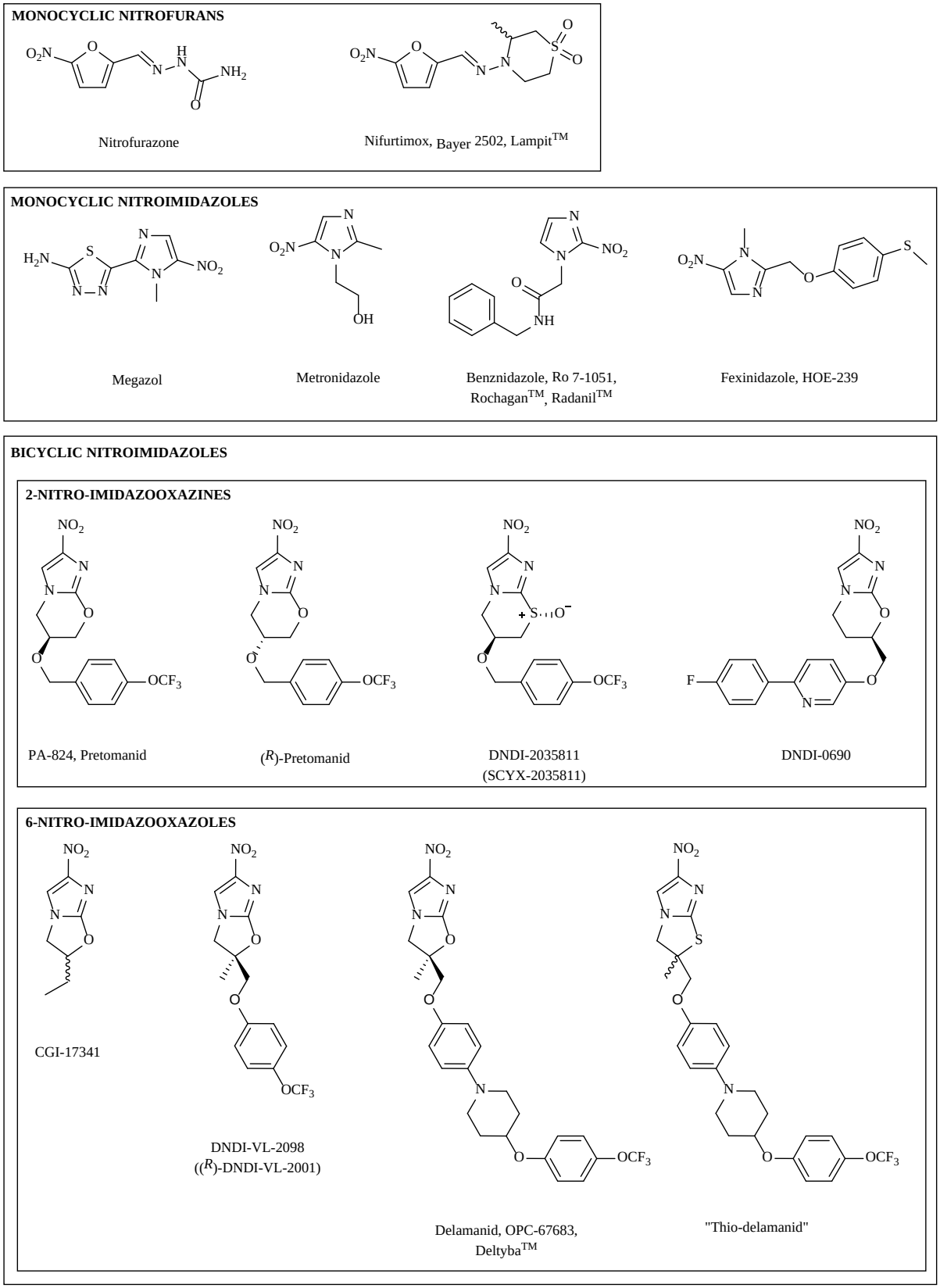


Figure 1 Structures of experimental and clinical nitro-drugs

upon cardiac deterioration, nearly 3000 adult patients with evidence of cardiomyopathy were enrolled onto a double-blind, placebo controlled trial; the BENEFIT project (benznidazole evaluation for interrupting trypanosomiasis) [10,11]. The trial concluded that benznidazole treatment had no effect upon the progression of CCC despite a reduction in circulating parasites. As such, there is still insufficient evidence to recommend benznidazole treatment in chronic-Chagas' patients.

As vector control improves, congenital transmission accounts for an increasing proportion of those infected with *T. cruzi*. To establish if drug treatment with either nifurtimox or benznidazole had an effect on the levels of congenital Chagas' disease, Fabbro and co-workers performed a retrospective, observational cohort study [12]. Their analysis compared the medical records of infected women who had, or had not, been treated for Chagas disease, prior to having children. The study found that treatment of women with chronic-Chagas was remarkably effective at preventing mother-to-child transmission; the level of transmission for treated and untreated women was 0% and 15.3%, respectively.

Current clinical guidelines state that children under 12 with early chronic, or congenital Chagas' disease should be treated with benznidazole. This drug treatment has proven to be safe and effective [13], however, the lack of pharmacokinetic (PK) data in children has meant that the amount of benznidazole taken is calculated by weight-based adjustment of the adult dose. Therefore, Altcheh and co-workers conducted a study (NCT00699387) to measure PK data for *T. cruzi* infected children aged 2-12 treated with benznidazole [14]. The concentrations of benznidazole in plasma were found to be lower in children than those reported in adults; however, the drug was still efficacious, with no detectable parasites in all participants who completed treatment. In addition, the incidence of adverse drug reactions was lower in children, with only a single child under the age of 7 displaying side effects. A similar study (NCT01549236) was conducted in children aged 0-2. As a result DND*i* and LAFEPE (Pernambuco State Pharmaceutical Laboratory) have supported the registration and implementation of a child-adapted benznidazole treatment.

The benznidazole PK study in children suggests that there may be some scope to reduce the adult dosage in order to lower the incidence of side effects whilst retaining efficacy, a hypothesis supported by a recent small adult PK study [15]. In addition, PKPD studies also suggest that there is scope to modify the treatment protocol for benznidazole (change from once-daily to intermittent treatment) whilst retaining efficacy [16]. As a result of these and other studies, a multicentre phase II clinical trial designed to evaluate the effectiveness of different benznidazole regimens as treatments for Chagas' disease in adults is currently in progress (NCT03191162). A greater understanding of the PK of benznidazole (and perhaps nifurtimox) will no doubt lead to improved treatment of Chagas' disease.

2.2 Nifurtimox eflornithine combination therapy NECT

Shortly after the discovery that nifurtimox could be used to treat Chagas' disease, further investigations demonstrated that the drug was also active against *T. brucei in vivo* [17]. This prompted a number of researchers to investigate the drug's potential to be repurposed for HAT [18,19]. Nifurtimox showed promise in an initial proof of concept trial for HAT, curing three out of four participants [17]. However, the cure rates in two further studies [20,21] were variable and attempts to produce consistent, high cure rates by increasing drug dosage only served to increase the incidence of unacceptable toxic side effects [22]. As such, nifurtimox was deemed unsuitable for use as a monotherapy and was only prescribed for HAT on compassionate grounds to treat patients who were refractory to approved treatment options [23].

As part of a strategy to counter the increasing incidence of HAT treatment failures, the efficacy of nifurtimox in combination with melarsoprol [24,25], or eflornithine (DFMO) [25] was investigated. Co-administration of nifurtimox and melarsoprol was found to be more effective than either drug as a monotherapy, but, unfortunately, the incidence of toxic side effects (including treatment-related death) was still unacceptably high. The nifurtimox-eflornithine combination therapy (NECT) arm did show promising results [24], which led to NECT being assessed against eflornithine monotherapy in a larger, phase III, non-inferiority trial [26] (NCT00146627). This study, conducted by Médecins Sans Frontières and DND*i* was a success; NECT, consisting of oral nifurtimox (15 mg/kg per day, every 8 h) and intravenous eflornithine (400 mg/kg per day, every 12 h) is non-inferior to eflornithine monotherapy. There were also fewer reported treatment failures, relapses and adverse effects in patients receiving the combination therapy. In addition to NECT's superior cure rate (96.5% versus

91.6% for eflornithine), the combination therapy also possesses logistical advantages. Specifically, a 4-fold decrease in the total number of intravenous eflornithine infusions reduces the complexity of drug administration, as well as the weight and volume of a treatment kit, both of which are important given the extremely limited medical and transport infrastructure in endemic areas [27]. In 2009, NECT was added to the WHO list of essential medicines, and the WHO supply this combination therapy to endemic countries at no cost via drug donations from Bayer and Sanofi. As a result, NECT is now the first-line treatment for stage 2 HAT, and is used to treat all new patients [27]. It is anticipated that access to NECT, the first new HAT therapy for over 30 years will significantly reduce the mortality and morbidity associated with this neglected disease.

3. NITRO-DRUGS IN THE PIPELINE

Nifurtimox and benznidazole are not the only nitroaromatic compounds to be recently considered for anti-trypanosomatid drug development. For example, the 5-nitroimidazole megazol (Figure 1) in combination with suramin was shown to be effective in stage II animal models of HAT [28,29]. Unfortunately, preclinical studies demonstrated that megazol was a potent inducer of *in vitro* and *in vivo* chromosomal aberrations [30] and so the compound was not further developed. Note, the genotoxic effect displayed by megazol is a known liability of this compound class, which together with other associated toxic effects [31] has discouraged some organisations from developing nitro-drugs.

3.1. Fexinidazole

As a result of the potent anti-trypanosomal activities of nifurtimox and megazol, DND*i* made a strategic decision to investigate the potential of related nitro-compounds. This strategy was realised by assembling a collection of >700 nitroheterocyclic compounds followed by screening in an anti-parasitic hit discovery programme. From this collection, the 5-nitroimidazole fexinidazole (Figure 1) was identified as having modest activity against *T. brucei in vitro* [32]. The anti-trypanosomal activity of fexinidazole (HOE 239) had been previously reported by Hoechst AG (now Sanofi) in 1978 [33]. *In vivo* studies conducted by Hoechst AG at the time had shown that fexinidazole co-administered with suramin effectively cured mice in a stage II HAT model [34]. However, Hoechst AG chose not to develop fexinidazole as a HAT therapy.

In vivo studies conducted by DND*i* demonstrated that orally-dosed fexinidazole was curative in both stage I and stage II murine models of HAT as a monotherapy. To allay concerns regarding the genotoxic potential of fexinidazole a number of toxicology assays were undertaken. Although fexinidazole was found to be a bacterial mutagen in the standard Ames test, all assays for mammalian genotoxicity were negative [35]. Further DMPK and safety pharmacology studies indicated that fexinidazole was safe and the compound then progressed into clinical trials. Phase I clinical trials (NCT00982904, NCT01340157, NCT01483170) showed that orally-dosed fexinidazole was generally well-tolerated, with good absorption when taken with food representative of the target population's diet [36]. These trials also showed that fexinidazole is metabolised to its sulfoxide and sulfone metabolites in man, as had been observed in mouse PK studies [32,37,38] (Figure 2). These metabolites also possess anti-trypanosomal activity and their observed blood concentrations post fexinidazole administration were sufficient to suggest that the candidate should be efficacious in stage 2 HAT patients.

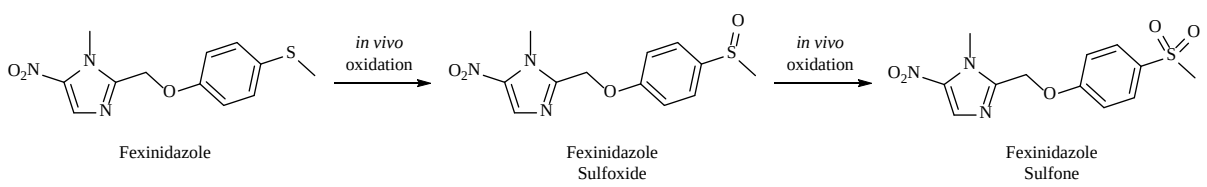


Figure 2. Metabolites of fexinidazole *in vivo*

Fexinidazole is currently undergoing phase III trials against HAT caused by *T. brucei gambiense* (NCT01685827, NCT02169557, NCT02184689, NCT03025789). These ongoing trials aim to assess the efficacy

of fexinidazole against the two disease stages in both adults, and in children aged 6-14. The most recently initiated trial is investigating the use of fexinidazole under real life conditions, with those enrolled being treated both in hospitals and on an out-patient basis. The recently published results of NCT01685827 indicate that fexinidazole is safe and efficacious for the treatment of late stage Gambian HAT [39]. Although less efficacious than NECT (91% versus 98%), oral fexinidazole is deemed to have logistical advantages over NECT due to ease of administration, possible home-based treatment rather than hospitalization and decreased risk of infection. It is hoped that fexinidazole will become the first oral therapy for both stage I and stage II HAT.

In *T. brucei* nifurtimox and fexinidazole are bio-activated by a type I nitroreductase (NTR) (see Mode of Action section below). The genome of *L. donovani* contains a homologous *NTR* gene [40], therefore, Wyllie and co-workers chose to investigate the anti-leishmanial potential of fexinidazole. *In vitro* assays demonstrated that the potency of fexinidazole against *L. donovani* promastigotes was comparable to that against bloodstream form *T. brucei* [41]. However, when assayed against the more clinically relevant intracellular amastigotes fexinidazole was found to be inactive. As fexinidazole is rapidly metabolised *in vivo* (Figure 2), its oxidised metabolites were also assayed and found to have promising activity against *both* extracellular and intracellular *L. donovani* amastigotes.

PK studies had shown that the free blood concentration of fexinidazole sulfone exceeds the compound's *L. donovani* EC_{99} value for 24 h in mice dosed orally with fexinidazole at a well-tolerated dose. Therefore, fexinidazole was progressed into a mouse model of VL, where it was found to have efficacy comparable to the drugs miltefosine and pentostam [41]. As the stage I clinical trials for fexinidazole had proven positive (see above), it was possible to initiate a phase II proof of concept trial to determine the efficacy of the candidate in VL patients (NCT01980199). The trial enrolled 14 patients, the majority of which were clear of parasites at the end of the treatment period. However, when the patients were re-examined after 6 months, only 3 remained cured, and the trial was terminated due to a lack of efficacy [42]. The DMPK in VL patients was similar to that observed in HAT patients and there was an association between higher exposure to fexinidazole sulfone with longer time to relapse in the leishmaniasis trial (Dr Graeme Bilbe, DND*i*, personal communication). The plan to extend treatment using fexinidazole in combination with miltefosine has been abandoned in favour of other promising oral chemical entities currently in pre-clinical development [43].

The preclinical work conducted by Hoechst AG had also demonstrated that fexinidazole was active against *T. cruzi in vivo* [33,44]. Post "rediscovery" DND*i* assessed the efficacy of fexinidazole in mice infected with *T. cruzi* strains with varying susceptibilities to benznidazole [45]. In all cases fexinidazole was found to be more effective than benznidazole, with cures rates greater than 70% in animals treated during the acute or chronic phase. In an additional preclinical study the efficacies of fexinidazole's metabolites were tested in a model of acute infection, and found to have higher cure rates than the parent compound [46]. Again, the preclinical and phase I trial data from the HAT drug development programme allowed fexinidazole to be rapidly progressed for a new indication. The efficacy of fexinidazole in Chagas disease patients was examined in a phase II, proof of concept dose ranging study in Bolivia (NCT02498782). Unfortunately, the study was interrupted due to safety and tolerability issues. Further analysis of the trial data identified good efficacy in those treated at the lowest dosing level used in the study [47]. Therefore, a new proof of concept study was initiated in Spain in 2017, with the results expected in 2019 (EudraCT Number 2016-004905-15). The recent poor phase II trial results for fosravuconazole (E1224) [48] and posaconazole [49,50] have left the Chagas disease drug pipeline worryingly underpopulated. It is hoped that fexinidazole will progress to provide a much-needed new drug for Chagas disease.

3.2. Bicyclic nitroimidazoles – (*R*)-PA-824, delamanid, DNDI-VL-2098, DNDI-0690.

A number of recent studies have also highlighted the therapeutic potential of nitroaromatics as anti-tubercular agents [51-54]. The anti-trypanosomatid activity of fexinidazole encouraged a number of research groups to investigate the potential of anti-tubercular nitroimidazoles as anti-parasitic agents. For example, the DND*i* entered into a product development partnership with the TB Alliance to facilitate the screening of a library of nitroimidazoles in anti-parasitic assays [55].

The nitroimidazole pretomanid (PA-824, Figure 1), a compound currently in stage II/III clinical trials for TB [56] was profiled against kinetoplastid parasites at the University of Dundee [38,57] and found to possess

promising *in vitro* activity against *L. donovani*, but poor activity against *T. brucei* and *T. cruzi*. *In vivo* studies demonstrated that pretomanid had some efficacy in a VL mouse model, but not sufficient to warrant further development. However, during hit expansion the enantiomer (*R*)-pretomanid was synthesised and found to possess sub-micromolar activity against intracellular *L. donovani* amastigotes, in addition to promising potency against *T. cruzi* amastigotes *in vitro*. Note, this differs to *M. tuberculosis*, where (*R*)-pretomanid is inactive [58]. *In vivo* studies demonstrated that (*R*)-pretomanid was well-tolerated by mice, and had a similar PK profile to pretomanid. Oral dosing of (*R*)-pretomanid at 100 mg/kg was subsequently found to suppress parasite infection by 99.9% in the murine VL model [57]. Unfortunately, it is the *S* and not the *R* enantiomer of pretomanid which is undergoing clinical development for TB. Therefore, (*R*)-pretomanid cannot be rapidly progressed into phase II clinical trials in the same manner as fexinidazole for VL / Chagas disease (see above), but must instead be considered a promising lead compound. Due to the promise shown by related compounds (see below), (*R*)-pretomanid has not been further developed for VL.

Independently, DND*i* investigated analogues of pretomanid as nitroimidazole backups for fexinidazole (HAT) [27]. From their work, they identified DNDI-2035811 (Figure 1) (the active enantiomer of SCYX-1227) as a promising lead, which was curative when orally dosed in a stage I HAT mouse model. However, in further studies DNDI-2035811 did not demonstrate adequate cure rates in the stage II CNS animal model [59]. In light of the progress made by fexinidazole (HAT) (see above) and the oxaborole SCYX-7158 [60], development of DNDI-2035811 was placed on hold in 2013, and the project subsequently terminated [61]. However, a recent report by Thompson and co-workers [59] outlines potential strategies to further develop this sub-series, including optimised dosing regimens of DNDI-2035811 and the design and synthesis of novel analogues.

DND*i* also investigated the anti-leishmanial potential of anti-tubercular nitroimidazoles; phenotypic screening against *L. donovani* amastigotes identified the racemic 6-nitroimidazooxazole DNDI-VL-2001 (Figure 1) as being sufficiently potent to progress to *in vivo* studies [62,63]. DNDI-VL-2001 was subsequently found to be highly efficacious in an acute mouse model of VL, with a once-daily 50 mg/kg oral dose of the inhibitor causing a 99.9% reduction in liver parasite levels. A collection of 72 DNDI-VL-2001 analogues across 4 structural sub-classes were then assayed against leishmania parasites *in vitro*, but none were found to be superior to DNDI-VL-2001 itself. Synthesis and assay of the two enantiomers of DNDI-VL-2001 revealed that the *R*-enantiomer, DNDI-VL-2098 (Figure 1) was more potent than the racemate. DNDI-VL-2098 was found to have satisfactory PK in hamster [64] and showed good efficacy in the chronic hamster VL model. However, during preclinical assessment a link between dose, length of treatment, and testicular toxicity was identified in three animal species, which resulted in the development of DNDI-VL-2098 being halted [65].

The anti-tubercular inhibitor CGI-17341 (Figure 1), another 6-nitroimidazooxazole structurally related to DNDI-VL-2098, has also been shown to possess anti-leishmanial activity, both *in vitro* and *in vivo* [66]. However, CGI-17341 is mutagenic, so is not suitable for further drug development.

The high degree of structural similarity between (*R*)-pretomanid, DNDI-VL-2098 and the recently approved [67] TB drug delamanid (OPC-67683, Deltyba™, Figure 1) prompted researchers at the University of Dundee to investigate the drug's anti-leishmanial potential. Delamanid was found to be a highly potent, cidal inhibitor of *L. donovani in vitro* [68]. Delamanid was then assessed in a mouse model of VL and found reduce parasite liver levels by ≥99.5% when orally dosed twice-daily at 30, or 50 mg/kg for 5 or 10 days. Further *in vivo* studies identified two unusual features of the pharmacokinetic/pharmacodynamic (PK/PD) relationship for delamanid in the animal model. First, due to delamanid's high plasma protein binding (PPB 99.5%) the free drug blood concentration is below the EC_{90}, which would ordinarily result in a compound displaying insufficient efficacy. Second, administration of delamanid at low doses revealed an unusual U-shaped dose-response curve, with twice-daily dosing at 1 mg/kg for 5 days being more efficacious than dosing at 3, or 10 mg/kg under the same regimen. Indeed, some mice administered a twice-daily oral dose of delamanid at 1 mg/kg for 10 days had no detectable parasites in their livers at the end of the study.

Mode of action investigations by Wyllie and co-workers [69] revealed that delamanid is enzymatically bio-activated (see below). Drugs that produce active metabolites that inhibit multiple targets do not require free blood concentrations >EC_{90} [70], thus bio-activation provides an explanation for delamanid's unexpected efficacy in the VL model. However, the cause of the U-shaped dose-response curve is currently unknown. *In vivo*, delamanid is known to undergo initial albumin-catalysed metabolism resulting in the degradation of the nitroimidazole moiety [71]. Further metabolism to seven other metabolites occurs via hydrolysis reactions and oxidation by cytochrome P450 enzymes (Figure 3), with some metabolites accumulating at significant

concentrations [72,73]. It has been hypothesised that one or more delamanid metabolites might possess biological activity antagonistic to delamanid's anti-leishmanial mechanism of action [68]. If the formation of this putative antagonistic metabolite occurred via a saturable process giving an S-shaped concentration curve, then this could result in the observed U-shaped dose-response curve for delamanid. Researchers at the University of Dundee are currently investigating this hypothesis in collaboration with Otsuka Pharmaceutical Company (OPC). It is hoped that a greater understanding of the *in vivo* mode of action of delamanid will allow the drug to be further developed and ultimately repurposed [18,19] for VL.

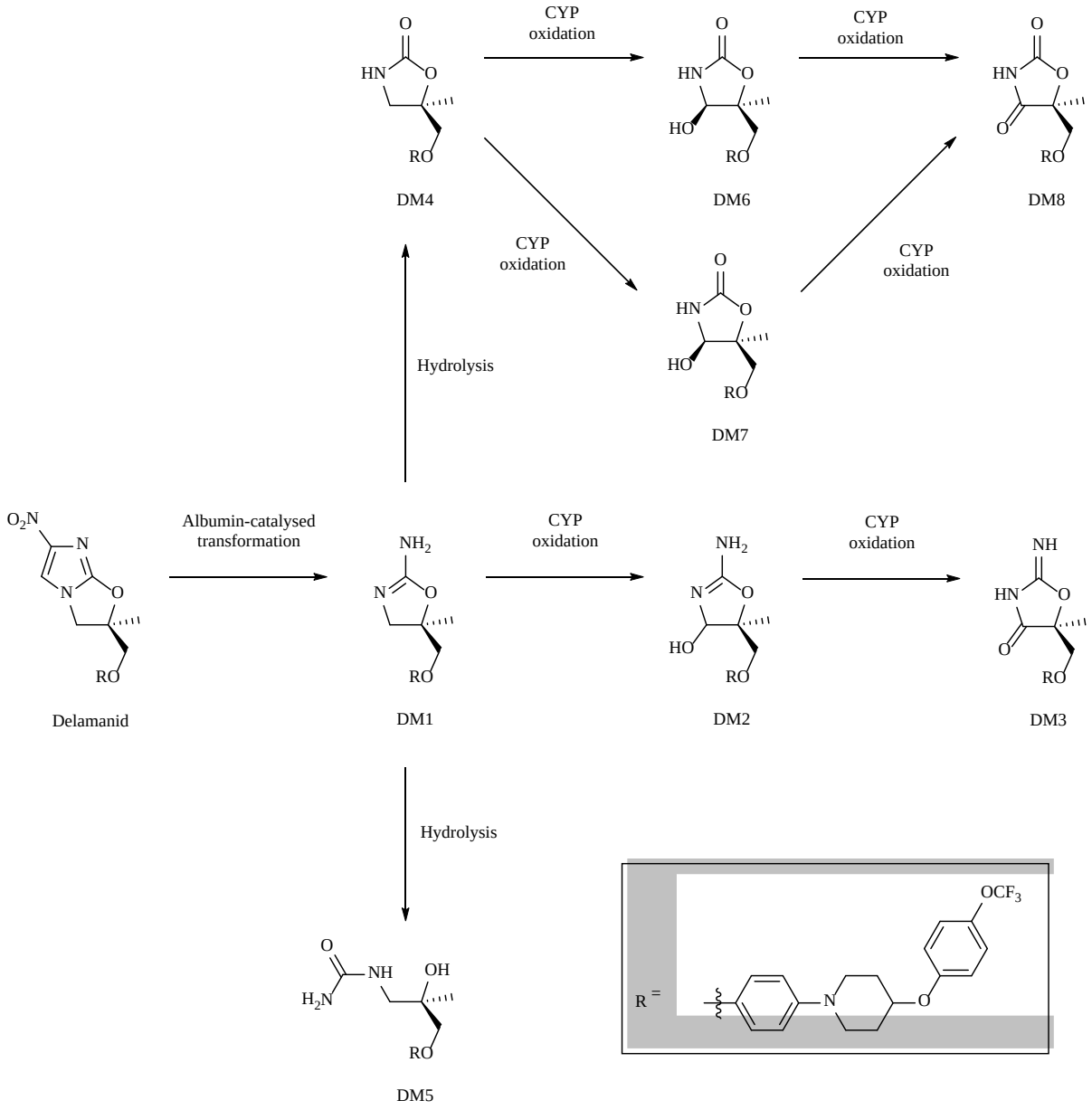


Figure 3. Metabolites of delamanid formed *in vivo*

Delamanid has also been shown to have good activity against *T. cruzi* intracellular amastigotes *in vitro* [63] (Manu de Rycker, personal communication). However, delamanid was not efficacious in a mouse model of Chagas disease [74] when orally dosed twice-daily at 50 mg/kg for 20 days (Laste Stojanovski & Prof Kevin Read, personal communication.). Without a detailed understanding of delamanid's antitrypanosomal mode of action it is not possible to establish the reason(s) for the lack of efficacy. It is possible that the potency of

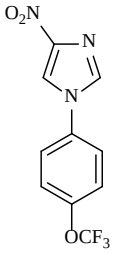
delamanid is insufficient; the EC_{50} for intracellular *T. cruzi* is 5-fold inferior to that for *L. donovani* (0.43 μM vs 0.087 μM, respectively [63,68]). The whole blood concentration of delamanid in infected animals does exceed the *in vitro T. cruzi* EC_{99} (3.37 μM, 1800 ng/mL) after extended oral dosing, however, the high plasma protein binding (fraction unbound = 0.045 [68]) means that the concentration of free drug is well below the EC_{90}. Additionally, delamanid was found to only be a slow cidal inhibitor of *T. cruzi* trypomastigotes *in vitro* (Dr Adam Roberts, personal communication), which may translate to a lack of efficacy *in vivo*. Encouragingly, researchers at the University of Auckland have prepared a number of delamanid analogues with improved potency against *T. cruzi* [75]. Of these, "thio-delamanid" (Figure 1) proved to be the most active, displaying potency comparable to that for delamanid against *L. donovani*. Therefore, it is hoped that it will be possible to identify a member of the bicyclic nitroimidazole class to advance into preclinical development for Chagas disease.

Following the advancement of DNDI-VL-2098 to preclinical candidate status DND*i* moved to develop a backup nitroimidazole with improved solubility and safety [63,76]. Towards this goal, Thompson and co-workers first optimised the sidechain of the molecule [63] and subsequently switched the pharmacophore from a 2-substituted 6-nitroimidazooxazole to a 7-substituted 2-nitroimidazooxazine [76] to great effect. The resultant molecule, DNDI-0690 (Figure 1) demonstrated excellent *in vitro* activity against *L. donovani*, *L. infantum* and *T. cruzi* (but not *T. brucei*). Critically, DNDI-0690 displayed good oral PK, was negative in the Ames mutagenicity test and had no measurable inhibition of the hERG channel. DNDI-0690 was found to be efficacious in a hamster VL model, with liver parasite levels being suppressed by 99.5% following twice-daily oral dosing at 12.5 mg/kg for 5 days. In light of the positive preclinical results, DND*i* aim to advance DNDI-0690 into phase I clinical trials for VL in 2018 [77]. Note, whilst this manuscript was under review Thompson and co-workers have reported the synthesis and profiling of an expanded collection of pretomanid analogues, which has led to the identification of an additional VL backup candidate, DNDI-8219 [78].

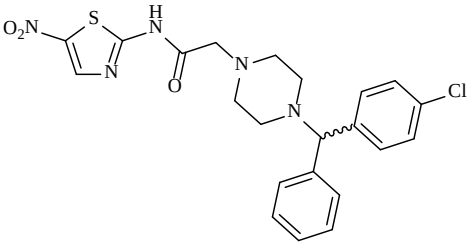
3.3. Other nitroaromatics

In addition to that described above, a number of recent publications have reported numerous novel nitroaromatics which possess promising *in vitro* activity against one, or more kinetoplastid parasites (Figure 4) [79-88]. These and other studies have established that the anti-kinetoplastid nitroaromatic pharmacophore is not limited to just nitroimidazoles and nitrofurans, but encompasses a range of ring systems, including nitrothiazoles (e.g. **II**), nitrothiophenes (e.g. **III**), nitrotriazoles (e.g. **V** & **VIII**), nitrobenzenes (e.g. **VII**), and nitrobenzene-containing bicycles (e.g. **VI** & **IX**) (Figure 4). Further preclinical work has also demonstrated that some of these compound series possess encouraging activity against kinetoplastids *in vivo*: compounds **V**, **VIII** & **IX** have been shown to reduce parasitaemia in acute Chagas disease models [81,84,85] and orally-dosed nitroimidazole **I** is curative in both acute and chronic murine models of HAT [87]. No doubt some of these compound series have the potential to progress from early- to late-stage drug discovery.

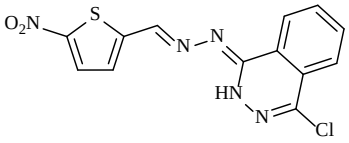
Due to the potential for cross-resistance between antiparasitic nitroaromatics (see below), the mechanism of action of these novel nitroaromatics (Figure 4) has also been investigated. It has been demonstrated that **V** and **VIII** are substrates for NTR (the enzyme which bioactivates nifurtimox, see below), whereas nitrothiazole **II** is not a substrate. Nitrofurans **IV** and **X** have both been shown to be active against nifurtimox-resistant *T. brucei*, suggesting that their modes of action are at least in part divergent.



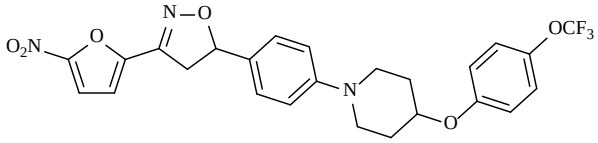
I
bsf *T. brucei* EC_{50} = 0.16 μM



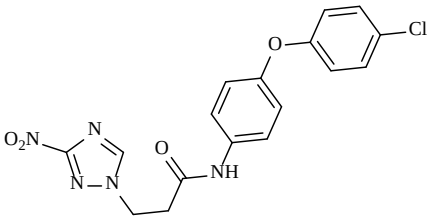
II
am. *T. cruzi* EC_{50} = 0.57 μM
ax. am. *L. donovani* EC_{50} = 3.4 μM



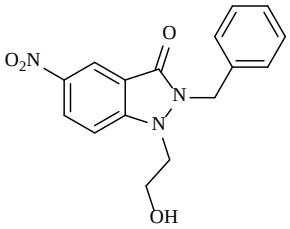
III
epi. *T. cruzi* EC_{50} = 7.2 μM



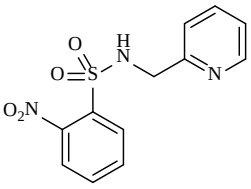
IV
bsf *T. brucei* EC_{90} = 0.015 μM



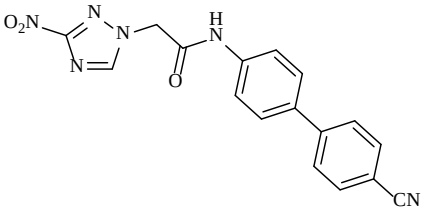
V
bsf *T. brucei* EC_{50} = 0.041 μM
am. *T. cruzi* EC_{50} = 0.005 μM
ax. am. *L. donovani* EC_{50} = 1.2 μM



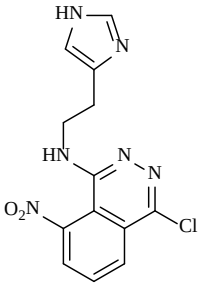
VI
epi. *T. cruzi* EC_{50} = 1.6 μM
am. *T. cruzi* EC_{50} = 0.22 μM



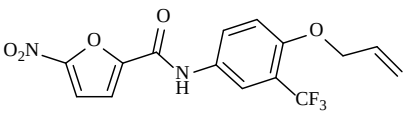
VII
pro *L. donovani* EC_{50} = 131 μM
in am *L. donovani* EC_{50} = 295 μM



VIII
bsf *T. brucei* EC_{50} = 0.34 μM
am. *T. cruzi* EC_{50} = 0.14 μM



IX
epi. *T. cruzi* EC_{50} = 5.6 μM
am. *T. cruzi* EC_{50} = 4.0 μM



X
bsf *T. brucei* EC_{50} = 0.0024 μM

Figure 4: The structures and biological activities of recently reported experimental nitroaromatic compounds possessing activity against one, or more kinetoplastids. Biological activity reported as EC_{50} or

EC_{90} values against blood stream form (bsf) *T. brucei* (*T. b. brucei*, or *T. b. rhodesiense*), intracellular *T. cruzi* amastigotes (am), extracellular *T. cruzi* epimastigotes (epi), *L.donovani* promastigotes (pro), *L. donovani* axenic amastigotes (ax am), and intracellular *L. donovani* amastigotes (in am). References; **I**, compound 16 from[87]; **II**, compound 6 from [88]; **III**, compound 3m from [80]; **IV**, compound 15 from [79]; **V**, compound 6 from [81]; **VI**, compound 20 from [82]; **VII**, compound 2NB from [83]; **VIII**, compound 2 from [85]; **IX**, compound 3 from [84]; and **X**, compound 22s from [86].

4. MODE OF ACTION

The modes of action of the monocyclic nitrofurans and nitroimidazoles, and the bicyclic nitroimidazo-oxazines and nitroimidazo-oxazoles are not fully understood. However, both classes require biological activation by enzymatic reduction using distinct nitroreductases. The enzymes involved in bio-activation differ between trypanosomatid species and caution is required in extrapolating from one parasite to another.

4.1 Nifurtimox

Early studies on the mode of action of the nitrofuran, nifurtimox, implicated redox cycling and radical damage in killing *T. cruzi* parasites [89,90], as well as being responsible for host toxicity [91]. The proposed mechanism involves one electron reduction of the drug by a type II nitroreductase followed by non-enzymatic re-oxidation by molecular oxygen to form superoxide anion ($\cdot O_2^-$) regenerating the parent compound. Subsequently, $\cdot O_2^-$ is converted to hydrogen peroxide (H_2O_2) under catalysis by superoxide dismutase (see review [92]) (Figure 5). If the capacity of the trypanosome to remove $\cdot O_2^-$ and H_2O_2 is exceeded then this results in the formation of the highly reactive hydroxyl radical ($\cdot OH$) via the Haber Weiss reaction (3) catalysed by transition metal ions:

$$\cdot O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad (2)$$

Net reaction $\cdot O_2^- + H_2O_2 \rightarrow O_2 + OH^- + \cdot OH$ (3)

In *T. cruzi*, both NADH- and NADPH-dependent flavoproteins appear to be involved in the activation of nifurtimox [89]. Possible candidates include the mitochondrial dihydrolipoamide dehydrogenase [93], flavoprotein cytochrome P-450 reductase [94] and trypanothione reductase [95]. Another somewhat serendipitous discovery was that the *T. cruzi* "Old Yellow Enzyme" not only has prostaglandin $F_{2\alpha}$ synthase activity, but also has redox cycling activity with anti-trypanosomal quinones such as β-lapachone [96]. Interestingly, these authors also found that Old Yellow Enzyme was able to carry out a two electron reduction of nifurtimox, but not benznidazole. The significance of this observation is not clear, because reduction of nifurtimox was observed only under anaerobic conditions, a physiological state unlikely to be found in the mammalian host.

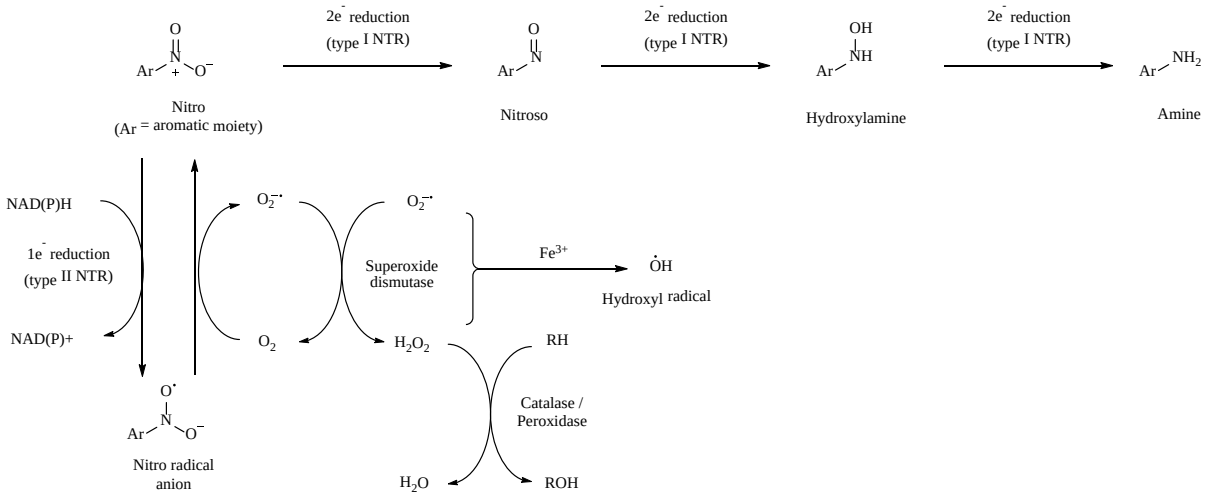


Figure 5. One and two-electron reduction pathways of nitro-compounds. Re-oxidation through the one electron route leads to the formation of reactive oxygen species in cells (oxidative stress). Reduction via the two electron route leads to the formation of reactive nitroso and hydroxylamine intermediates (chemical stress). Adapted from [97] and [92]

The "TriTryp" genome sequencing projects led to the identification and characterisation of a candidate nitroreductase present in all three trypanosomatids [98-100]. These nitroreductases (NTR) showed homology to type I bacteria-like nitroreductases that carry out sequential two electron reduction of nitro-compounds without formation of reactive oxygen species (Figure 5). Loss of a single copy of NTR in *T. cruzi* or *T. brucei* through drug selection for resistance or by gene knockout led to decreased susceptibility to nifurtimox and related nitro-drugs such as benznidazole [101]. However, *T. cruzi* epimastigotes lacking NTR activity were only 4- and 10-fold less susceptible to nifurtimox and benznidazole, respectively, indicating that other activating enzymes must be involved, such as those mentioned above. An additional study in transgenic *T. brucei* overexpressing either NTR, two putative cytochrome P450 reductases or a prostaglandin F-synthase (an aldo-keto reductase distinct from Old Yellow Enzyme) showed that only overexpression of NTR caused an increase in susceptibility to nifurtimox [102]. Furthermore, these authors demonstrated that recombinant NTR and NADH were able to completely metabolise nifurtimox to an open chain unsaturated nitrile via a two electron reduction pathway (Figure 6).

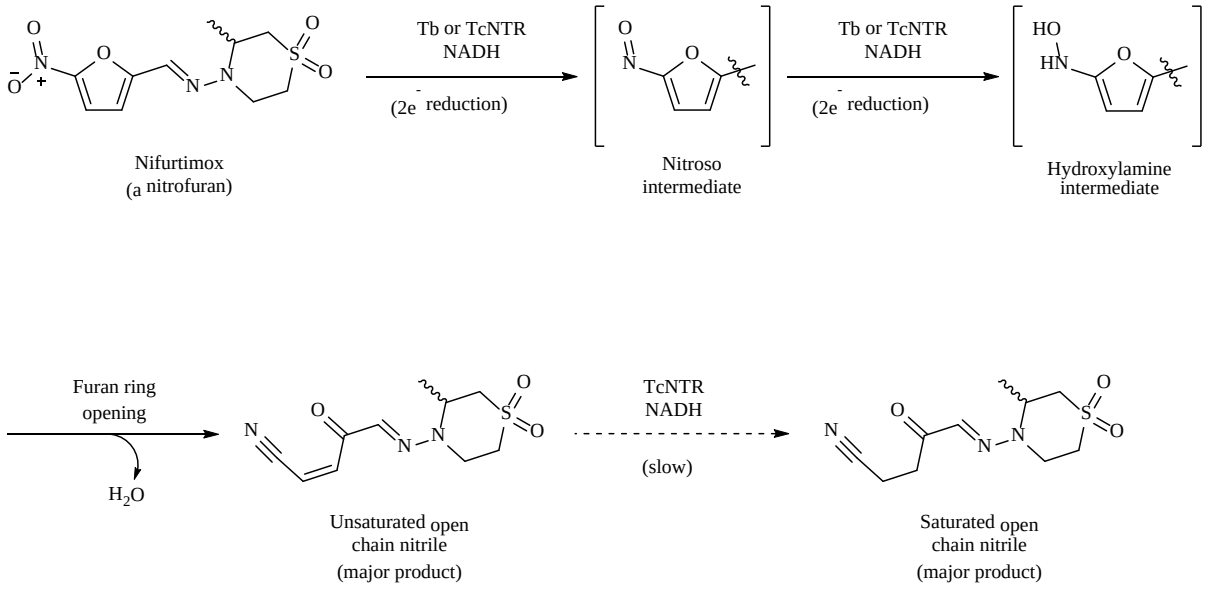


Figure 6. Metabolic products of nifurtimox formed by type I nitroreductases. Modified from [97]

The selective toxicity of nifurtimox and other redox cycling quinones between the host and parasite was originally ascribed to deficiencies in anti-oxidant defences, because medically important trypanosomatids lacked key enzymes such as catalase and selenium dependent glutathione peroxidase. However, with the discovery of trypanothione [103] and its robust ancillary antioxidant systems [104,105], the role of oxidant stress in the mode of action of nifurtimox became less clear [106]. Certainly, in early studies, exposure of parasites to nifurtimox was found to cause marked decreases in the level of intracellular thiols [107], with evidence of DNA damage [108] and lipid peroxidation [109]. The formation of the above highly reactive open chain nitrile could certainly account for loss of thiols and DNA damage observed in earlier studies. However, formation of such DNA adducts remains to be demonstrated.

4.2 **Benznidazole**

The mode of action of benznidazole is considered distinct from nifurtimox in that redox cycling producing reactive oxygen intermediates is not involved; rather reduction of benznidazole is via reduced reactive intermediates that alkylate macromolecules such as DNA, lipids and proteins [110]. However, like nifurtimox, benznidazole metabolites react with the intracellular thiol metabolites glutathione, glutathionylspermidine and trypanothione [111]. Benznidazole is metabolised by recombinant *T. cruzi* NTR via a 4-electron reduction to form 4,5-dihydro-4,5-dihydroxy-imidazole via the chemically reactive nitroso and hydroxylamine intermediates (Figure 7) [112]. 4,5-Dihydro-4,5-dihydroxy-imidazole can also slowly break down to form *N*-benzyl-2-guanidinoacetamide and the dialdehyde, glyoxal [112], although the equilibrium is unfavourable [113]. It is proposed that glyoxal adducts formed with guanosine *in vivo* occur via direct exchange with 4,5-dihydro-4,5-dihydroxy-imidazole rather than with free glyoxal [113]. Studies on whole cells have confirmed the presence of these NTR catalysed metabolites, including *N*-benzyl-2-guanidinoacetamide, but not glyoxal, free or as an adduct with nucleotides, nitrogenated bases or amino acids [114]. Multiple benznidazole-derived adducts were found with redox active thiols such as trypanothione and glutathione, supporting earlier observations [107]. Further work is required to establish which of these effects are important for parasite killing.

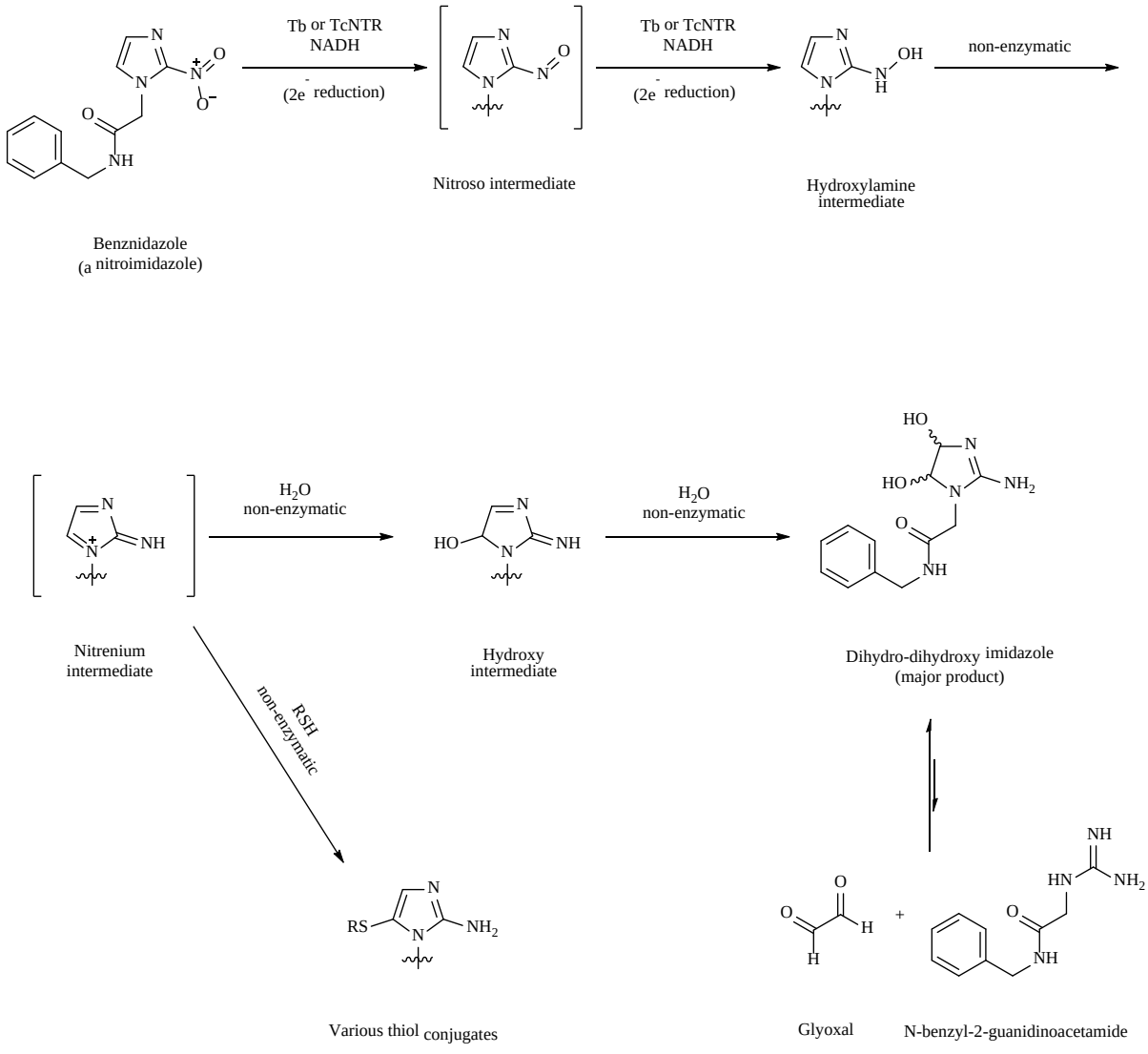


Figure 7. Metabolic products of benznidazole reduction identified *in vitro* or in whole cells. Modified from [97]

4.3 **Fexinidazole**

Indirect evidence indicates that fexinidazole and its metabolites are metabolised by *T. brucei* and *T. cruzi* NTR, but the identity of the drug metabolites has not been reported to date. No information is available on the possible effects of these metabolites on DNA, RNA, protein, lipid or thiol metabolism.

4.4 **Bicyclic nitroimidazoles**

Bicyclic nitroimidazoles with promising *in vivo* anti-leishmanial activity include (*R*)-PA-824 (the opposite enantiomer of pretomanid) and delamanid. Pretomanid and delamanid are anti-tubercular prodrugs that are activated by a deazaflavin-dependent nitroreductase (Ddn) [53,54,115], an enzyme which is absent in *Leishmania spp*. Unlike nifurtimox and fexinidazole, transgenic parasites overexpressing the leishmania nitroreductase (NTR1) are not hypersensitive to either of these compounds, indicating that NTR1 is not the primary target of these compounds [57,68]. The des-nitro analogues were inactive, underlining the potential importance of the nitro-substituent and the possibility of a second nitroreductase. Whole genome sequencing and quantitative proteomics identified loss of a putative FMN-containing NAD(P)H oxidase as the activating nitroreductase (NTR2) [69]. Parasites lacking both copies of *NTR2* were viable and completely resistant to (*R*)-PA-824 delamanid and the (now abandoned) preclinical candidate DNDI-VL-2098. Conversely, parasites overexpressing *NTR2* were hypersensitive to these compounds. Wyllie *et al.* also showed that these compounds were metabolised by wild-type and NTR2 overexpressing parasites, but not in transgenic NTR2 null mutants [69]. The identity of these drug metabolites and their potential targets are being actively pursued in the Fairlamb laboratory and include those identified in *M. tuberculosis* [116] (Figure 8).

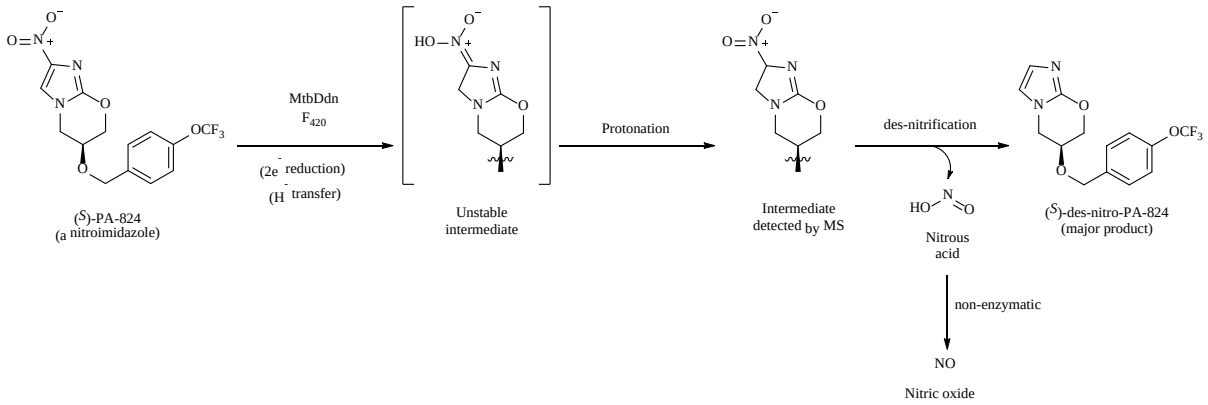


Figure 8. Metabolism of pretomanid (PA-824) in *M. tuberculosis.* Modified from [97]

It is noteworthy that both classes of nitro-compounds require biological activation using distinct nitroreductases resulting in chemical and oxidative stress. Thus, the divergent mechanisms of activation for monocyclic and bicyclic nitroaromatics means that they could potentially be used in combination to reduce the dose and/or duration of treatment.

5. DRUG RESISTANCE

Drug resistance occurs when an organism no longer responds to a concentration of drug to which it was previously susceptible. It is important to differentiate failure of clinical treatment from clinical drug resistance by testing for drug susceptibility *in vitro* or in animal models. Unfortunately, this information is often not so clear cut due to the technical limitations of the assay methods used to detect ongoing infection in patients and drug susceptibility. Moreover, as pointed out in a recent review [117], the reporting of half-maximal effective concentrations (EC_{50} values) without reporting Hill slope of the growth inhibition curve makes it impossible to calculate the more relevant parameters EC_{90} or EC_{99}, the nearest equivalent of minimal inhibitory concentration (MIC) used in bacteriological susceptibility testing. It is also relevant to note that laboratory-derived and clinical resistance mechanisms can differ because laboratory-derived mutants may show decreased fitness in terms of transmissibility or virulence in real life settings.

Clinical response to treatment of Chagas' disease with nifurtimox or benznidazole is reported to vary geographically and with morphologically different strains of *T. cruzi* [118,119]. Cross-resistance to both drugs has also been frequently observed in clinical isolates [119,120]. These studies used prolonged experimental chemotherapy in mice (20-60 days oral treatment with benznidazole or nifurtimox) with criteria of cure defined by negative xenodiagnosis and negative haemoculture to distinguish resistant and susceptible parasite strains. In contrast, *in vitro* drug susceptibility testing does not distinguish strains that show resistance in mice [121] or were derived from patients suffering therapeutic failure [122]. The reasons for this discrepancy are not clear and deserves further investigation. Possibilities include the need for a host immune response to achieve complete parasite clearance [123-125] or residual infection at privileged sites with poor drug penetration [74,126,127].

Experimentally derived resistance to nifurtimox and benznidazole in *T. cruzi* is clearly associated with a reduction in type I NTR gene copy number or point mutations in NTR resulting in decreased or loss of NTR activity [101,128-130]. The potential of NTR as a diagnostic test for resistance in clinical settings remains to be determined. Proteomic and genomic studies have identified many additional drug resistance candidates such as Old Yellow Enzyme [96,131,132], cytochrome P450 reductase [133], tryparedoxin peroxidase [132,134], superoxide dismutase [135,136] and aldo-keto reductase [137,138]. However, one study reported contradictory findings suggesting upregulation of Old Yellow Enzyme rather than downregulation and found no changes in enzyme levels of cytochrome P450 reductase [130] or superoxide dismutase [130]. P-glycoprotein efflux pumps have also been implicated in drug resistance by some investigators [139] but not others [140]. Clearly further investigations are required to clarify some of these conflicting findings.

Clinical efficacy of treatment with nifurtimox monotherapy for second stage HAT is low [22] suggesting that some isolates of *T. b. gambiense* may be inherently resistant to this drug, or nifurtimox fails to achieve sufficiently high therapeutic levels in the central nervous system. Susceptibility of clinical isolates to nifurtimox ranges over 10-fold (EC_{50} values from 0.3 – 3.8 μM) [141,142], similar to the predicted serum concentration (3 μM) in patients [38]. Unfortunately, the concentration of nifurtimox in the central nervous system of patients is not known, so it is difficult to define the pharmacodynamics for stage II sleeping sickness. However, studies in mice suggest that nifurtimox readily crosses the blood-brain barrier [143]. Laboratory selection for resistance to nifurtimox is rapid, stable in the absence of drug selection and cross-resistant to fexinidazole (and vice versa) [38]. NTR was identified and subsequently confirmed as a key resistance determinant along with other as yet unidentified mechanisms [144]. Should resistance arise as a consequence of improperly administered NECT therapy, this could have serious implications for fexinidazole, currently in Phase II/III clinical trials. No data is available on the susceptibility of clinical isolates of *T. b. gambiense* to fexinidazole and therefore the possibility of natural resistance in the parasite population.

In leishmania, NTR1 plays a pivotal role in the activation of fexinidazole or its mammalian metabolites [40,41]. Gene deletion experiments suggest that NTR1 is essential for parasite survival and that deletion of one copy of NTR1 results in only a mild decrease in susceptibility to fexinidazole [40], similar to that observed for *T. brucei* NTR [144]. NTR appears to be dispensable in *T. cruzi* epimastigote stages; however, this results in decreased infectivity of mammalian cells and lack of a patent infection in mice [101,129]. Taken together, these data indicate that the possibility of clinical resistance through changes in NTR expression is limited.

6. TOXICOLOGY OF NITRO-DRUGS

In addition to their use as anti-infectives [4] nitro-aromatic compounds are also approved drugs for the treatment of several other indications [145]. Despite this, the pharmaceutical industry generally avoids the *de novo* development of compounds which include a nitroheterocyclic moiety, and the nitro group is classified as a 'structural alert' in early stage drug discovery [31]. This proactive avoidance is due to the discovery of a number of toxicities linked to the chemistry of the nitro group, specifically, its ability to undergo bio-reduction.

Nitroaromatics can undergo bio-reduction via either a one-, or two-electron process (see section 4.1, Figure 5). One-electron reduction leads to the formation of radical species, which are known to cause modifications to cellular macromolecules [146]: for example, the hydroxyl radical can cause DNA-strand breaks [147], and is one of the mechanisms by which nitro drugs can cause DNA damage [145]. As a result, some nitroheterocycles are mutagenic (and hence genotoxic and potentially carcinogenic), which renders those specific

compounds unsuitable for drug development. In addition, nitroaromatic treatment can cause the production of reactive oxygen species at levels which exceed the antioxidant capacity of a cell, resulting in oxidative stress. Drug-induced oxidative stress has itself been implicated as a cause of tissue and organ toxicity [146]. The nitroso and hydroxylamine intermediates resulting from the two-electron reduction of nitroaromatics (Figure 5) have also been implicated in DNA damage mechanisms [reviewed in [145]].

Thus, lead compounds with a nitro-group structural alert need to be assessed early in development using the Ames *Salmonella*/microsome mutagenicity assay [148]. Although the test has a high positive predictive value in rodent carcinogenicity tests, certain compounds with low redox potentials are only activated by the *Salmonella* nitro-reductases and the use of either the Comet Assay [149] or *in vivo* micronucleus test [150] or a combination of both assays [151] can be important discriminatory tests of mammalian toxicity.

Despite the irrefutable toxicity concerns associated with nitroaromatics, this compound class should still be explored as a source of potential drugs for the kinetoplastid diseases. There are no human homologues of the kinetoplastid NTR1, or leishmanial NTR2 responsible for bio-activating nitro prodrugs in these parasites. Therefore, there is the potential for *selective* bio-activation, and hence kinetoplastid selective toxicity of nitroheterocycles. It is anticipated that an increased understanding of the substrate specificity of NTR1 and NTR2 will assist in the design of selectively activated compounds.

7. CONCLUSION AND FUTURE PROSPECTS

The repurposing of nifurtimox to treat HAT as part of NECT demonstrated that it is possible to develop safe, efficacious nitro drugs for kinetoplastid diseases. The success of NECT heightened interest in this compound class and has resulted in a number of nitro-aromatic compounds being investigated and subsequently developed for VL, HAT and Chagas' disease. Fexinidazole in particular shows considerable promise, and has advanced into clinical trials for all three indications. Recently, a number of anti-tubercular bicyclic nitroimidazoles have also been found to possess anti-parasitic activity in rodent models of VL. Two of these bicyclic compounds, DNDI-VL-2098 and DNDI-0690 have progressed to pre-clinical candidate status, although development of the former has been abandoned due to toxicity.

As nitro-aromatics have advanced through the drug discovery pipeline researchers have investigated the mode of action of both these newer compounds and the existing monocyclic drugs. Advancements in genome sequencing and kinetoplastid proteomics methodology facilitated the discovery of two parasite nitroreductase enzymes, NTR1 and NTR2. These two enzymes have different substrate specificities; NTR1 reductively activates monocyclic nitrofurans/nitroimidazoles, whereas NTR2 bio-activates bicyclic nitroimidazole prodrugs. Structural determination of these proteins would greatly aid in drug design. The metabolic products of the NTR1-catalysed reduction of nifurtimox and benznidazole have been identified, and these reactive metabolites have been implicated in the mechanism of cell killing. The anti-parasitic mode of action of the newer nitroimidazoles remains to be characterised at the molecular level, but could identify new targets in biochemical pathways for drug discovery. There is also evidence that additional enzymes are capable of reducing monocyclic nitro-drugs, suggesting that there may be secondary modes of action for this inhibitor class (particularly in *T. cruzi*).

There is no doubt that nitroaromatics have significant potential to be developed into drugs for kinetoplastid diseases. The identification of kinetoplastid nitroreductases absent in humans, offers the potential to develop compounds that are selectively bio-activated and thus display selective parasite toxicity. Given the number of nitroaromatics in the development pipeline it is likely that this compound class will deliver at least one new therapy.

CONFLICT OF INTEREST

The authors declare no actual or potential conflicts of interest.

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