J Antimicrob Chemother 2018; **73**: 265–279
doi:10.1093/jac/dkx351 Advance Access publication 25 October 2017

Journal of Antimicrobial Chemotherapy

Metronidazole: an update on metabolism, structure–cytotoxicity and resistance mechanisms

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Metronidazole, a nitroimidazole, remains a front-line choice for treatment of infections related to inflammatory disorders of the gastrointestinal tract including colitis linked to *Clostridium difficile*. Despite >60 years of research, the metabolism of metronidazole and associated cytotoxicity is not definitively characterized. Nitroimidazoles are prodrugs that are reductively activated (the nitro group is reduced) under low oxygen tension, leading to imidazole fragmentation and cytotoxicity. It remains unclear if nitroimidazole reduction (activation) contributes to the cytotoxicity profile, or whether subsequent fragmentation of the imidazole ring and formed metabolites alone mediate cytotoxicity. A molecular mechanism underpinning high level (>256 mg/L) bacterial resistance to metronidazole also remains elusive. Considering the widespread use of metronidazole and other nitroimidazoles, this review was undertaken to emphasize the structure–cytotoxicity profile of the numerous metabolites of metronidazole in human and murine models and to examine conflicting reports regarding metabolite–DNA interactions. An alternative hypothesis, that DNA synthesis and repair of existing DNA is indirectly inhibited by metronidazole is proposed. Prokaryotic metabolism of metronidazole is detailed to discuss new resistance mechanisms. Additionally, the review contextualizes the history and current use of metronidazole, rates of metronidazole resistance including metronidazole MDR as well as the biosynthesis of azomycin, the natural precursor of metronidazole. Changes in the gastrointestinal microbiome and the host after metronidazole administration are also reviewed. Finally, novel nitroimidazoles and new antibiotic strategies are discussed.

Introduction

The nitro functional group features in >200 naturally occurring compounds.[1] 5-nitro antibiotics share a nitro functional group ($-NO_2$) at the fifth position of a planar 5-membered ring, with a nitrogen (nitroimidazole), oxygen (furan) or sulphur (thiazole) at position 1 (see examples in Figure 1). A variety of side chains may be appended to the imidazole. The heterocycles are aromatic, sharing a conjugated system of single and double bonds, in which a lone pair of electrons from the nitrogen, oxygen or sulphur are delocalized in the ring. The nitro group of these prodrugs is required for cytotoxicity.[2,3] Interestingly, some nitroimidazoles have structural similarity to uncouplers of oxidative phosphorylation (reviewed elsewhere),[4] containing an electron-withdrawing (nitro) group, a weak acid (for example, hydroxethyl side chain) and a hydrophobic ring (imidazole).

Metronidazole is a bactericidal synthetic derivative of azomycin[5] (Figure 1), originally detected in cultures of *Streptomyces* spp. during the 1950s.[6] Azomycin is produced by Actinobacteria including *Streptomyces eurocidicus*[7–9] and *Nocardia mesenterica*[6,10] as well as Proteobacteria (*Pseudomonas fluorescens*).[11] It was not until 1962 during treatment for vaginitis associated with the protozoan *Trichomonas vaginalis*, that it was realized in the same patient that metronidazole was also effective in treating gingivitis.[12] Metronidazole has since been used against microaerophiles including *Helicobacter pylori*, protozoa including *Entamoeba histolytica* (amoebic dysentery) and *Giardia lamblia* (giardiasis) as well as methanogenic archaea.[13,14] Metronidazole is also used as a tool for targeted cell ablation in developmental studies.[15] Metronidazole remains a good choice for colitis associated with *Clostridium difficile*[16,17] and for infections of the oral cavity, such as periodontitis.[18,19] Nitroimidazole prodrugs cross these taxonomic boundaries because susceptible microorganisms encode enzymes of sufficiently negative redox potential to permit reduction of the nitro group, which may function albeit poorly, under low oxygen concentrations. Thus, effective use of nitroimidazoles is limited to anaerobic and nanaerobic bacteria[20] (herein referred to as anaerobic bacteria), protozoans and microaerophiles.

Pharmacodynamics of metronidazole in microorganisms

A receptor for metronidazole uptake has not been described and uptake is considered a passive process. However, nitro group

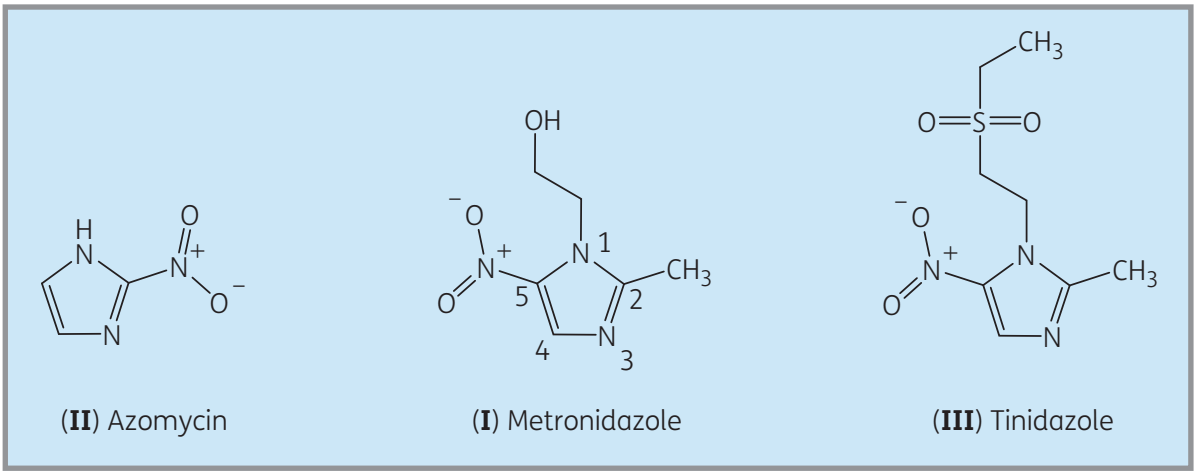

Figure 1. A variety of aromatic nitroimidazoles sharing the common nitro functional group (-NO_2) at position two or five of the imidazole. Azomycin, the 2-nitroimidazole biological precursor of metronidazole is included. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

reduction and associated cytotoxicity is correlated with the rate of metronidazole uptake[21] indicating uptake may not be passive. Indeed, the oxidative phosphorylation inhibitor, carbonyl cyanide *m*-chlorophenyl hydrazone slows metronidazole uptake in *H. pylori* and *Clostridium pasteurianum* suggesting uptake is dependent on a proton gradient.[22,23] This gradient is probably dependent on electron transport as uptake is also inhibited by potassium cyanide and oxygen.[22,23] Inhibitors of glycolysis, sodium fluoride, arsenate and iodoacetic acid, slow the uptake of metronidazole.[23,24] Uptake by *C. pasteurianum*,[23] trichomonads[25,26] and *Entamoeba invadens*[26] is rapid, as assessed with radiolabelled metronidazole. In agreement, gas chromatography indicates *Clostridium* spp., *Bacteroides* spp. and *Fusobacteria* spp. rapidly take up metronidazole.[21] Other than *Escherichia coli* and *Enterococcus faecalis*, it is generally considered that few facultative anaerobes take up metronidazole.[21,27,28] However, uptake of metronidazole by facultative anaerobic bacteria is likely to be underestimated, as discussed in this review.

Being a prodrug, metronidazole is inactive until taken up and reduced. Reduction of the nitro group of metronidazole occurs by two routes, with important consequences for survival (Figure 2). Reductive activation of the nitro group can lead to activation of metronidazole resulting in imidazole fragmentation and cytotoxicity. Alternatively, reduction of the nitro group to the non-toxic amino derivative can occur (referred to herein as 'reductive inactivation', overviewed in Figure 2). The reductive inactivation of metronidazole to the stable and non-toxic amino derivative is oxygen insensitive, occurring in two-electron steps, consuming a total of six electrons.[29,30] Reductive inactivation of metronidazole is one mechanism of resistance discussed further under resistance mechanisms. The reductive activation of metronidazole consumes four electrons and is proposed to occur in a series of one- or two-electron steps leading to ring-fission and formation of transient cytotoxic derivatives.[31–34] It has been proposed that the process of reductive activation is cytotoxic, as metronidazole acts as an alternative electron acceptor, inhibiting the proton motive force and diminishing ATP production.[27,31] The first step of the reductive activation of metronidazole is proposed to form the nitro free radical, followed by the nitroso, nitroso free radical and hydroxylamine derivatives (compounds **II-VII**; Figure 2). Oxygen has a higher affinity for electrons than metronidazole. It has been proposed that removal of an electron from the nitroso radical by oxygen regenerates metronidazole in a process termed 'futile cycling'.[23] The presence of oxygen generates oxygen radicals that may induce DNA strand breaks. Metronidazole may indirectly accelerate this process via futile cycling. Alternatively, oxygen may inhibit metronidazole uptake.[22,23]

A thermodynamic logic proposed in the 1970s, describing the flow of electrons leading to the reductive activation of metronidazole, remains relevant.[35] The thermodynamic logic employs the midpoint redox potential (E_0', the electrochemical property of a redox active compound relative to the H^+/H_2 couple with 1 M H^+ saturated with H_2 under atmospheric pressure), describing the tendency of a more positive carrier to accept electrons from a more negative carrier. During fermentation, pyruvate is oxidatively decarboxylated by pyruvate:ferredoxin oxidoreductase (PFOR) forming acetyl phosphate, liberating carbon dioxide and, in turn, reducing ferredoxin (summarized in Figure 3). During fermentation, electrons flow from pyruvate through PFOR (containing three iron-sulphur (FeS) clusters, the most negative of which is $E_0' = -540$ mV)[36] to reduced ferredoxin [$Fd_{(ox)}/Fd_{(red)}$, $E_0' = -420$ mV]. Electrons proceed to other low potential redox carriers, such as hydrogenase 1 and to protons ($2H^+/H_2$, $E_0' = -414$ mV).[37]

Originally, it was proposed that PFOR in concert with ferredoxin were the only couple in anaerobic bacteria with a sufficiently low redox potential to reductively activate metronidazole [$E_0' = -470$ to -510 mV].[2,31,38–40] Later it was discovered that effectors with very negative midpoint redox potentials, including purified ferredoxin, flavodoxin[34] and hydrogenase[31,34] from *C. pasteurianum* were able to reductively activate metronidazole directly.[34] More recently, the expression of flavodoxin from *Clostridium acetobutylicum* was used to render *E. coli* susceptible to metronidazole.[41] In addition, transposon mutagenesis of the gene encoding pyruvate-formate lyase activating enzyme (*pflA*) in *C. acetobutylicum* renders this bacterium less susceptible to metronidazole, although PflA is unlikely to mediate directly the reductive activation of

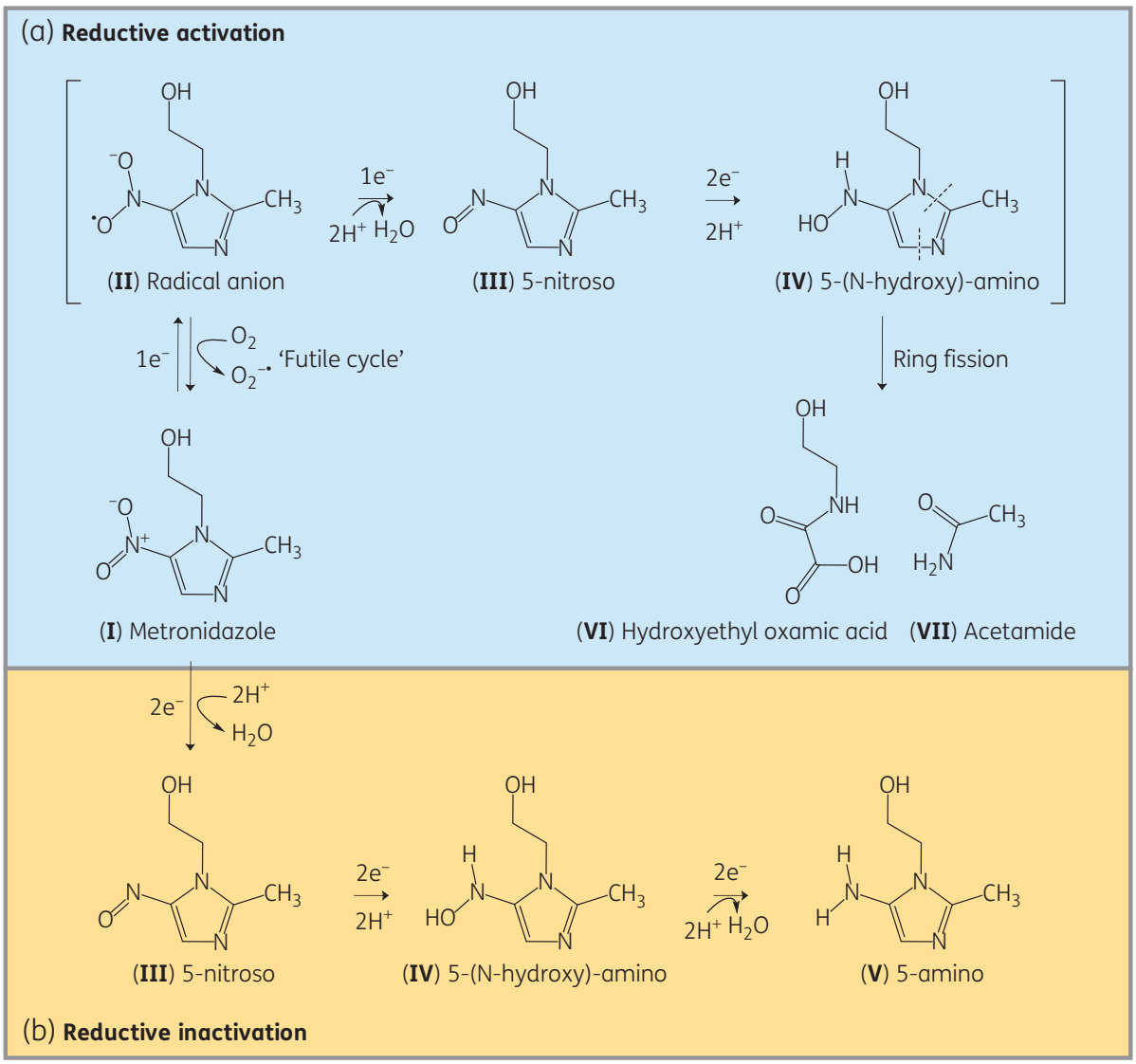

Figure 2. Showing reductive activation of metronidazole leading to heterocycle fission, transient compound formation and cytotoxicity (a) or reductive inactivation of metronidazole forming the 5-amino derivative (b). Reductive activation of metronidazole, driven by pyruvate:ferredoxin oxidoreductase (PFOR), ferredoxin, flavodoxin, hydrogenase and effectors of the dissimilatory sulphate pathway, is hypothesized to form transient intermediate compounds **II**–**IV**, resulting in heterocycle fission (marked with dashed lines in compound **IV**). Only two products of ring fission are shown (compounds **VI–VII**). Presence of oxygen is proposed to regenerate metronidazole in the so-called 'futile cycle' (a). Reductive inactivation of the nitro group to the 5-amino derivative (compound **V**) is a resistance mechanism proposed to occur via oxygen-insensitive nitroreductases and *nim* genes, rendering metronidazole non-toxic (b). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

metronidazole.[42] Metronidazole also competes with sulphite (SO_3^{2-}) for reducing equivalents originating from ferredoxin in the dissimilatory sulphate pathway, as noted in studies on *C. pasteurianum*.[33,43] Loss of function mutants of PFOR in *Clostridium perfringens* and *Trichomonas* spp. are up to 100-fold more resistant to metronidazole although are still killed.[44–46] Similarly, a *Bacteroides fragilis* double mutant of PFOR and flavodoxin is ~10-fold more resistant to metronidazole, although is still killed[47] confirming activation of metronidazole occurs, albeit less efficiently, independently of PFOR. Other candidates that may function in the reductive activation of metronidazole are the poorly characterized oxygen-sensitive (type II) nitroreductases.[30] Lastly, metronidazole is also reductively activated by sodium dithionite ($E_0' = -660$ mV)[48] in a reaction consuming four electrons, which is irreversible because it induces heterocycle fission (cleavage).[34]

It is generally considered that only selected facultative anaerobic bacteria including *E. coli*, *E. faecalis* and *Klebsiella pneumoniae* take up metronidazole.[21,27,28] It has been proposed that metronidazole taken up by facultative bacteria such as *E. coli* is not activated. The proposed mechanism is linked to the activity of NAD^+ coupled pyruvate-formate lyase instead of PFOR during anaerobic cleavage of pyruvate to acetyl-coenzyme A and formate.[49,50] However, the metronidazole-mediated killing of *E. coli* is documented.[51,52] The rate of kill and liberation of acetamide, a major

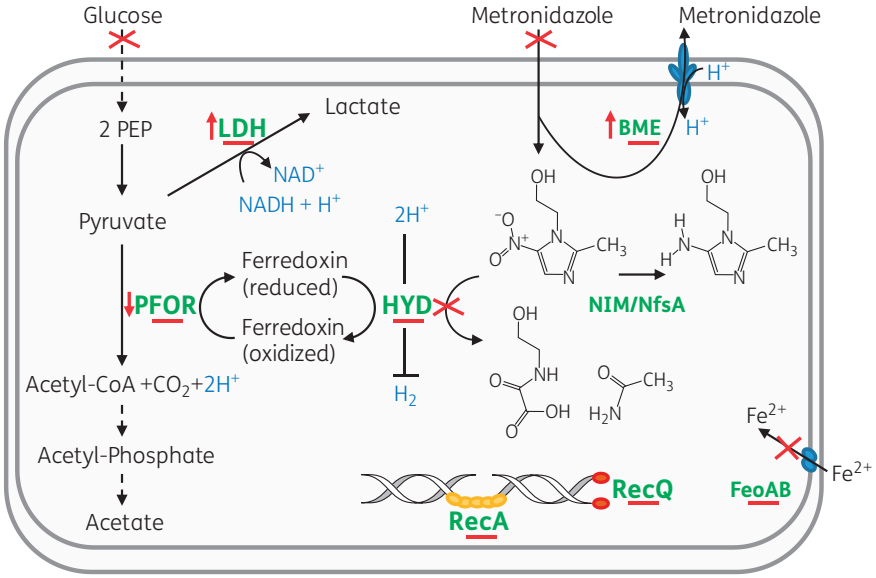

Figure 3. Summary of reductive activation/inactivation and resistance mechanisms to metronidazole and other nitroimidazoles. Red arrows indicate a change in gene expression that confers resistance. Underlined enzymes indicate that loss of function mutants affect susceptibility to metronidazole. Crosses indicate reduced activity or uptake. Enzymes shown in green include pyruvate:ferredoxin oxidoreductase (PFOR), lactate dehydrogenase (LDH) and hydrogenase (HYD) as well as DNA repair effectors recombinase A (RecA) and DNA helicase (RecQ). Metronidazole efflux is facilitated by *Bacteroides* multidrug efflux pump system (BME) and the ferrous iron transporter (FeoAB) imports iron. *nim* genes (NIM) and oxygen-insensitive nitroreductase (NfsA) are proposed to reductively inactivate the nitro group appended to an amino derivative. See text for details on pyruvate-formate lyase activating protein (*pflA*), glutamate cysteine ligase (*gshA*), effectors of the SOS response (*lexA*/*recA*) and transcriptional regulators (*AraC*/*XylS*) which are not shown. Fermentations culminating in butyrate, formate, ethanol and butanol, or the dissimilatory sulphate pathway, characteristic of metabolism in some clostridia or other bacteria, are not shown. Only two of the metabolites of reductively activated metronidazole are shown. See text for further details. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

product formed after the reduction of metronidazole and heterocycle fission, is higher in *B. fragilis* than *E. coli*,[53] suggesting heterocycle fission and cytotoxic products are less efficiently formed in *E. coli*. Importantly, even under strict anaerobic conditions the cytotoxic concentration of metronidazole for facultative anaerobes is 10–1000-fold higher than corresponding concentrations for anaerobic bacteria.[21,27,51,52,54–56] Indeed, anaerobes are the first to be killed in biofilms.[57–59] As noted, reduction of the nitro group to the stable amino derivative by nitroreductases renders metronidazole inactive. The NfsA, NfsB and YdjA nitroreductases of *E. coli* are type I oxygen-insensitive, reducing metronidazole to the amino derivative.[30] Type I oxygen-insensitive nitroreductases may account for higher rates of uptake in facultative bacteria such as *E. coli*. Pyruvate:flavodoxin oxidoreductase (YdbK) from *E. coli*[60] and related enzymes in other facultative bacteria, as well as type II oxygen-sensitive nitroreductases are candidates for the reductive activation of metronidazole and resulting cytotoxicity. Being relatively ineffective against facultative anaerobic bacteria, in the clinical setting metronidazole is not recommended for the treatment of these microorganisms.

Pharmacokinetics and pharmacodynamics of metronidazole in humans and murine models

Metronidazole is rapidly absorbed in humans,[61] mostly entering the gastrointestinal tract directly through the mucosa, rather than via the enterohepatic circulation.[62] Metronidazole is found in all tissues in mice and rats, after oral or intravenous administration. The highest concentrations are found in liver, bladder, kidneys, vagina and gastrointestinal tract.[62–64] The majority of metronidazole in human or murine models is excreted in urine unchanged or as oxidized derivatives (compounds **VIII**–**XIII**; Figure 4).[61,62,65] High concentrations of unchanged metronidazole are detected throughout the gastrointestinal tract of rats, except for the caecum,[62] indicating this region, corresponding to the highest number of anaerobic microorganisms,[66] is the major site of nitroimidazole reductive activation.[62] Reduced products of metronidazole (Figure 4) are detected in urine of conventional rats and not germ-free rats, confirming microorganisms are required to reduce metronidazole.[67] In humans, metronidazole has been reported to not alter faecal short-chain fatty acid profile.[68] In rats, metronidazole also enhances microbial colonization of intestinal crypts and increases the thickness of the colonic mucus layer.[69] In mice, metronidazole alters goblet cell function and promotes intestinal inflammation (upregulation of RegIII and IL-25) in the colon and a reduction in MUC2.[59] Also in mice, metronidazole reduces colonization resistance to *Citrobacter rodentium*[59] and temporarily to *C. difficile*, *K. pneumoniae* and *E. coli*.[70] It has been proposed that the induction of colitis may be strain dependent, as observed in a mouse model using *Salmonella enterica*.[71] Recently, in mice colonized with *E. coli*, metronidazole induced intestinal inflammation and increased expression of antimicrobial peptides (*Reg3β* and *Reg3γ*).[72] As *E. coli* or metronidazole treatments alone did not drive this shift, this study also indicates the initial microbiome is

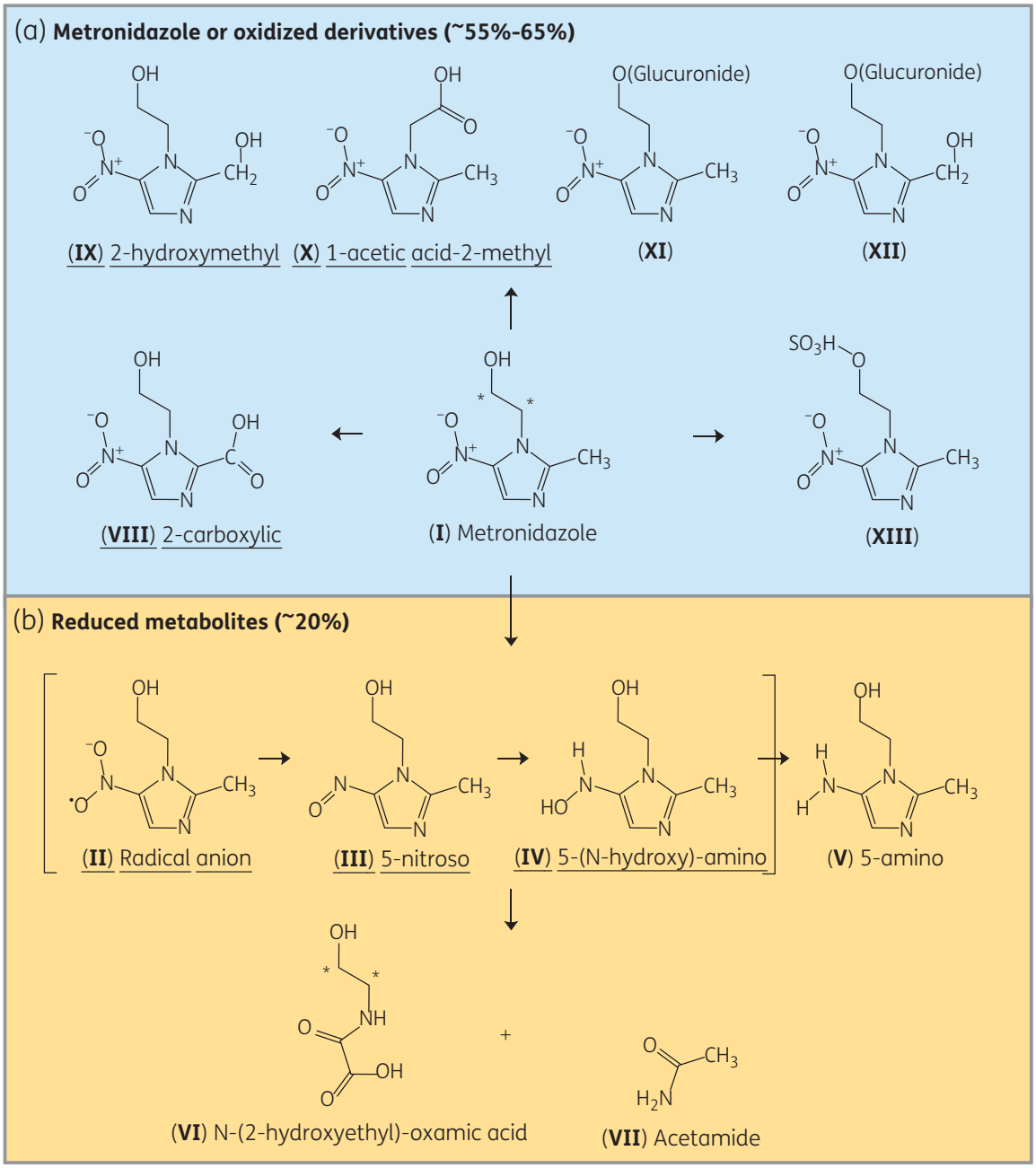

Figure 4. Metabolites of metronidazole and approximate proportions excreted in urine and faeces of mice,[81] humans[81] and rats.[62,67,80] Metabolites of metronidazole proposed to exert cytotoxicity are underlined. Note that intermediates in brackets (compounds **II**–**IV**) are presumptive and have not been isolated. Compounds **XI** and **XII** were found conjugated to glucuronide via an ether linkage. Carbons linking the hydroxyl functional group appended to metronidazole (**I**) and oxamic acid (**VI**) are marked with an asterisk for clarity. Only two products of ring fission are shown [compounds (**VI**–**VII**)]. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

important in host and microbiome responses to metronidazole. Adverse effects of metronidazole are rare. However, prolonged high doses of metronidazole may result in optic neuropathy,[73] peripheral neuropathy[74,75] and encephalopathy.[76]

Determining how metabolites of metronidazole exert cytotoxicity after reductive activation is challenging, as intermediates are short-lived.[23] From studies *in vitro* of the oxidized products of metronidazole, the 'alcohol' and 'acid' (**IX** and **X**, respectively, in Figure 4) have been estimated to account for 30%–65% and 0%–5%, respectively, of the toxicity exerted by metronidazole for *Clostridium* spp. and *Bacteroides* spp.[77–79] In the urine of rats treated with metronidazole, *N*-(2-hydroxyethyl)-oxamic acid[62,80] (compound **VI**; Figure 4) and acetamide[67] (compound **VII**; Figure 4) were detected. These metabolites are consistent with fission of the imidazole between positions 1 and 2 plus positions 3 and 4; however, they do not account for all of the carbon and

nitrogen in metronidazole. Together these metabolites recovered from urine account for only 20% of the total metronidazole administered to rats.[67,80] Importantly, about half of the reduced derivatives of total administered metronidazole are unaccounted for in the excreta of humans and rodents[67,80–82] (see Figure 4).

Various *in vitro* models have been used to mimic biological reductive activation of metronidazole. An enzymatic model consisting of xanthine oxidase and metronidazole produced similar concentrations of *N*-(2-hydroxyethyl)-oxamic acid (compound **VI**; Figure 4) and acetamide (compound **VII**; Figure 4) to those detected in rat studies.[83] Metronidazole was also reduced electrolytically forming at least 12 products, including ethanolamine, *N*-acetylglycine, glycine and acetate, in agreement with a fragmentation between carbons 2 and 4 of the imidazole ring.[84] The metabolites produced in these models of reductive activation of metronidazole broadly agree. Terminal reductively activated products of metronidazole are not toxic.[3,84]

Conflicting reports exist as to how the 'intermediate' metabolites produced during the reductive activation of metronidazole mediate cytotoxicity. Specifically, the capacity of hydroxylamine or nitroso 'intermediate' products (**III** and **IV** of Figure 4) to exert cytotoxicity by direct DNA damage is contested.[23,85] For example, the release of thymidine from radiolabelled DNA during the electrolytic reductive activation of various nitroimidazoles, indicates that intermediate metabolites do damage DNA.[86] Reductively activated metabolites of metronidazole and dimetronidazole have also been found to cause GC to CG transversions within plasmids of *B. fragilis*[87] as well as DNA degradation.[88] Other investigators found metronidazole reductively activated by sodium dithionite formed covalent bonds with guanine and cytosine bases of bacterial and mammalian DNA.[89] However, these covalent links caused no detectable DNA damage.[90] Indeed, another report found only 0.02% of radiolabelled metabolites of reductively activated metronidazole associated with DNA from *E. coli*.[91] In addition, no degradation of chromosomal DNA or single/double-stranded 'nicking' of an endogenous plasmid were detected in *B. fragilis* exposed to metronidazole.[85] Importantly, other investigations found RNA and protein synthesis in cultures of *B. fragilis* and *Clostridium bifermentans* continued at linear rates even after 80 min exposure to metronidazole.[85,92] Indeed, prior inhibition of protein synthesis by chloramphenicol did not alter the bactericidal rate for *Bacteroides* spp. subjected to metronidazole.[27] The variation in the concentration of metronidazole used in these *in vitro* studies accounts for these discrepancies in relation to DNA damage. Importantly, these concentrations are much higher than those achievable *in vivo*. Together, these studies confirm that reductively activated metabolites of metronidazole may associate with DNA, although the incidence is probably too low to directly mediate cytotoxicity. It is more likely that metronidazole indirectly inhibits DNA synthesis and repair.

Several reports concur that bacteria containing various DNA-repair defects are more susceptible to metronidazole. For instance, *E. coli* mutants deficient in *recA* and *lexA*, which control the SOS DNA repair system, were 10-fold more susceptible to metronidazole than parental strains.[51] *E. coli uvrB* mediates DNA repair by unwinding DNA in unison with ABC exinuclease. *E. coli uvrB* mutants are also more susceptible to metronidazole.[55] Double mutants affecting glutamate-cysteine ligase (*gshA*) and *uvrD*/*recG*/*recC* are also up to 40-fold more susceptible to metronidazole.[52] A transcriptional regulator (AraC/XylS) involved in DNA repair has been reported to aid survival after metronidazole challenge in *B. fragilis*.[93] In addition, *B. fragilis* recombinase A (*recA*) DNA repair mutants[94] and DNA helicase mutants (*recQ*)[95] are more susceptible to metronidazole. However, the increased susceptibility of DNA repair mutants does not prove that metronidazole or derivatives directly damage DNA *in vivo*. Ribonucleotide reductase converts ribonucleotide triphosphates (adenosine triphosphate, cytosine triphosphate, guanosine triphosphate and thymine triphosphate) to corresponding deoxyribonucleotide triphosphates, the monomers required for DNA synthesis and repair. The reductive activation of metronidazole alters normal metabolic functions including fermentation. These observations are consistent with the alternative hypothesis that failure to replicate DNA is an indirect response following primary drug toxicity. Specifically, it is hypothesized that reductive activation of metronidazole alters the redox state and carbon flow, which in turn inhibits ribonucleotide reductase. The formation of covalent adducts with thioredoxin reductase and other proteins, derived from reductively activated metronidazole, may contribute to the inhibition of ribonucleotide reductase.[96]

Metronidazole resistance

The mechanisms of metronidazole resistance are complex, manifesting as reduced rate of uptake, by efflux or by reducing the rate of metronidazole reductive activation, for instance by altering pyruvate fermentation. Inactivating resistance determinants (summarized in Figure 3) and increased DNA repair efficiency provide additional mechanisms. Clinical isolates are typically cultured under strict anaerobic conditions, although recently, agar media supplemented with glutathione and ascorbic acid have been used to examine the susceptibility of anaerobic bacteria to metronidazole under aerobic conditions.[97,98] Metronidazole resistance was first described in a clinical isolate of *B. fragilis* in 1978.[99] Recent worldwide rates of reduced susceptibility to metronidazole amongst clinical isolates of *Bacteroides* tend to be low.[100] For example, none of 451 clinical *Bacteroides* spp. isolated from 2011 to 2012 in the United States was resistant to metronidazole.[101] In another study in the United States, one clinical *Bacteroides thetaiotaomicron* isolate was resistant to metronidazole out of 65 strains (1.5%) collected during 2011–14.[102] Of 824 *Bacteroides* spp. isolates with collected from 13 European countries during 2008–09, 4 (0.5%) were found resistant to metronidazole.[103] More recently in Hungary, 1.7% (1 of 60) of *Bacteroides* spp. isolated during 2012 were found to be metronidazole resistant,[104] while in a recent study in the Netherlands, none of the 283 isolates was found resistant (2011–13).[105] Recent rates of clinical *Bacteroides* spp. isolates with resistance to metronidazole are highest in the Middle East, at 5.1% and 8%, respectively, in Iran (8 of 157 strains collected in 2011)[106] and Turkey (4 of 50 strains collected in 2012–13).[107] Notably, in Norway (2009–13),[108] China (2011–12)[109] and the United States (2008–09),[110] recent rates of metronidazole resistance amongst clinical isolates of *Prevotella* spp. are high, from 15% to 21%. The rate of reduced susceptibility to metronidazole was also high in clinical isolates of *Prevotella* spp. from Korea in 2012.[111]

The rate of metronidazole resistance amongst clinical isolates of *C. difficile* tends to be more variable and higher than for

Table 1. Metronidazole MDR clinical isolates

Ref.	Year published[a]	Isolate region	Patient/sample details [age (years), gender]	Strain	Strain no. (%)	MIC (mg/L)	Associated antibiotic resistance[b]	*nimA-I*[c]	*nimJ*[c]	Method[c,d]	Reduced-susceptibility genes
122	1995	England	bacteraemia (blood, F, 38)	*B. fragilis*	1	8	AMC, PEN, TET	NP	NP	NP	NP
			bacteraemia (pus, F, 38)	*B. fragilis*	1	8	AMC, IPM, PEN, TET	NP	NP	NP	NP
178	1999	India	P1: paracolic abscess (pus, F, 75)	*B. fragilis*	1	>32	FOX, PIP	NP	NP	NP	NP
		Bangladesh	P2: peritoneal swab (pus, 40, M)	*B. fragilis* *Bacteroides ovatus*	1	>32	FOX, PIP	NP	NP	NP	NP
					1	>32	FOX, PIP	NP	NP	NP	NP
		Syria	P3: necrotizing pancreatitis (37, M)[b]	*Bacteroides distasonis*	2	>32	FOX, IPM, MEM, TZP	NP	NP	NP	NP
132,145, 165	2005	England	sepsis (blood, F, 48)	*B. fragilis*	1	>256	AMC, CIP, CLI, ERY, FOX, IPM, MEM, NOR, PEN, TET	ND	1	RT-PCR, S, G	*cfiA*, *ermF*, *tetQ* (plasmid), *gyrA* mutation, *bme* efflux pumps (2)
179	2006	Greece	atelectasis (drainage fluid, M, 75)	*Bacteroides vulgatus*	1	>256	CLI, ETP, IPM, FOX, PEN, TZP, TET, TIM	ND	NP	PCR	*cfiA*
131,145	2007	United States	appendicitis (paediatric)	*B. fragilis*	1	≥64	AMP, CFP	ND	3	RT-PCR, S, G	*bme* efflux pumps (3)
145,180	2011	United States (in Afghanistan)	leg wound/bacteraemia (blood, M, 23)	*B. fragilis*	1	>64	CLI, IPM, FOX, MEM, PEN, TZP, SAM, TGC	*nimE* (plasmid pWAL610, IS*Bf6* upstream)	ND	RT-PCR, S, G	
123	2011	South Africa	urology/plastic surgery (pus)	*B. fragilis*	1/23 (4.3%)	>256	FOX, IPM, TET	ND	NP	PCR	
181,182	2012	Denmark	colon cancer (blood/abdominal fluid, M, 84)	*B. fragilis*	1	16	CLI, IPM, MEM, TZP	*nimD* (plasmid pIP421, with activation by IS*1169*)		PCR, G	*bexB*, *cfiA* (IS*Bf12* upstream), *ermF*, *tetQ*, *tetX*
120,183	2013	United States	intra-abdominal abscesses (blood, M, 71)	*Bacteroides* sp.	1	>256	CLI, CTT, IPM, MXF, TZP, SAM	ND	ND	G	*cfxA*, *cfiA13*, *ermF*, *tetQ*, *gyrA* (mutation), putative nitroimidazoles (2, non *nimA-J*)
104	2014	Hungary	abdominal fluid	*B. fragilis*	1/60 (1.7%)	4	CLI, FOX, IPM	nimA (plasmid, IS*1168* upstream)	NP	PCR, S	*cfiA*
121,182	2015	Denmark	peritoneal carcinomatosis/bacteraemia (blood, F, 61)	*B. fragilis*	1	64	CLI, CST, IPM, MEM, PEN, TZP	*nimE* (IS*Bf6* upstream)	ND	G	*bexB*, *cfiA*, *ermF*, *tetQ (2)*,

Continued

Table 1. *Continued*

Ref.	Year published[a]	Isolate region	Patient/sample details [age (years), gender]	Strain	Strain no. (%)	MIC (mg/L)	Associated antibiotic resistance[b]	*nimA-I*[c]	*nimJ*[c]	Method[c,d]	Reduced-suscepibility genes
182	2015	Denmark	blood culture	*B. fragilis*	1	>32	CLI, IPM, MEM, TZP	ND	1 (partial IS*614B* upstream)	G	*bexB*, *cfiA*, *tetQ*
184	2015	Hungary	colonic tumour (stoma pus, M, 57)	*B. fragilis*	1	2	AMC, CLI, ERY, FOX, IPM, MEM, PEN, TZP, TET, TGC	*nimA* (plasmid, IS*1168*)		RT-PCR	*cepA*, *erm*, *cfiA* (IS*1187*), *cfi*AUP, *tetQ*
102	2015	United States	pleural empyema (M, 43)	*B. thetaiotaomicron*	1	32	CLI, ETP, MEM, TZP, TGC	nimD (plasmid, portion IS*1169* upstream), putative nim gene (1)	ND	G	*tetX* (2), *tetQ*, *ermF*, cat (2), *mef*, efflux pumps [ABC transporter (2), RND family (2), AcrB/AcrD/AcrF, efflux pump]
185	2015	Russia	gastric cancer (wound)	*B. fragilis*	1	>256	AMC, IPM	NP	NP	NP	NP
186	2015	India	pyopneumothorax (pus, M, 40)	*B. fragilis*	1	NA	CLI, CRO, FEP, LVX, OFX, TZP	NP	NP	NP	NP
124	2015	Holland	clinical isolates	*P. bivia*	2/113 (1.8%)	8, 64, >256	AMX, CLI, TET	ND	NP	PCR	
					1/113 (0.9%)	8, 64, >256	AMX, TET	ND	NP	PCR	
162	2016	Sweden	clinical isolates	*B. fragilis*	1	>256	FOX, MEM, TZP, TET	*nimB* (IS*11860* upstream)	ND	PCR	*cfiA*, *tetQ*, IS*4351*
					1	>256	CLI, FOX, MEM, TZP, RIF, TET	*nimB* (IS*11860* upstream)	ND	G,PCR	*cfiA*, *ermF*, *tetQ*, *tetX*, IS*4351*
					1	>256	FOX, MEM, MXF, TZP, RIF, TET	*nimB* (IS*11860* upstream)	ND	PCR	*cfiA*, *tetQ*, IS*4351*
161	2016	United States	paraspinal/psoas abscesses (blood, M, 82)	*B. fragilis*	1	32	CLI, FOX, ETP, IPM, MEM, PEN, SAM	ND	ND	G	*cfiA*, *cfxA*, *ermF*, *ermB*, *sul2*

[a]When a study is extended, the first publication year is listed.
[b]AMC, amoxicillin/clavulanate; AMP, ampicillin; CFP, cefoperazone; CIP, ciprofloxacin; CLI, clindamycin; CRO, ceftriaxone; CST, colistin; CTT, cefotetan; ERY, erythromycin; ETP, ertapenem; FEP, cefepime; FOX, cefoxitin; IPM, imipenem; LVX, levofloxacin; MEM, meropenem; MXF, moxifloxacin; NOR, norfloxacin; OFX, ofloxacin; PEN, penicillin; PIP, piperacillin; RIF, rifampicin; SAM, ampicillin/sulbactam; TET, tetracycline; TGC, tigecycline; TIM, ticarcillin/clavulanic acid; TZP, piperacillin/tazobactam.
[c]PCR for *nim* genes not performed (NP). PCR or genome sequencing did not detect *nim* genes (ND).
[d]Methods used to assess genetics of strains include real-time PCR (RT-PCR), Sanger sequencing (S) and genome sequencing (G).

Bacteroides spp. For example, during 2008–09 one study found 46.2% of clinical *C. difficile* isolates (12 of 26) from the United States had resistance to metronidazole.[110] The rate of metronidazole resistance of *C. difficile* isolates collected from the same regions during 2011–12 and 2012–13 were 3.6% (33 of 925)[112] and 0% (0 of 196),[113] respectively. In contrast, no resistant *C. difficile* were found in another region of the United States during 2008–09 (316 isolates).[114] In addition, none of the 114 *C. difficile* isolates collected from Swedish (2008–11)[115] patients and only 0.1% of the isolates from a European study (1 of 916, 2011–12) were resistant to metronidazole. Lastly, none of the clinical isolates of *C. difficile* was metronidazole resistant in Australia (0 of 91; 2005–14),[116] Thailand (0 of 107; 2015)[117] and China (0 of 101; 2012–15).[118]

An MDR isolate has acquired reduced susceptibility to an antibiotic in three or more antimicrobial categories.[119] Metronidazole-MDR clinical isolates of *Bacteroides* spp. and *Prevotella* spp. are considered rare and metronidazole-MDR *C. difficile* have not been isolated.[100,120,121] The first report of a metronidazole-MDR isolate was of *B. fragilis* in 1995 (Table 1).[122] The rate of MDR-metronidazole *Bacteroides* spp. and *Prevotella* spp. isolates is increasing, with >25 strains discovered to date (Table 1), the majority of which are *Bacteroides* spp. The rate of MDR-metronidazole *Bacteroides* in South Africa between 2003 and 2005 was 4.3%[123] while in Hungary the rate was 1.7% during 2012.[104] In the Netherlands, the rate of MDR-metronidazole *Prevotella* spp. isolates from 2011 to 2013 was 2.7%[124] (Table 1). Amongst *Bacteroides* spp. the most frequent antibiotic co-resistance was imipenem (55.6%) followed by piperacillin/tazobactam (51.9%, Table 1). Cefoxitin, clindamycin and meropenem (all 48.1%) were frequently associated with metronidazole-MDR *Bacteroides* spp. isolates, as were tetracycline (40.7%) and penicillin (29.6%, Table 1). Of three MDR-metronidazole *Prevotella* spp. clinical isolates, all were *Prevotella bivia* with co-resistance to amoxicillin and tetracycline. Two metronidazole-MDR *P. bivia* isolates were also resistant to clindamycin (Table 1).

As previously mentioned, the mechanisms of metronidazole resistance are complex. Metronidazole uptake is one parameter involved in resistance to this drug. Generalized defects in metronidazole transport, accompanied by changes in cell wall structure, have been described in *B. fragilis*.[125] *E. coli* that survived high-concentration metronidazole challenge exhibited an elongated filamentous morphology and colonies were anaerogenic.[51] It is possible that the uptake rate of metronidazole was lower than WT as previously detected by others.[27] Sublethal metronidazole also induced elongation of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.[126,127] *E. coli* mutants lacking the ability to reduce nitrate and chlorate, presumably reducing the rate of metronidazole uptake, are also resistant.[55] A reduced growth rate is also associated with metronidazole resistance in *C. perfringens*,[44] *C. difficile*[128] and *B. fragilis*.[129] *Bacteroides* resistance–nodulation–division (RND) efflux pumps (*bme*) also reduce susceptibility to metronidazole (Figure 3).[130–132]

Changes in type of metabolism or downregulation of metronidazole activators are also associated with resistance. The reduction of PFOR activity and upregulation of LDH activity is commonly associated with metronidazole resistance in *C. perfringens* and *Bacteroides* spp.[44,47,133–136] In *B. thetaiotaomicron*, the upregulation of rhamnose catabolism regulatory protein (RhaR) results in a similar rerouting and increased resistance to metronidazole.[134] Sublethal concentrations of metronidazole also induce catabolism of raffinose by *F. nucleatum* as well as suppressing arginine dehydrolase.[126] In addition, downregulation of flavodoxin in *B. fragilis* decreases susceptibility to metronidazole.[47] Transposon mutants of *C. acetobutylicum pflA* are also more resistant to metronidazole.[42] In genomic screens of metronidazole-susceptible or -resistant *C. difficile* strains, mutations in *hemN* (oxygen-independent coproporphyrinogen-III oxidase), *thiH* (thiamine biosynthesis), glycerol-3-oxidoreductase (*glyC*) and pyruvate-flavodoxin oxidoreductase (*nifJ*)[128] were associated with resistance, consistent with a reduced rate of electron transport and alterations in intracellular redox. Deletion of the gene encoding the ferrous transport fusion protein (*feoAB*) also decreases susceptibility to metronidazole.[137] In *H. pylori*, thioredoxin reductase, alkyl hydroperoxide reductase and superoxide dismutase are involved in resistance to metronidazole.[138]

Various enzymes inactivate metronidazole, primarily by reductive inactivation of the nitro group to the amino derivative. Nitroreductases consist of oxygen-insensitive NAD(P)H pairing (type I) and oxygen-sensitive (type II) types.[30] Type I nitroreductases are the primary enzymes described in reductive inactivation of metronidazole, while type II nitroreductases may reductively activate metronidazole, leading to cytotoxicity (Figures 2 and 3). The biological role of nitroreductases is unclear although they may function in redox maintenance, by dissipating excess reducing power.[30] Early studies of *S. faecalis*[139] and *E. coli*[3] indicated nitroreductases were able to inactivate metronidazole. For example, *E. coli* reduce ∼20% of administered metronidazole to the amino derivative (**V** of Figure 2).[3] The oxygen-insensitive major and minor (*nsfA* and *nfsB*, respectively) flavoprotein nitroreductases of *E. coli*, were shown to reduce nitrofurans and later, metronidazole.[15,140] These nitroreductases share a similar structure to the *nim* genes described below. Mutants of both *nfsA*/*nfsB* in *E. coli* show reduced susceptibility to metronidazole. A 2-nitroimidazole nitrohydrolase (NnhA) was also shown to render *E. coli* resistant to 2-nitroimidazoles.[141] Metronidazole was not inactivated, although other enzymes may function similarly to render bacteria resistant to metronidazole.

Low-level resistance to nitroimidazoles is often ascribed to the *nim* genes.[87] The *nim* genes are best characterized in *Bacteroides* and other genera of the Bacteroidetes phylum, although they are also encoded within genera of the Clostridia class, Proteobacteria and Archaea.[142–144] Currently nine *nim* genes are described in *Bacteroides*, including *nimA*–*nimH* and *nimJ*, which are chromosomally or plasmid borne.[129,145,187] A 'silent' nim gene, *nimI*, is encoded by *Prevotella* spp.[146] Of 1502 isolates of *Bacteroides* spp., some 2% were found to carry *nim* genes.[147] More recent worldwide rates of detection of *nim* genes in *Bacteroides* spp. has found carriage rates of 0%–2.8%.[104,145,148–151] Recent worldwide carriage rates of *nim* genes in *Prevotella* spp. range from absent to 8%.[107,109,124,152] In 38 clinical *Bacteroides* spp. isolates from the United States and South Africa, *nimJ* was absent.[123,145] Whether chromosomal or on a plasmid, resistance is transferable in some *Bacteroides* spp.,[153] including by conjugation between *Bacteroides* spp. and *Prevotella* spp.[154] In *B. fragilis*, *nimA* encodes a 5-nitroimidazole reductase reducing the metronidazole analogue dimetronidazole (1,2-dimethyl-5-nitroimidazole) to the amino derivative, preventing ring fission and associated

toxicity.[155] Presumably, other *nim* genes encode 5-nitroimidazole reductases, although the exact mechanism of *nim* resistance is not known[145] and it has not been demonstrated that the *nim* system produced the amino derivative in metronidazole. Structural analysis of NimA from *Deinococcus radiodurans* (from the Bacteroidetes phylum) indicates the protein is a homodimer, with two binding sites with weak affinity for nitroimidazoles including metronidazole.[156] Two nitroreductases from *C. difficile* are also homo-dimeric and both bind flavin mononucleotide.[157]

The role of *nim* genes in metronidazole resistance is controversial. It has been established that overexpression of a NimA homologue from *B. fragilis* induces a 3-fold increase in metronidazole resistance in *E. coli*.[158] In addition, overexpression of *nimE* and *nimJ* on pMCL140 in *B. fragilis* induces a 4–6-fold increase in metronidazole resistance.[145] However, several non-*nim*-containing *Bacteroides* spp., *Prevotella* spp. and *C. difficile* and other genera within the Clostridia class are resistant to metronidazole whereas *nim*-containing isolates within these clades are susceptible to metronidazole.[123,129,135,143,146,158–162] In addition, expression of the NimA-D proteins does not correlate with metronidazole resistance in *B. fragilis*.[163] The possibility that insertion sequences upstream of *nim* genes may control expression is also contested.[147] Lastly, up to a 256-fold increase in resistance to metronidazole can be induced in the absence of the *nim* genes,[146,159,163] suggesting other factors also feature in metronidazole resistance.[145,146,159,163,164] An explanation for these conflicting results is that very high levels of the Nim proteins are required to confer metronidazole resistance and that other factors determine high-level metronidazole resistance.

Current developments in nitroimidazole drugs and concluding remarks

The human gastrointestinal tract hosts complex microbial populations. Broad-spectrum antibiotics kill off-target microorganisms,[165,166] promote overgrowth of certain clades[57] and alter the physiology of those remaining.[167] Changes in the number of microbes within particular clades may persist longer term[165,168] reducing resistance to colonization by pathogenic microorganisms or allowing overgrowth of endogenous pathogens such as *C. difficile*.[59] To circumvent drug resistance and the possibility of, e.g. *C. difficile* overgrowth, new nitroimidazoles have been synthesized.[169,170] Other novel nitroimidazoles include metronidazole/triazole conjugates.[171]

Alternative strategies for eliminating pathogens include the use of narrow-spectrum or selective antibiotics. Selectively targeting pathogens may circumvent increases in rates of resistance, if fewer bacteria take up a drug.[172–176] Using this strategy, our group synthesized deuteroporphyrin–nitroimidazole adducts designed to be taken up by porphyrin receptors of *P. gingivalis*.[174,176] In principle, selective killing may also be achieved by activating cryptic alternative pathways, thereby usurping activation of nitroimidazole pro-drugs.[177] Continued development of new nitroimidazoles and novel killing strategies will be useful to have in the antibiotic arsenal to combat resistant microorganisms or in targeting specific pathogens.

In relation to metronidazole, facets of the uptake and cytotoxicity profile remain unanswered. It is clear that the ultimate fate of metronidazole depends on whether it is reductively activated or inactivated. In addition, several enzymes that reductively activate metronidazole have been described. How metronidazole halts DNA synthesis and how high levels of resistance are achieved remain important questions. Solving these questions is likely to aid the control of pathogenic microorganisms by informing rational design of new compounds and novel antibiotic strategies.

Acknowledgements

Authors thank Michael Malamy for providing critical comments during preparation of this review.

Transparency declarations

None to declare.

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