The treatment of helicobacter pylori infection and its sequelae with emphasis on nitroimidazole resistance
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Chapter 4

Anaerobic incubation of metronidazole resistant *Helicobacter pylori*: understanding the mechanism of metronidazole resistance.

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Submitted
**Background & Aims:** Previous observations showed that metronidazole resistant strains become susceptible after anaerobic incubation in the presence of this drug. The clinical significance of this reversibility in resistance is still unknown. The aim of this study was to describe this phenomenon in more detail to gain more insight in the mechanism of metronidazole resistance.

**Methods:** Nine metronidazole resistant clinical isolates and a reference strain were incubated under anaerobic conditions with or without a metronidazole containing E-test strip for 0, 0.25, 0.5, 1, 2, 4 and 8 hours. Plates were stored under micro-aerophilic conditions and read after 3 days. The experiment was repeated in broth with or without the protein synthesis inhibitor chloramphenicol.

**Results:** The minimal inhibitory concentrations of metronidazole decreased slowly under anaerobic conditions. Anaerobic incubation without metronidazole did not decrease the viability of the organism. Chloramphenicol did not affect this loss of resistance.

**Conclusions:** In resistant isolates there was still reduction of metronidazole to active components. The enzymes required for this reduction are already present under micro-aerophilic conditions. This is in consistence with previous observations showing that several nitroreductases are present in *H. pylori* but that resistance in the presence of oxygen is related to absence of one of them: the oxygen-insensitive nitroreductase (*rdxA*). The large variation in nitroimidazole susceptibility between different isolates can be easily explained by variable activity of these enzymes between these isolates. Furthermore, repetitive testing of one isolate may result in a lack of reproducibility due to variability in expression of these enzymes. Finally, our data suggest that metronidazole is still of therapeutic value in resistant strains.
Introduction.
Metronidazole is one of the key drugs in treatment regimens for *Helicobacter pylori* (*H. pylori*) infection (1). It has been shown that the prevalence of metronidazole resistance is increasing in the Western world (2-7). Although resistance of the microorganism to this drug affects the efficacy of metronidazole containing regimens it does not make them completely ineffective (8). Therefore, insight in the mechanism of metronidazole resistance is important as it may lead to new treatment regimens that are better able to overcome this resistance.

Recently, the genetic background of metronidazole resistance has been elucidated. Null mutations in a gene encoding for an oxygen-insensitive nicotinamide adenine dinucleotide phosphate nitroreductase (*rdxA*) are associated with resistance (9). Whether this mechanism accounts for resistance in all clinical isolates is still much debated (10-13). Moreover, the discovery of the *rdxA* gene did not clarify previous observations that metronidazole resistant strains turn into susceptible ones during anaerobic incubation in the presence of metronidazole (14-15). To shed more light on this phenomenon, the duration of incubation needed for this transformation was examined and it was studied whether *H. pylori* required de novo protein synthesis for this phenomenon to occur.

Methods.
*Strains and growth conditions.* Nine different clinical isolates of *H. pylori* obtained from nine different dyspeptic patients and the reference strain ATCC 43504 (16) were used. All strains were metronidazole resistant (MIC > 8 µg/mL) as determined by E-test. The strains were stored frozen at -80°C in brain heart infusion broth with 20% glycerol until use. Bacteria were cultured on Columbia agar plates supplemented with 7% lysed, defibrinated horse blood with or without 0.004% tetrazolium chloride and 0.0025% Dent supplement or in Brucella broth supplemented with 2% newborn calf serum and 0.0025% Dent supplement. All cultures were incubated under micro-aerophilic conditions (5% O2, 10% CO2 and 85% N2) and anaerobic conditions (10% H2, 5% CO2 and 85% N2) at 37°C.

The influence of different periods of anaerobic incubation in the presence of metronidazole on metronidazole resistance. Columbia agar plates without supplements were inoculated with a suspension of multiple *H. pylori* colonies using an inoculum
approximating a McFarland 3-4. A metronidazole containing E-test strip (AB-Biodisk, Solna, Sweden) was applied according to the instructions of the manufacturer. Thereafter, the plates were incubated under anaerobic conditions for 0, 0.25, 0.5, 1, 2, 4, 8 hours, respectively. After each incubation period the strains were stored under micro-aerophilic conditions and read after three days. To determine whether metronidazole needs to be present during the anaerobic phase for this transformation, plates were also incubated under anaerobic conditions for 480 minutes without application of the E-test strip. Thereafter, the E-test strip was applied and the plates were stored under micro-aerophilic conditions. Metronidazole susceptibility was read after three days. All experiments were performed in triplicate.

*The influence of chloramphenicol on metronidazole resistance during anaerobic incubation.*

It has been suggested that the induction of compensatory metabolic pathways are needed for *H. pylori* to survive under anaerobic conditions. As it is unknown if the killing of bacteria by metronidazole under anaerobic incubation requires these metabolic pathways the novo protein synthesis was prevented through the addition of chloramphenicol. Bacteria harvested from freshly grown, almost confluent Columbia agar plates were inoculated in Brucella broth (one plate per 100 ml) and incubated overnight with gentle agitation under micro-aerophilic conditions. At the onset of the experiment, 0.1 % fumaric acid was added to the overnight culture to make strict anaerobic conditions possible (13). Thereafter, the culture was split into 10 ml portions that were incubated under micro-aerophilic or anaerobic conditions either with or without 16 µg/mL metronidazole and with or without 10 µg/ml of the protein synthesis inhibitor chloramphenicol for 0, 2, 4, 6 and 8 hours. Thereafter, 1ml of the broth was taken and washed twice with phosphate buffered saline to remove the antibiotics. In order to determine the number of viable bacteria, 50µL of undiluted and 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ dilutions in phosphate buffered saline was plated on Columbia agar plates, and incubated under micro-aerophilic conditions. After four days colonies were counted and data are expressed as colony forming units per mL (CFU/ml). All experiments were performed in triplicate.
Results.
Without anaerobic incubation the median MIC as determined by the E-test was 224 µg/ml (range 24 to >256 µg/ml). With increasing anaerobic incubation time the MIC decreased slowly for all strains tested. After 2 hours all strains were still resistant. After 8 hours, however, all strains had become susceptible (Figure 1). A decrease in MIC was never seen when the E-test strip was applied immediately after the anaerobic phase (data not shown). From this we concluded that there is slow killing of the resistant bacteria by metronidazole during the anaerobic phase of the experiment.
For all strains tested in broth both metronidazole and anaerobic incubation were needed for killing. Neither metronidazole alone nor anaerobic incubation alone resulted in a decrease in CFU/ml. Moreover, the addition of chloramphenicol could not prevent the loss of resistance during anaerobic incubation in the presence metronidazole. A representative example of the results in one isolate is shown in Figure 2. All the other isolates reproducibly gave similar results. Therefore, the addition of chloramphenicol did not prevent the decrease in MIC, suggesting that for killing of the bacteria induction of new metabolic pathways is not required.

Figure 1. The influence of anaerobic incubation in the presence of metronidazole on the susceptibility to metronidazole in 10 resistant strains.
Figure 2. The influence of chloramphenicol (chlor) on metronidazole (met) susceptibility under micro-aerophilic (micro) and anaerobic conditions in one of the 10 metronidazole resistant strains.

Discussion.
Strains of \textit{H. pylori} grow best under micro-aerophilic conditions (17). Therefore, susceptibility testing for antibiotics in \textit{H. pylori} is usually performed under such conditions. Under anaerobic conditions, however, \textit{H. pylori} strains remain viable, although they do not grow (17). Metronidazole, one of the nitroimidazoles, is a prodrug that needs to be activated by a redox-reaction, in which metronidazole is the electron acceptor (18,19). This reduction step works out most effectively in an environment with a low redox-potential such as the anaerobic cell (20). Nitroimidazoles, therefore, are antimicrobial agents that are primary active against \textit{anaerobic} bacteria (18,19).

Surprisingly, however, they are also active against the majority of \textit{H. pylori} strains, a \textit{micro-aerophilic} pathogen. This is explained by the presence in \textit{H. pylori} of an oxygen-insensitive nitroreductase encoded by the \textit{rdxA} gene. In the majority of resistant isolates tested so far resistance to metronidazole was associated with null-mutations in this gene (9,12,13,16). We used the ATCC 43504 reference strain as it was shown previously that in this strain resistance was due to the insertion of a mini-IS605 transposable element in
the \textit{rdxA} gene and that this strain had no mutations in other genes previously associated with the metabolism of nitroimidazoles (16). Although we did not establish the exact mechanism of resistance in the clinical isolates, the results of our experiments were similar to that of the reference strain with a known defective \textit{rdxA} gene. This suggests a similar mechanism of resistance.

It has been shown previously that resistant strains become susceptible to metronidazole during anaerobic incubation (13-15). Our study confirms these observations and indicate that this transformation is a slow process and that susceptibility to metronidazole is induced during the anaerobic phase. It has previously been suggested that the loss of resistance is due to the induction of compensatory metabolic pathways required for the survival of \textit{H. pylori} under anaerobic conditions (20,21). The results of our study using chloramphenicol as a protein synthesis inhibitor, however, indicate that compensatory metabolic pathways are not involved as the inhibition of de novo protein synthesis did not prevent the loss of susceptibility. Therefore, our data suggest that during anaerobic incubation of metronidazole resistant \textit{H. pylori} strains, metronidazole is slowly reduced by preexisting mechanisms.

This is in concurrence with metronidazole uptake studies showing that under anaerobic conditions there is a slow uptake of metronidazole in resistant strains that is absent under micro-aerophilic conditions (21,22). As it is known that metronidazole diffuses passively into the bacterium (19), uptake of metronidazole is only possible if a diffusion gradient persists because of the metabolism of metronidazole to active metabolites. These observations shed more light on the mechanism of metronidazole resistance. In metronidazole susceptible strains the reduction of metronidazole by the \textit{rdxA} encoded nitroreductase leads to a nitrosoderivate by direct transfer of two electrons (MET + 2e$^{-}$ \rightarrow MET$^{2-}$). This highly toxic nitrosoderivate causes DNA damage and subsequent killing of the bacterium (Figure 3) (9,19). These toxic nitrosoderivates cannot be re-oxidized by molecular oxygen due to the chemical nature of the two-electron transfer step catalyzed by this enzyme, and the nitroreductase is, therefore, called \textit{'oxygen-insensitive'} (19). In contrast to the situation in susceptible strains, a functional \textit{'oxygen-insensitive'} nitroreductase is not available in metronidazole resistant strains. In \textit{H. pylori}, however, several other nitroreductases are present (23,25-27). These nitroreductases can reduce metronidazole, all be it to a free radical anion by a one-electron transfer step (MET + e$^{-}$ \rightarrow MET$^{-}$) (23). This toxic free radical anion can, in contrast to the nitrosoderivate that is produced by the \textit{rdxA} encoded nitroreductase, be
Figure 3. Suggested metabolism of metronidazole in susceptible strains. MET represents metronidazole, R represents the electron donor, and e represents an electron.

Figure 4. Suggested metabolism of metronidazole in resistant strains under microaerophilic conditions. MET represents metronidazole, R represents the electron donor, e represents an electron, and SOD represents super oxide dismutase.
Figure 5. Suggested metabolism of metronidazole in resistant strains under anaerobic conditions. MET represents metronidazole, R represents the electron donor, and e represents an electron.

Re-oxidized by molecular oxygen to the original compound, a process called 'futile cycling' (MET + O₂ → MET + O₂⁻) (13,19). These other nitroreductases are, therefore, called 'oxygen-sensitive'. The toxic superoxide (O₂⁻), produced in this process, can easily be eliminated by superoxide dismutase and catalase, two enzymes present in large amounts in *H. pylori* (Figure 4) (28). Moreover, it has been shown that *H. pylori* reduces the activity of several of these 'oxygen-sensitive' nitroreductases in the presence of metronidazole (24,29,30), probably with the aim to reduce the production of free radical anions. Both 'futile cycling' and reducing the activity of 'oxygen-sensitive' nitroreductases may be responsible for metronidazole resistance in the absence of a functional *rdxA* encoded nitroreductase as long as molecular oxygen is present. In the
absence of molecular oxygen, however, 'futile cycling' is not possible. The free radical anion can then exert its toxic effects or can be further reduced (Figure 5) (19,28). This results in loss of metronidazole resistance under anaerobic conditions.

Our observations have important clinical implications. It has been shown that there is a wide variation in MIC for metronidazole both among strains and among resistant strains (31). It was, therefore, suggested that resistance could not be the result of mutations in only one gene, but that mutations in several genes had to be involved (11). In contrast, our data suggest that susceptibility to metronidazole might very well be dependent on the presence of one enzyme only, the \( rdxA \) encoded nitroreductase and that the large variation in MIC's can be explained by 'background' variation in the activity of other nitroreductases. This could also explain the lack in reproducibility in MIC when one isolate is repeatedly tested of metronidazole susceptibility, although a strain remains either susceptible or resistant (32). Our present observations suggesting that several other nitroreductases are slowly reducing metronidazole and that the activity of these nitroreductases are regulated by environmental factors may explain these variations in MIC. These variations are, therefore, real variations in nitroreductase activity and not merely methodological artifacts as suggested previously (33).

Our study shows that reduction of metronidazole to active components is still possible in resistant strains suggesting that the addition of metronidazole to a regimen may, therefore, increase the efficacy of this regimen in resistant strains. Administration of metronidazole may, therefore, have therapeutic value even in patients infected with a resistant strain. The clinical significance of these observations is probably underestimated and clinical trials are warranted. If this observation proves to be of clinical relevance it may become of great importance as multiple resistant \( H. pylori \) strains are an emerging problem.

In summary, our data are in concurrence with previous observations showing that resistance is related to mutations in the \( rdxA \) gene, encoding for an oxygen-insensitive nitroreductase. In the absence of a functional \( rdxA \) gene, however, there is still a slow reduction of metronidazole. These mechanisms become operative in the absence of oxygen when re-oxidation of the metronidazole metabolites becomes impossible. This slow reduction of metronidazole may be responsible for the large 'background' variation in MIC that is observed between and within clinical isolates of \( H. pylori \) and may explain why administration of metronidazole may still have therapeutic value in patients infected with a resistant strain.
References.
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