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A review on metronidazole: an old warhorse in antimicrobial chemotherapy

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Abstract

The 5-nitroimidazole drug metronidazole has remained the drug of choice in the treatment of anaerobic infections, parasitic as well as bacterial, ever since its development in 1959. In contrast to most other antimicrobials, it has a pleiotropic mode of action and reacts with a large number of molecules. Importantly, metronidazole, which is strictly speaking a prodrug, needs to be reduced at its nitro group in order to become toxic. Reduction of metronidazole, however, only takes place under very low concentrations of oxygen, explaining why metronidazole is exclusively toxic to microaerophilic and anaerobic microorganisms. In general, resistance rates amongst the pathogens treated with metronidazole have remained low until the present day. Nevertheless, metronidazole resistance does occur, and for the treatment of some pathogens, especially *Helicobacter pylori*, metronidazole has become almost useless in some parts of the world. This review will give an account on the current status of research on metronidazole's mode of action, metronidazole resistance in eukaryotes and prokaryotes, and on other 5-nitroimidazoles in use.

Introduction

Metronidazole is a 5-nitroimidazole drug that has become the mainstay in the treatment of anaerobic infections worldwide and ranks amongst the 'essential medicines' as defined by the WHO. It was developed in 1959 (Cosar and Julou, 1959) specifically for the treatment of trichomoniasis, an infection of the genital tract caused by the microaerophilic parasite *Trichomonas vaginalis* that was notoriously difficult to treat at that time. Although metronidazole is a synthetic drug, its basic structure derives from 2-nitroimidazole, or azomycin, which had been isolated from *Streptomyces* sp. or other closely related bacteria a few years earlier (Maeda *et al.* 1953). Several independent studies quickly confirmed the imposing effectivity of metronidazole, then already being sold under its brand name Flagyl®, against *T. vaginalis* (Durel *et al.* 1960; Nicol *et al.* 1960; Rodin *et al.* 1960). Soon thereafter, the suitability of metronidazole for the treatment of other microaerophilic parasites, i.e. *Giardia lamblia* (Schneider, 1961) and *Entamoeba histolytica* (Powell *et al.* 1966), was demonstrated. Metronidazole proved to be active against anaerobic and microaerophilic bacteria as well, as shown for *Clostridium* spp. (Freeman *et al.* 1968; Füzi and Csukás, 1969*a*), *Fusobacterium fusiforme* (Füzi and Csukás, 1969*b*), *Bacteroides fragilis* (Nastro and Finegold, 1972) and against *Helicobacter pylori* (Hirschl *et al.* 1988). Indeed, metronidazole is active against the vast majority of anaerobic and microaerophilic pathogens, rendering it an indispensable weapon in our antimicrobial arsenal (Table 1).

Despite its frequent use over such a long period of time, metronidazole has remained a reliable drug for the treatment of most anaerobic/microaerophilic infections, thereby setting it apart from most other antimicrobials to which resistance develops much more quickly (Holmes *et al.* 2016). This is undoubtedly attributable to its pleiotropic mode of action as it targets a large number of molecules in the cell, rather than only a few or even just a single one, as most antimicrobials do. In fact, metronidazole's mode of action is fiendishly simple: it enters the cell without the help of any transporting mechanisms and unfolds its destructive potential after having been reduced to its nitro group, a reaction which occurs only under very low oxygen concentrations.

Nevertheless, metronidazole resistance does occur in some pathogens more frequently than in others; and despite its overall high tolerability, metronidazole can cause unpleasant side-effects. Further, metronidazole and other 5-nitroimidazoles are still under discussion as being potentially carcinogenic. The present review will summarize the most important aspects of metronidazole and gives a comprehensive overview of resistance and safety issues.

Mode of action

Metronidazole uptake occurs without any specific mechanisms such as transporters but depends on metabolic activity ensuring an energized membrane (Müller and Gorrell, 1983; Edwards, 1993). It is, as such, a prodrug which is poorly if at all reactive (Edwards, 1993). However, if the nitro group is reduced (Fig. 1) metronidazole is transformed into a reactive intermediate that reacts with multiple targets in the cell (Müller and Gorrell, 1983). To

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Table 1. Human infections treated with metronidazole

Pathogens	First report
Protist parasites	
Trichomonas vaginalis	Cosar and Julou (1959)
Entamoeba histolytica	Powell *et al.* (1966)
Giardia lamblia	Schneider (1961)
Balanthidium coli	Zaman and Natarajan (1969)
Bacteria	
Helicobacter pylori	Hirschl *et al.* (1988)
Campylobacter spp.	Chow *et al.* (1978)
Clostridium spp.	Freeman *et al.* (1968)
Bacteroides spp.	Nastro and Finegold (1972)
Fusobacterium spp.	Füzi and Csukás (1969*b*)
Gardnerella vaginalis	Ralph *et al.* (1979)
Desulfovibrio spp.	Lozniewski *et al.* (2001)

date, it is still not fully clear which intermediate, determined by the number of electrons transferred to the nitro group, is the actual toxic form. Several propositions were made, ranging from the nitroradical anion stage (one electron transferred) (Lindmark and Müller, 1976; Edwards, 1993; Kulda, 1999) to the nitroso stage (two electrons transferred) or the hydroxylamine stage (four electrons transferred) (Wardman, 1985; Leitsch *et al.* 2007, 2009, 2012*a*, *b*). Importantly, metronidazole has a very low midpoint redox potential (−486 mV) (Smith and Edwards, 1995), thus well below the midpoint redox potential of NADPH and NADH (approximately −320 mV each), resulting in very small amounts of metronidazole being reduced in aerobes. Moreover, oxygen can re-oxidize the metronidazole nitroradical anion in a redox cycling reaction (Mason and Holtzman, 1975), leading to the generation of superoxide anions and the re-established prodrug. In microaerophiles and anaerobes, however, intracellular oxygen concentrations are low and factors exist in abundance that are able to reduce metronidazole and, thereby, activate it to its toxic form. In the last three to four decades, several such factors were identified in different microaerophilic or anaerobic organisms. The first enzyme suggested to be relevant for metronidazole reduction was pyruvate:ferredoxin oxidoreductase (PFOR) (Lindmark and Müller, 1976), which transfers, *via* its iron–sulphur clusters, electrons derived from pyruvate to the electron carrier protein ferredoxin, which also contains iron–sulphur clusters. Ferredoxin, in turn, has a very low midpoint redox potential (−430 mV) and can transfer electrons to the nitro group of metronidazole, thereby generating metronidazole nitroradical anions as can be readily measured by electron paramagnetic resonance spectroscopy (Moreno *et al.* 1983, 1984; Chapman *et al.* 1985; Lloyd and Pedersen, 1985). Since the PFOR pathway exists in almost all anaerobes susceptible to metronidazole (Narikawa, 1986), with possibly the exception of bifidobacteria, it was an obvious candidate for metronidazole activation in the living organism. About the same time, however, it was observed that also rat liver microsomes (Pervez-Reyes *et al.* 1980) or certain flavin enzymes, such as xanthine oxidase (Kedderis *et al.* 1988), can reduce metronidazole under anaerobic conditions. Indeed, several flavin enzymes have been described in microaerophiles and anaerobes to be involved in metronidazole reduction, including thioredoxin reductase (TrxR) in *T. vaginalis* (Leitsch *et al.* 2009), *E. histolytica* (Leitsch *et al.* 2007) and *G. lamblia* (Leitsch *et al.* 2011) and nitroreductase RdxA in *H. pylori* (Olekhnovich *et al.* 2009). Many studies were conducted to identify the main activation pathways in anaerobic and microaerophilic pathogens. Surprisingly, downregulation or deactivation of PFOR in *T. vaginalis* (Leitsch *et al.* 2009), *Tritrichomoas foetus* (Sutak *et al.* 2004) or *B. fragilis* (Diniz *et al.* 2004) had only a minimal effect, if any, on the susceptibility to metronidazole. An appreciable negative effect on metronidazole susceptibility, however, could be observed when PFOR was downregulated in *G. lamblia* (Dan *et al.* 2000). In turn, overexpression of TrxR rendered *G. lamblia* somewhat more susceptible to metronidazole (Leitsch *et al.* 2016). It has, however, proven impossible so far to pinpoint reduction of metronidazole to one single enzymatic pathway. Interestingly, even non-enzymatic reduction of metronidazole under anaerobic conditions by cysteine and ferrous iron was reported (Willson and Searle, 1975). It is, therefore, safe to conclude that reduction of metronidazole in microaerophiles and anaerobes is performed by several factors, arguably some of which are non-enzymatic. This circumstance reduces the likelihood of emergence of metronidazole resistance in most organisms considerably. The only exception might be RdxA in *H. pylori*, which was identified as the major activating enzyme of metronidazole in several independent studies (Debets-Ossenkopp *et al.* 1999; Jenks *et al.* 1999*a*, *b*; Kwon *et al.* 2001; Latham *et al.* 2002).

A fairly motley picture is also evident regarding the targets of metronidazole in susceptible organisms. Damage to DNA, including strand breaks, was reported from bacteria (Plant and Edwards, 1976) as well as parasites, e.g. *T. vaginalis* (Ings *et al.* 1974) and *G. lamblia* (Uzlikova and Nohynkova, 2014). In addition, 5-nitroimidazoles were shown to form adducts with nucleotides (LaRusso *et al.* 1978; Ludlum *et al.* 1988) and cysteine (Wislocki *et al.* 1984; Leitsch *et al.* 2007), an amino acid which is highly abundant in many anaerobes, both as non-protein thiol buffer and as constituent of proteins. Non-protein thiol buffers can be depleted in metronidazole-treated parasites through adduct formation (Leitsch *et al.* 2007, 2009, 2011; Williams *et al.* 2012), thereby causing oxidative stress. Further, metronidazole–cysteine adducts can negatively affect the activity of certain enzymes, such as the disulphide/thioredoxin reductase activity of TrxR (Leitsch *et al.* 2007, 2009; Williams *et al.* 2012). Thus, TrxR is a special case in this context as it functions, both, as an activator and as a target of metronidazole. Importantly, TrxR was identified as a target of metronidazole in four microaerophilic parasites, i.e. *E. histolytica* (Leitsch *et al.* 2007), *T. vaginalis* (Leitsch *et al.* 2009), *Spironucleus vortens* (Williams *et al.* 2012) and *G. lamblia* (Leitsch *et al.* 2012*b*), whereas the other proteins affected by metronidazole treatment varied strongly between the parasites studied. The majority of these, however, were reported to interact with thioredoxin in anaerobes and other organisms, e.g. enolase, malate dehydrogenase and ribonucleotide reductase in *T. vaginalis* (Leitsch *et al.* 2009), thereby underscoring a correlation between metronidazole action and the thioredoxin system. It is also interesting to note that metronidazole treatment in *G. lamblia* leads to the degradation of translation elongation factor 1-γ, a factor likely to be essential for cell viability (Leitsch *et al.* 2012*b*).

Pharmacokinetics and safety issues

Mostly, metronidazole is administered intravenously or orally, either in large single doses of 2 g or in smaller repeated doses (Ralph *et al.* 1974). Treatment regimens vary with the condition treated. After a 2 g oral dose, the peak serum level in a female patient was 40 μg mL^{-1} and the half-life of elimination approximately 7 h (Wood and Monro, 1975). When smaller doses are administered, peak serum levels are clearly lower, i.e. 11·5 μg mL^{-1} after oral administration of 500 mg and 6·2 μg mL^{-1} after oral administration of 250 mg (Ralph *et al.* 1974). However, metronidazole can also

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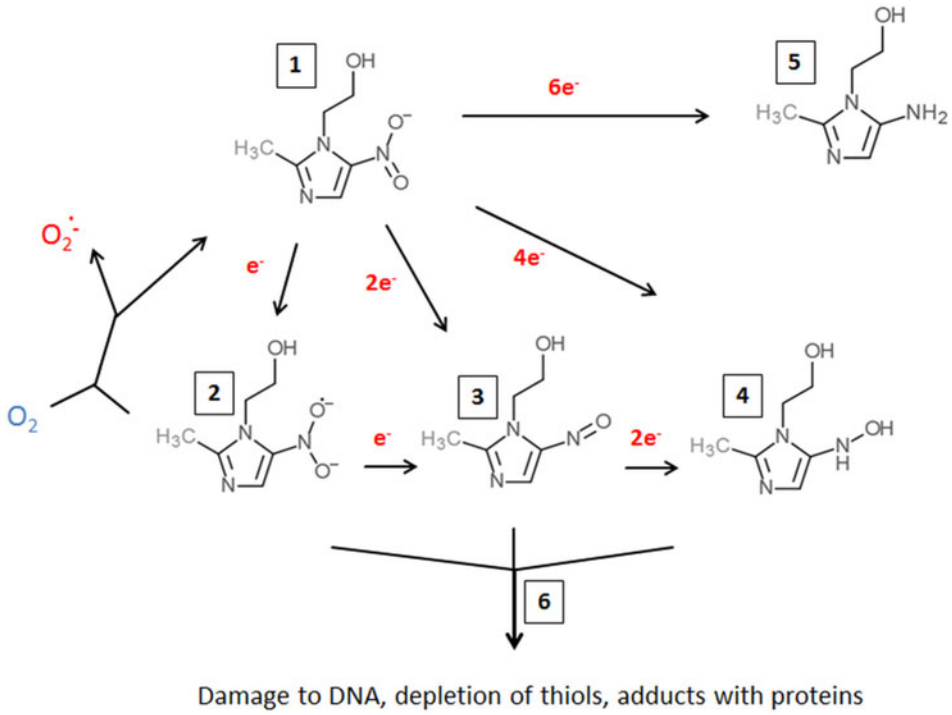

Fig. 1. Metronidazole reduction and toxicity in microaerophiles and anaerobes. Metronidazole enters the cell (**1**). Depending on the number of electrons transferred to the nitro group, a nitroimidazole radical anion (**2**), a nitrosoimidazole (**3**) or a hydroxylaminimidazole (**4**) is formed. Reduction can be either sequential, (**2→3→4**) or catalysed in one step. If oxygen is present, the nitroimidazole radical anion (**2**) is re-oxidized and the original metronidazole prodrug (**1**) re-established. Some enzymes (e.g. nitroreductase 2 from *Giardia lamblia* or Nim proteins from *Bacteroides* spp.) are proposed to detoxify metronidazole by transferring six electrons to the nitro group, thereby generating a non-reactive aminoimidazole (**5**). Reactive metronidazole intermediates (**2–4**) damage cell constituents such as DNA and proteins, and deplete thiol pools (**6**).

be applied topically in ointments, e.g. for the treatment of rosacea (Korting and Schöllmann, 2009), a chronic inflammatory skin condition which is treatable with metronidazole for, as yet, unknown reasons.

In most cases, metronidazole is fairly well tolerated, but adverse effects, especially neurological, are not rare. Consumption of alcohol during metronidazole treatment and several days thereafter should be strictly avoided because it can strongly exacerbate side-effects such as nausea or stomach cramps. Indeed, the discomfort resulting from simultaneous intake of metronidazole and alcoholic beverages is so great that metronidazole was used as an adjuvant in the treatment of alcoholism (Semer *et al.* 1966). Due to its reactivity with DNA (LaRusso *et al.* 1978), metronidazole was soon assumed to be carcinogenic and teratogenic (Voogd, 1981). A teratogenic effect of metronidazole could not be established (Koss *et al.* 2012), but it was found to be carcinogenic in rodents after extended durations of highly dosed treatment. In man, results were less clear and often conflicting (Dobiás *et al.* 1994). With regard to short-term treatment with metronidazole, originally no correlation between metronidazole intake and cancer was found (Falagas *et al.* 1998), but more recent studies report on a limited correlation (Friedman *et al.* 2009). As a consequence, metronidazole is officially classified as 'reasonably anticipated to be a human carcinogen'.

Metronidazole resistance

Due to metronidazole's multifaceted and pleiotropic mode of action and its ability to enter cells without the need for a specific transport mechanism, the emergence of resistance is, on the whole, far less common than seen with other antimicrobials (Holmes *et al.* 2016). However, metronidazole resistance is observed in the field, with varying frequency and depending on the pathogen concerned. Importantly, treatment failures with metronidazole are not necessarily due to drug resistance as such but can also be attributable to reinfections or caused by poor drug availability in the host (Nash, 2001). Other microbes inhabiting the same niches as the pathogens can also modulate the efficacy of metronidazole treatment (Nagy and Földes, 1991). In laboratory research, resistance to metronidazole can be generated in stocks of most parasites or bacteria. This, however, can give rise to phenotypes which are not viable in the host (Tejman-Yarden *et al.* 2011). Interestingly, several features of metronidazole resistance seem to be shared in bacteria and protists, despite the large evolutional distance between these kingdoms of life. Unfortunately, however, there has been little cooperative research between bacteriologists and protistologists on this particular issue, so that our understanding of metronidazole resistance has remained fairly incomplete despite the strong efforts undertaken by a large number of individual research groups. Nevertheless, in several microorganisms underlying mechanisms have been described in detail and, gradually, a more complete picture is evolving (Tables 2 and 3).

Parasites: T. vaginalis

In *T. vaginalis*, the mechanisms underlying metronidazole resistance are complex. Importantly, two different types of resistance have been established in the literature: 'aerobic' or clinical resistance (Meingassner *et al.* 1978; Meingassner and Thurner, 1979), and 'anaerobic' or laboratory-induced resistance (Cerkasovová *et al.* 1984; Kulda, 1999). The former is caused by defective oxygen scavenging mechanisms in the parasite (Yarlett *et al.* 1986), leading to higher intracellular oxygen concentrations which counteract metronidazole activation through redox cycling (Mason and Holtzman, 1975) and, consequently, increase the tolerance of *T. vaginalis* to the drug. Under normal growth conditions, as applied in laboratory culture, these strains exhibit no or just minimal resistance (Müller and Gorrell, 1983). In the presence of oxygen, however, susceptibilities can be reduced up to several orders of magnitude. This startling effect is hard to observe in the laboratory because stably elevated oxygen levels are hard to achieve in sealed culture flasks filled with commonly used growth media developed for *T. vaginalis*. In the human host, however, a steady state of decreased oxygen concentrations is readily established in certain body parts. At the mucosal epithelium of the human vagina, the niche of *T. vaginalis*, oxygen concentrations range from 15 to 56 μM (Ellis *et al.* 1992), well below the approximate

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Table 2. Overview over established factors involved in metronidazole resistance in protist parasites

Factor	Organism	Putative role	Supportive observations	Contradicitng observations	References
PFOR/ferredoxin (hydrogenosomal malate deydrogense/ferredoxin)	All microaerophilic and anaerobic protists (only trichomonadids)	Reduction to toxic intermediates	Not expressed in many resistant lines Direct inhibition in *Giardia* causes resistance	Absence in iron-depleted trichomonads (PFOR and hydrogenosomal MDH) has no effect on metronidazole susceptibility Fully functional in some resistant *Giardia* lines	Cerkasovová *et al.* (1984); Kulda *et al.* (1993); Dan *et al.* (2000); Rasoloson *et al.* (2002); Sutak *et al.* (2004); Hrdy *et al.* (2005); Leitsch *et al.* (2009); Leitsch *et al.* (2011)
TrxR	*Entamoeba histolytica* *Trichomonas vaginalis* *Giardia lamblia*	Reduction to toxic intermediates	Inactive in anaerobic-resistant *T. vaginalis* Overexpression in *G. lamblia* causes enhanced susceptibility TrxR downregulated in resistant *E. histolytica*	TrxR not downregulated or less active in clinical resistance in *T. vaginalis* Not downregulated in resistant *G. lamblia*	Leitsch *et al.* (2007); Leitsch *et al.* (2009); Leitsch *et al.* (2011); Leitsch *et al.* (2012*a*); Leitsch *et al.* (2016); Ansell *et al.* (2017)
Nitroreductase 1	*G. lamblia*	Reduction to toxic intermediates	Downregulated in resistant *G. lamblia* Overexpression causes enhanced susceptibility	None so far	Müller *et al.* (2007); Nillius *et al.* (2011); Müller *et al.* (2013); Müller *et al.* (2015)
Nitroreductase 2	*G. lamblia*	Reduction to non-toxic aminoimidazole	Overexpression renders *G. lamblia* and *Escherichia coli* more resistant to metronidazole	None so far	Müller *et al.* (2007); Müller *et al.* (2013): Müller *et al.* (2015)
Flavin reductase 1	*T. vaginalis*	Oxygen scavenging	Activity decreased or absent in all resistant *T. vaginalis* studied Overexpression of FR1 in resistant strain cancels resistance	None so far	Leitsch *et al.* (2009); Leitsch *et al.* (2010); Leitsch *et al.* (2012*a*); Leitsch *et al.* (2014*a*)

Table 3. Overview over established factors involved in metronidazole resistance in bacteria

Factor	Organism	Putative role	Supportive observations	Contradicitng observations	References
PFOR/ ferredoxin	Many microaerophilic and anaerobic bacteria	Reduction to toxic intermediates	Loss of PFOR activity in resistant *Clostridium perfringens*	Knock-out of PFOR has no effect on susceptibility in *Bacteroides fragilis*	Sindar *et al.* (1982); Diniz *et al.* (2004)
RdxA	*Helicobacter pylori* *Campylobacter jejuni*	Reduction to toxic intermediates	Mutated in almost all resistant clinical isolates Mutated in *H. pylori* with induced resistance Reduction of metronidazole by RdxA under anaerobic conditions shown in assays Mutation of *rdxA* in *C. jejuni* causes resistance	RdxA-deficient strains are only resistant in the presence of oxygen, although RdxA reduces metronidazole only under anaerobic conditions. This contradicts RdxA's role as a metronidazole activating enzyme	Jenks *et al.* (1999*a*); Jenks *et al.* (1999*b*); Debets-Ossenkopp *et al.* (1999); Kwon *et al.* (2001); Latham *et al.* (2002); Gerrits *et al.* (2004); Olekhnovich *et al.* (2009); Ribardo *et al.* (2010); Binh *et al.* (2015)
FrxA	*H. pylori*	Reduction to toxic intermediates	FrxA mutated in many resistant strains Mutations in *frxA* enhance resistance caused by mutations in *rdxA*	FrxA-deficient strains only resistant in the presence of oxygen. This contradicts FrxA's role as a metronidazole activating enzyme.	Kwon *et al.* (2000); Kwon *et al.* (2001); Gerrits *et al.* (2004)
Nim proteins	*Bacteroides* spp.	Reduction to non-toxic aminoimidazole	Introduction of *nim* genes can cause metronidazole resistance *nim*-positive *B. fragilis* reduces dimetridazole to aminodimetridazole Most resistant strains are *nim*-positive Resistance can be more easily induced in *nim*-positive strains	With increasing resistance, Nim levels do not increase Transfer of *nim*-gene from a resistant strain to a susceptible one renders the latter resistant but to a lesser degree Only few *nim*-positive strains are resistant	Breuil *et al.* (1989); Sebald (1994); Haggoud *et al.* (1994); Carlier *et al.* (1997); Gal and Brazier (2004); Löfmark *et al.* (2005); Leitsch *et al.* (2014*b*); Veeranagouda *et al.* (2014)

200 μM as found in oxygen-saturated water but much higher than in growth media. It is certain, however, that this mechanism is not the only one contributing to treatment failures with metronidazole in trichomoniasis patients. Although a correlation between measurable aerobic metronidazole resistance and treatment failure does exist (Müller *et al.* 1988), it seems to be rather weak (Schwebke and Barrientes, 2006), suggesting that the interplay between host and parasite has a decisive role. This interplay has not been studied as yet but likely involves a large number of factors and processes. It is interesting to speculate that one of the factors could be the oxygen concentration in the vagina which varies individually and during different phases of the menstrual cycle. It is important to note, however, that clinical resistance to metronidazole varies strongly between different parts of the world, ranging from the single-digit percentage area (Wendel and Workowski, 2007) to almost 20% (Upcroft *et al.* 2009), indicating that there are genetically distinct subpopulations of *T. vaginalis* with varying metronidazole susceptibility. This is further emphasized by the division of the species into two similarly large, globally occurring populations, of which the second comprises far more metronidazole-resistant isolates than the first (Conrad *et al.* 2012).

Anaerobic metronidazole resistance can only be induced in the laboratory and has not been observed with clinical isolates, with the possible exception of one strain, i.e. B7268 (Voolmann and Boreham, 1993; Upcroft and Upcroft, 2001). This form of resistance can be very strongly pronounced and allows growth of *T. vaginalis* at metronidazole concentrations up to 1000-fold higher than the minimum lethal concentration (MLC) observed with the parent cell line (Kulda *et al.* 1993; Leitsch *et al.* 2009). It is, however, accompanied by fundamental changes in the parasite's physiology. Most importantly, cell lines exhibiting anaerobic resistance lack central hydrogenosomal pathways including PFOR and hydrogenase (Kulda *et al.* 1993; Rasoloson *et al.* 2002). Consequently, they produce no hydrogen, the usual end product of the hydrogenosome. Rather, they produce lactate as the major metabolic end product, formed by cytoplasmic lactate dehydrogenases which are strongly upregulated in expression (Kulda *et al.* 1993). A very similar phenotype can be observed in metronidazole-resistant *T. foetus* (Cerkasovová *et al.* 1984), a related parasite of cattle. The main fermentative end product in resistant *T. foetus*, however, is not lactate but ethanol. Further, highly resistant *T. vaginalis* cell lines have very low levels of flavins (Leitsch *et al.* 2009), rendering flavin-dependent pathways, including TrxR, inactive. This is accompanied by a marked increase in expression of antioxidant enzymes (Leitsch *et al.* 2009), possibly in an attempt to counterbalance the loss of TrxR activity, which is central to the antioxidant defence. Nevertheless, these cell lines are highly sensitive to oxygen and, therefore, difficult to grow. These physiological changes were interpreted as being in line with the hypothesis that PFOR and ferredoxin are critical for the activation of metronidazole because the absence of this pathway was assumed to abolish metronidazole reduction (Kulda, 1999). Results from several studies, however, suggest that this pathway is unlikely to be decisive for metronidazole reduction in *T. vaginalis*. First, the deletion of the ferredoxin 1 gene, the main interaction partner of PFOR, did not lead to a decreased susceptibility to metronidazole, although the expression of PFOR was concomitantly decreased by 95% (Land *et al.* 2004). Further, withdrawal of intracellular iron with the iron chelator bipyridyl caused a near-to-complete shutdown of PFOR expression but did not increase tolerance to metronidazole (Leitsch *et al.* 2009). A similar observation was made in *T. foetus* (Sutak *et al.* 2004). Possibly, downregulation of PFOR and other hydrogenosomal enzymes is a consequence of low flavin levels. Evidence for this assumption is provided by a study on the effect of diphenyleneiodonium (DPI), a flavin inhibitor which covalently binds to reduced flavins, on metronidazole susceptibility in *T. vaginalis* (Leitsch *et al.* 2010). Strikingly, 10 μM of DPI rendered *T. vaginalis* completely insensitive to metronidazole. This was accompanied by a total loss of TrxR and PFOR activities and strongly increased expression of antioxidant enzymes, quite comparable to the situation in cell lines with induced metronidazole resistance. It is important to note, however, that protein levels of PFOR were not decreased upon addition of DPI but, to the contrary, increased, probably as an attempt by the cell to compensate for the sudden loss of PFOR activity. Unfortunately, the continued culture of *T. vaginalis* in the presence of DPI was not possible due to its antiproliferative effect on the parasite, so that the long-term effect of DPI on PFOR expression could not be monitored.

Although aerobic resistance and anaerobic resistance have been established as two distinct phenomena, they have several traits in common. Most importantly, the expression of flavin reductase 1 (FR1) is decreased or even abolished in cells exhibiting either form of resistance (Ellis *et al.* 1992; Leitsch *et al.* 2012*a*). FR1 reduces oxygen to hydrogen peroxide *via* its FMN and NADPH cofactors and, arguably, constitutes a major pathway for oxygen scavenging in *T. vaginalis* (Chapman *et al.* 1999; Linstead and Bradley, 1988; Leitsch *et al.* 2014*a*). Accordingly, the introduction of a functional episomal *fr1* gene under the control of a strong promoter into a highly resistant clinical strain, B7268, re-established metronidazole susceptibility (Leitsch *et al.* 2014*a*). It is, therefore, likely that FR1 is a central factor in the emergence of aerobic resistance. Since FR1 was also found to be inactive in an anaerobic-resistant cell line, it is likely that loss of this pathway is also a necessary for the development of anaerobic resistance (Leitsch *et al.* 2009). This hypothesis is supported by the observation that an aerobic resistance-like phenotype constitutes an early intermediate stage in the development of anaerobic resistance (Tachezy *et al.* 1993).

Other factors modulating metronidazole resistance in *T. vaginalis* also do exist, most notably nitroreductases (Pal *et al.* 2009). Recently, a clear correlation of stop mutations in two nitroreductase genes, *ntr4* and *ntr6*, and clinical resistance was found (Paulish-Miller *et al.* 2014). However, since clinical strains do not exhibit resistance in the absence of oxygen, it is questionable if these nitroreductases directly reduce metronidazole. Their importance is, nevertheless, also suggested by a recent large-scale genomic study in which 102 isolates were included (Bradic *et al.* 2017). Ntr6, amongst other nitroreductases, was found downregulated in metronidazole-resistant strains, as was FR1. In addition, a thioredoxin family protein was upregulated, and three iron–sulphur flavoproteins, two multidrug resistance pumps, four r2r3-Myb transcription factors, and a metal ABC transporter downregulated in metronidazole-resistant *T. vaginalis*. These results provide good confirmation of previously made observations, but also suggest the existence of hitherto unstudied mechanisms, although it is hard to reconcile reduced drug export due to decreased levels of efflux pumps with resistance. The same study also identified a number of single-nucleotide polymorphisms associated with metronidazole resistance. Interestingly, a large number of these were found in intergenic regions, raising the possibility that they are located in sequences modulating expression of adjacent genes. This is consistent with the observation that the amino acid sequence of FR1 is unchanged even in the most resistant strains studied (Leitsch *et al.* 2014*a*), suggesting that metronidazole resistance in *T. vaginalis* is not caused by mutations in genes but by their differential expression. In addition, and to make things even more complicated, different alterations might lead to the same phenotype. For example, metronidazole resistance is also strongly correlated with a

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decreased activity of alcohol dehydrogenase 1 (ADH1), a zinc-dependent enzyme that oxidizes secondary alcohols and reduces ketones (Leitsch *et al.* 2012*a*, 2013). In some resistant strains ADH1 expression levels are downregulated, but in others, the decrease of ADH1 activity is caused by low intracellular zinc concentrations (Leitsch *et al.* 2012*a*). These issues add to the astounding complexity of metronidazole resistance in *T. vaginalis* and warrant further research.

Parasites: G. lamblia

Treatment regimens of giardiasis with metronidazole are failing fairly often, with varying rates being reported from different sources (Nash, 2001; Mørch *et al.* 2008; Carter *et al.* 2017); but in contrast to clinical metronidazole resistance in *T. vaginalis*, no 'aerobic' type of resistance has been observed so far. Rather, *G. lamblia* isolates from patients who are refractory to metronidazole treatment are normally fully susceptible to metronidazole (Smith *et al.* 1982). This, however, might also be due to non-optimized conditions applied during drug susceptibility testing. *Giardia lamblia* displays a lower tolerance to oxygen as compared with *T. vaginalis* (Mastronicola *et al.* 2011), rendering metronidazole susceptibility testing in the presence of oxygen hardly feasible if its concentration is not precisely tuned (Gillin and Reiner, 1982). Thus, it is presently not possible to rule out the existence of a form of clinical metronidazole resistance in *G. lamblia*, which resembles aerobic resistance in *T. vaginalis*. In fact, the results from a study in which *G. lamblia* isolates from refractory cases were tested in a mouse model suggest that true clinical resistance does indeed exist as the parasites also retained their tolerance to metronidazole in the mice (Lemée *et al.* 2000). In any case, further endeavours are needed in the future to optimize assay conditions and to gain more clarity on whether treatment failure is caused by a resistance mechanism in the parasite or by other factors which could be, at least partly, host-derived.

Induction of metronidazole resistance in laboratory stocks of *G. lamblia* is easily achievable and has been reported from several laboratories. Different approaches have been applied, including prolonged culture in the presence of sublethal but increasing doses of the drug (Boreham *et al.* 1988; Townson *et al.* 1992; Müller *et al.* 2007) and mutagenesis with UV-light (Townson *et al.* 1992). As a rule of thumb, the tolerance to metronidazole in *G. lamblia* can be enhanced by about 100-fold. Interestingly, strongly decreased susceptibility to metronidazole was also observed after knocking down PFOR levels with hammerhead ribozymes (Dan *et al.* 2000). This contrasts with the results of similar studies performed in trichomonadids in which (very) low levels of PFOR activity did not alter metronidazole susceptibility (Land *et al.* 2004; Sutak *et al.* 2004; Leitsch *et al.* 2009). Importantly, however, the knock-down of PFOR also rendered *G. lamblia* tolerant to oxygen (Dan *et al.* 2000), indicating a large-scale shift in the parasite's physiology due to the methodology applied. Results from other studies are rather conflicting as to the role of PFOR in metronidazole resistance. In one cell line, 106-2ID$_{10}$, exhibiting metronidazole resistance induced by prolonged exposure of the cells to sublethal doses of the drug, PFOR was found to be strongly downregulated (Leitsch *et al.* 2011). In a cell line with metronidazole resistance induced by mutagenesis with UV-light, however, the PFOR pathway was fully intact (Leitsch *et al.* 2011). Other factors potentially involved in metronidazole resistance were also studied, including nitroreductase 1 (NR1) (GL50803_22677, now annotated as nitroreductase Fd-NR2) which also modulates metronidazole susceptibility (Nillius *et al.* 2011). In a transfectant cell line expressing elevated levels of NR1, metronidazole susceptibility was found to be enhanced twofold to threefold. Recent data from a transcriptomic study, measuring overall mRNA expression in three resistant strains (106-2ID$_{10}$, 713-M3 and WB-M3) and their respective susceptible parent strains (Ansell *et al.* 2017), further emphasize the role of NR1 in metronidazole resistance. In two resistant strains, expression levels were decreased and in the third line about a third of the NR1 transcripts had a non-sense mutation, effectively reducing the copy number of functional NR1 in the cell. In addition to NR1, also other nitroreductases could have a role in metronidazole resistance. Surprisingly, nitroreductase 2 (NR2) (GL50803_6175; now annotated as nitroreductase family protein fused to ferredoxin domain Fd-NR1), might have exactly the opposite, i.e. protective effect if overexpressed (Müller *et al.* 2013). It is possible that NR2 transfers as many as six electrons to the nitro group of metronidazole, thereby forming a non-toxic aminoimidazole. However, further research will be necessary in order to frame a reliable hypothesis regarding NR2 function. A third nitroreductase, GL50803_8377, was found to be downregulated in two of three resistant strains assayed (Ansell *et al.* 2017). Nitroreductase activity, however, is not necessarily only exerted by enzymes designated as nitroreductases. TrxR, for example, can reduce nitro compounds, including nitroimidazoles in several microaerophilic parasites, including *G. lamblia* (Leitsch *et al.* 2011). A potential role for TrxR in metronidazole activation in *G. lamblia* was demonstrated recently when a cell line strongly overexpressing TrxR (Leitsch *et al.* 2016) was found to exhibit moderately increased metronidazole susceptibility. Importantly, TrxR is not downregulated in metronidazole-resistant strains (Leitsch *et al.* 2011; Ansell *et al.* 2017) but it is currently unclear if it is active. Loss of TrxR activity but not expression was observed before in a *T. vaginalis* strain with 'anaerobic' resistance and was caused by the loss of the enzyme's FAD cofactor (Leitsch *et al.* 2009). Measuring TrxR activity in *G. lamblia*, however, is currently unfeasible because a functional thioredoxin has not yet been identified in this parasite.

In accordance with metronidazole-resistant *T. vaginalis*, reduction of flavins was also found to be decreased in cell extracts of metronidazole-resistant *G. lamblia* cell lines as compared with their parent cell lines (Ellis *et al.* 1993; Leitsch *et al.* 2011), mirroring the observations made in *T. vaginalis* (Leitsch *et al.* 2009, 2012*a*, 2014*a*). A homologue of *T. vaginalis* FR1 does not exist in the *G. lamblia* genome but potential candidate enzymes which could exert this activity, three FMN-dependent oxidoreductases (GL50803_9719; GL50803_17150; GL50803_17151), were downregulated in metronidazole-resistant strains (Ansell *et al.* 2017). Quite confusingly, however, a closely related enzyme (GL50803_15004), termed diaphorase (Sánchez *et al.* 2001), was upregulated in two of the three strains. It was hypothesized that diaphorase exerts a different activity, i.e. detoxification of metronidazole (Ansell *et al.* 2017), but experimental data with the purified enzyme are needed to support this claim.

Taken together, metronidazole resistance in *G. lamblia* is currently not as well understood as in *T. vaginalis*, mainly due to the lack of clinical metronidazole-resistant strains available to the research community. There is strong evidence for an involvement of NR1, but further research on a larger number of resistant isolates is warranted.

Parasites: E. histolytica

Clinical metronidazole resistance in *E. histolytica* has not been reported in the field and, therefore, poses no problem for the treatment of amoebic liver abscess. Intriguingly, it is also very difficult to induce metronidazole resistance in the laboratory with only a few successful attempts documented (Samarawickrema *et al.* 1997; Wassmann *et al.* 1999; Penuliar *et al.* 2015). Moreover, the extent of the resistance induced is far smaller

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than observed in *T. vaginalis* and *G. lamblia*, and ranges from twofold (Samarawickrema *et al.* 1997; Penuliar *et al.* 2015) to about 10-fold (Wassmann *et al.* 1999) of the normal MLC. This low-level metronidazole resistance is associated with increased expression of superoxide dismutase (Samarawickrema *et al.* 1997; Wassmann *et al.* 1999) and peroxiredoxin (Wassmann *et al.* 1999), and decreased expression of ferredoxin 1 and TrxR (Wassmann *et al.* 1999), which is strongly reminiscent of the changes reported for metronidazole-resistant *T. vaginalis* (Leitsch *et al.* 2009). However, levels of PFOR were reported to be unchanged in another cell line with reduced susceptibility to metronidazole, whereas 88 genes in total were reported to be differentially regulated at the mRNA level (Penuliar *et al.* 2015). This set of genes also did not include TrxR or two NADPH-dependent oxidoreductases which had been previously discovered by the same investigators to render *E. histolytica* slightly more susceptible to metronidazole if overexpressed (Jeelani *et al.* 2010). Instead, DNA polymerase, several other factors involved in DNA metabolism, and several iron–sulphur flavoproteins were upregulated, whereas several leucine-reach repeat proteins and cysteine proteases were downregulated. The significance of these observations, however, is presently unclear.

Bacteria: H. pylori

By a large margin, metronidazole resistance occurs most often in *H. pylori* infections, for which metronidazole is often used in combination with other antimicrobials such as clarithromycin (De Francesco *et al.* 2017). Indeed, metronidazole resistance in *H. pylori* has become so widespread in some parts of the world, mainly in South Asia and Africa (De Francesco *et al.* 2010), that metronidazole has been practically rendered useless in the treatment of peptic ulcer. Resistance is, almost invariably, caused by mutations in the *rdxA* gene (Debets-Ossenkopp *et al.* 1999; Jenks *et al.* 1999*a*; b; Kwon *et al.* 2001; Latham *et al.* 2002), encoding a nitroreductase harnessing FMN and NADH as cofactors (Goodwin *et al.* 1998; Olekhnovich *et al.* 2009). In several independent studies on metronidazole-resistant clinical isolates as well as on laboratory stocks with induced resistance, the *rdxA* gene contained non-sense and missense mutations (Kwon *et al.* 2001; Latham *et al.* 2002). According to observations in some studies, metronidazole resistance can be further enhanced through mutations in the *frxA* gene, encoding another nitroreductase (Kwon *et al.* 2000, 2001; Justino *et al.* 2014). This notion was further supported by a careful genomic study (Binh *et al.* 2015) in which mutations were found in the *rdxA* and *frxA* genes in a laboratory strain with induced resistance but not its susceptible parent. Thus, at a first glance, a very clear correlation seems to exist between abolished reduction of metronidazole and resistance. At a second glance, however, the picture becomes less clear because RdxA- and FrxA-deficient clinical strains are only resistant in the presence of oxygen but not under anaerobic conditions (Gerrits *et al.* 2004). This resembles 'aerobic' resistance in *T. vaginalis* and is incompatible with the notion that RdxA and FrxA are the only factors capable of reducing metronidazole in *H. pylori*. Possibly, RdxA and FrxA do not reduce metronidazole *in vivo* at all because metronidazole reduction by RdxA was only observed under anaerobic but not aerobic conditions in assays with the purified enzyme (Olekhnovich *et al.* 2009). It is interesting to note that in strains with laboratory-induced metronidazole resistance, several enzyme activities, including disulfide reduction (possibly catalysed by a TrxR), NADH oxidation and nitroreduction were strongly decreased in metronidazole-resistant cell lines as compared with the sensitive parent cell lines (Trend *et al.* 2001). Unfortunately, these enzymes have not been further characterized but the involvement of these activities resembles metronidazole resistance in parasites (Kaakoush *et al.* 2009). To conclude, it is well established that clinical metronidazole resistance in *H. pylori* is mostly caused by mutations in the *rdxA* and *frxA* genes, at least in most cases (Marais *et al.* 2003), but the exact mechanism of resistance remains unresolved.

Bacteria: B. fragilis *and other* Bacteroides *spp.*

Bacteroides fragilis, together with *H. pylori*, is the prokaryote in which metronidazole resistance has been most extensively studied. This is somewhat surprising considering resistance rates are very low (about 1%) (Urbán *et al.* 2002; Aldridge *et al.* 2003; Hedberg and Nord, 2003; Sóki *et al.* 2013; Snydman *et al.* 2017), although alarmingly high metronidazole resistance rates (between 5 and 10%) have been reported in the UK (Brazier *et al.* 1999), Brazil (Vieira *et al.* 2006), Lebanon (Yehya *et al.* 2014) and Pakistan (Sheikh *et al.* 2015). Of great interest, however, is a metronidazole resistance mechanism, possibly specific for *B. fragilis* and still incompletely understood: Nim protein-mediated resistance. Nim proteins were discovered in 1989 as transmissible, mainly plasmid-borne metronidazole resistance determinants (Breuil *et al.* 1989; Haggoud *et al.* 1994; Sebald, 1994), which are normally preceded by an insertion element to enable transcription (Sóki *et al.* 2006). They are assumed to be the major cause of metronidazole resistance in the field and predicted to contain a FMN-binding domain and a pyridoxamine 5′-phosphate oxidase domain. Currently, nine homologues of Nim proteins have been described in *Bacteroides* spp. (NimA to NimJ, with NimI occurring in *Prevotella*, a closely related genus.) Interestingly, proteins with the same designation also exist in other organisms but the nomenclature is confusing because the different Nim homologues of *Bacteroides* are more closely related to each other than to homologues with the same designation in other genera, e.g. NimB in *B. fragilis* and *Clostridium difficile*. Interestingly, Nim proteins also exist in *T. vaginalis* and *E. histolytica* (Pal *et al.* 2009). These homologues are only distantly related to the Nim proteins in *B. fragilis* but seem to have a similar function because they render *Escherichia coli* more insensitive to metronidazole when introduced on a plasmid (Pal *et al.* 2009).

It has been proposed that Nim proteins act as nitroreductases which reduce metronidazole to non-toxic aminoimidazoles (Carlier *et al.* 1997) by transferring six electrons to the drug's nitro group. However, direct proof of this activity with purified Nim is lacking and data from more recent studies are hard to reconcile with this hypothesis. Expression levels of Nim proteins are not increased in *nim*-positive strains after the induction of high-level metronidazole resistance and, thus, are independent of the degree of metronidazole resistance (Leitsch *et al.* 2014*b*). This is at odds with the notion that Nim proteins are nitroreductases because higher concentrations of metronidazole would require larger amounts of the reducing enzyme in order to detoxify all metronidazole. Further, *nim* genes only confer very modest levels of resistance if transferred from highly resistant *nim*-positive to *nim*-negative recipient strains (Husain *et al.* 2013). It is also interesting to note that the occurrence of *nim* genes in *B. fragilis* by far exceeds the proportion of metronidazole-resistant isolates (Gal and Brazier, 2004; Löfmark *et al.* 2005). Thus, most isolates carrying a *nim* gene are not metronidazole resistant. By contrast, it was repeatedly shown that high-level metronidazole resistance can be much more easily induced in *nim*-positive strains (Gal and Brazier, 2004; Löfmark *et al.* 2005; Leitsch *et al.* 2014*b*) than in *nim*-negative strains, although the latter is still possible (Schaumann *et al.* 2005). It is, therefore, certain that Nim proteins

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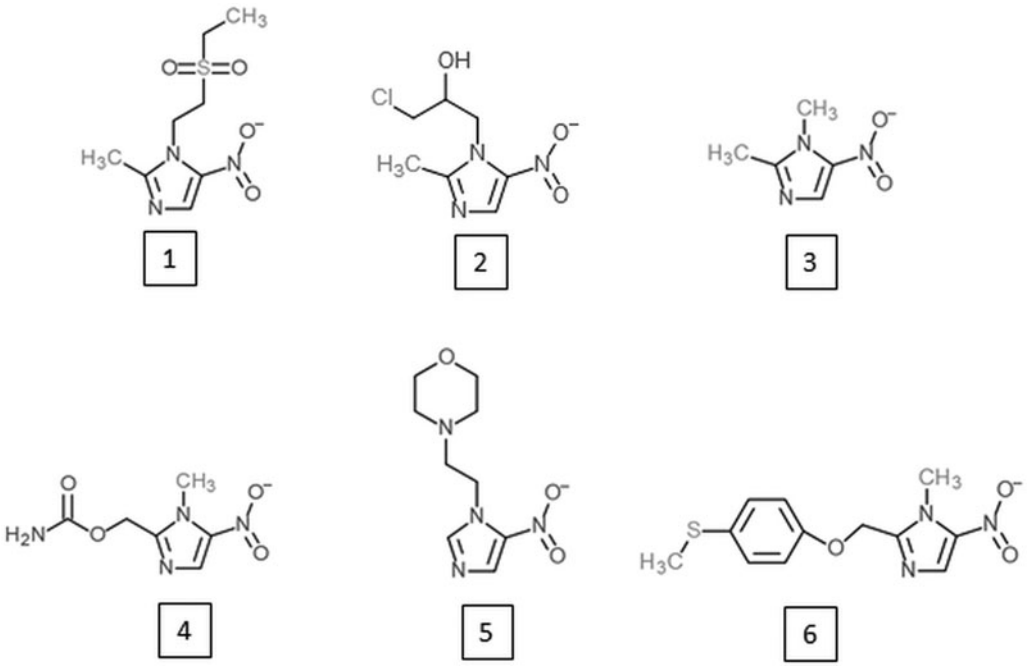

Fig. 2. 5-nitroimidazoles developed as alternatives to metronidazole or as novel treatment option against African trypanosomiasis. (**1**) Tinidazole; (**2**) ornidazole; (**3**) dimetridazole; (**4**) ronidazole; (**5**) nimorazole; (**6**) fexinidazole.

are correlated with metronidazole resistance but the underlying mechanism remains to be discovered.

In addition to Nim proteins, other factors potentially involved in metronidazole resistance were studied. Importantly, a knock-out of PFOR (Diniz *et al.* 2004) had little or no effect at all on metronidazole susceptibility. However, it was demonstrated that the deletion of the iron transporter gene *feoAB* leads to reduced susceptibility of *B. fragilis* to metronidazole (Veeranagouda *et al.* 2014), but it is presently unclear if the iron import is also reduced in metronidazole-resistant *B. fragilis* clinical isolates. A forced efflux of metronidazole through efflux pumps of the RND family (Pumbwe *et al.* 2006, 2007) could also have a certain role in metronidazole resistance although their role in clinical metronidazole resistance remains to be established.

Bacteria: Clostridia

Fairly little is known about metronidazole resistance in clostridia despite their great medical importance. Metronidazole, together with vancomycin, has remained the treatment option of choice for *C. difficile* infections (Peng *et al.* 2017) but treatment failures seem to occur more frequently lately (Leffler and Lamont, 2015). It is important, however, to emphasize that treatment failures are not necessarily caused by resistance as such, as discussed previously. Nevertheless, some of the refractory strains are definitely metronidazole-resistant, as determined in appropriate susceptibility assays. In a careful proteomic study (Chong *et al.* 2014), overall protein expression in one such isolate was compared to a normally metronidazole susceptible isolate, revealing numerous changes in the expression profile. Interestingly, several thioredoxin reductases and thioredoxins were differentially expressed and ferredoxin was downregulated approximately 2·5-fold. In contrast, a Nim homologue, NimB, was expressed more strongly (upto threefold). The significance of these changes remains unclear, but the same candidate factors emerge in *C. difficile* with respect to metronidazole resistance as seen in other microbes. Metronidazole resistance in clostridia can also be induced in the laboratory. In a study on *Clostridium perfringens*, metronidazole resistance was induced by mutagenesis using N-methyl-N′-nitro-N-nitrosoguanidine lactate (Sindar *et al.* 1982). Quite in accordance with the observations in *T. vaginalis*, resistance was accompanied by a total loss of PFOR activity and a shift of metabolic end products from acetate to pyruvate and lactate.

Other 5-nitroimidazoles and outlook

Research on alternative 5-nitroimidazoles began soon after the introduction of metronidazole in order to develop alternatives with similar potential but improved characteristics such as patient compliance, serum half-life and safety. Tinidazole (Fig. 2) has emerged as the most successful of these alternative 5-nitroimidazoles and is superior to metronidazole in several aspects. It has the same spectrum as metronidazole (Fung and Doan, 2005) but a longer half-life, i.e. 12·5 *vs* 7·3 h (Wood and Monro, 1975), and is better tolerated (Fung and Doan, 2005). Most importantly, tinidazole can be used to overcome metronidazole resistance in many cases. In metronidazole refractory trichomoniasis patients, for example, cure rates with tinidazole were as high as 92% (Sobel *et al.* 2001). Despite these advantages, tinidazole was not approved in the USA before 2004 (Nailor and Sobel, 2007), and in many countries metronidazole has even yet remained the only approved 5-nitroimidazole for the treatment of anaerobic infections in man. Nevertheless, other 5-nitroimidazoles are in use, such as ornidazole, nimorazole, ronidazole and dimetridazole. Ronidazole and dimetridazole were originally widely used in food-producing animals but were banned in the USA and the EU due to their suspected carcinogenic potential. The use of 5-nitroimidazoles, however, is still legal for the treatment of anaerobic infections in companion animals, such as ronidazole for the treatment of trichomoniasis in cats (Gookin *et al.* 2017). The mode of action of the various 5-nitroimidazoles seems to be very similar. Along with DNA (Zahoor *et al.* 1987), proteins and thiols seem to be affected by all 5-nitroimidazoles studied so far. Tinidazole, for example, was found to bind the same proteins as metronidazole in the parasites *E. histolytica* (Leitsch *et al.* 2007), *T. vaginalis* (Leitsch *et al.* 2009) and *G. lamblia* (Leitsch *et al.* 2012*b*) and to inhibit TrxR to a similar extent as metronidazole (Leitsch *et al.* 2007, 2009). Moreover, tinidazole, ornidazole and ronidazole also

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decrease non-protein thiol levels, with ronidazole exhibiting the strongest effect (Leitsch *et al.* 2007, 2009, 2012*b*).

Despite the reluctance of the authorities to approve alternative 5-nitroimidazoles, obviously due to the deficient safety profile of this drug class, research on novel 5-nitroimidazoles has never stopped. There are many promising candidates amongst newly developed 5-nitroimidazoles which could enable more effective treatments with reduced mutagenicity and an improved management of metronidazole resistance in the future (Crozet *et al.* 2009; Dunn *et al.* 2010; Jarrad *et al.* 2016). Interestingly, another 5-nitroimidazole which was developed in 1983, fexinidazole (Jennings and Urquhart, 1983; Raether and Seidenath, 1983), might revolutionize the notoriously difficult treatment of African trypanosomiasis or sleeping sickness in the near future (https://www.dndi.org/diseases-projects/portfolio/fexinidazole/). Probably, fexinidazole has a different mode of action than other 5-nitroimidazoles because trypanosomatids are not microaerophilic. This example shows that the well-studied drug class of 5-nitroimidazoles might still have some surprises in store for us.

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