Chapter 8

Nitroimidazole resistance in *Helicobacter pylori*: a review.

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Abstract.
The efficacy of a nitroimidazole containing regimen for the treatment of *Helicobacter pylori* infection is decreased by nitroimidazole resistance. Nitroimidazoles are metabolized by *Helicobacter pylori* by several nitroreductases of which an oxygen-insensitive NADPH nitroreductase encoded by the *rdxA* gene is the most important one. Null mutations in this gene are associated with resistance.

Susceptibility testing to nitroimidazoles may give variable results. This is not only related to the slow growth under specific conditions, but also related to variability in the activity of the other nitroreductases and the ability to deactivate toxic metabolites of a nitroimidazole and to repair DNA damage. Moreover, co-infections with resistant and susceptible bacteria are frequently found. The presence of nitroimidazole resistance is related to the previous use of this drug. The prevalence of resistance is rising and nowadays 10-50% of the isolates are resistant. Resistance reduces the efficacy of a treatment regimen to a variable degree. This is related to efficacy of the other components of the regimen and the treatment duration. Whether a nitroimidazole is still effective in resistant strains remains unresolved. When nitroimidazole resistance is present a nitroimidazole containing regimen should be avoided or a regimen with other highly effective components should be used.
Introduction.

*Helicobacter pylori* (*H. pylori*) is a curved, micro-aerophilic, Gram negative rod that colonizes the stomachs of approximately half the world's population (1,2). In all subjects infected with this micro-organism it results in an active, chronic, antrum-predominant gastritis. Although this gastritis is asymptomatic in the majority of the infected subjects, in the Western world approximately 10% develop a duodenal or gastric ulcer (3). Furthermore, it has been suggested that in a subgroup of *H. pylori* infected patients with functional dyspepsia the infection causes the symptoms (4-6). This, however, is still a much debated topic (5,7,8). Finally, in a minority of *H. pylori* infected subjects the chronic gastritis leads to gastric cancer (9).

There is international consensus that in patients with proven peptic ulcer disease treatment of the *H. pylori* infection is warranted as it cures the disease (5,10). As endoscopy is gradually replaced by non invasive *H. pylori* testing as the primary diagnostic tool in dyspeptic patients, peptic ulcer disease is often not diagnosed with certainty anymore and *H. pylori* may be eradicated in all infected dyspeptic patients (5,11). This would result in a substantial increase in the number of patients subjected to *H. pylori* eradication. Furthermore, it has even been suggested that screening and subsequent treatment of the whole population is cost-effective as it may prevent gastric cancer (12) and may lead to a reduction of dyspeptic symptoms in the population (13). This strategy would lead to an enormous increase in the use of anti-*H. pylori* therapy.

An effective widespread use of anti-*H. pylori* regimens, however, is only possible if these treatment regimens are safe, simple, and highly effective. For the treatment of *H. pylori* infection a combination of several drugs, including antibiotics, acid-suppressants, and/or bismuth compounds, is needed (14). A nitroimidazole, such as metronidazole and tinidazole, is often included in these drug combinations (14). Resistance of *H. pylori* to nitroimidazoles, however, is frequently encountered and is, therefore, an important threat to the efficacy of these regimens (15). Recently, new information on the mechanism of resistance has become available. In this review these data are discussed together with the techniques to determine nitroimidazole resistance in *H. pylori* and its epidemiology and clinical significance.

**Mechanism of nitroimidazole resistance.**

Nitroimidazoles are prodrugs and need to be activated intracellularly to become effective. After entering the cell by passive diffusion (16,17), the nitroimidazole is
metabolized by a reduction step in which the drug is the electron acceptor (18,19). For this reduction step several nitroreductases are present in *H. pylori* (20-23), but recently it has been suggested that an oxygen-insensitive NADPH nitroreductase encoded by the *rdxA* gene is the most important one (24). Oxygen-insensitive denotes in this case that the reduction of the nitroimidazole results in a nitrosoderivate by the simultaneous transfer of two electrons. This nitrosoderivate can not be re-oxidized by molecular oxygen and the nitroreductase facilitating the two-electron transfer step is, therefore, called oxygen-insensitive. The nitrosoderivate leads to DNA damage and subsequent cell death (figure 1). The other nitroreductases present in *H. pylori* reduce a nitroimidazole by a one-electron transfer step to a toxic free radical anion that can be metabolized in two ways (figure 2). First, the free-radical anion can be re-oxidized to the original compound by molecular oxygen with the production of superoxide. Thereby, molecular oxygen reverts the reduction step and these nitroreductases are, therefore, called oxygen-sensitive. Theoretically, this process of reduction and re-oxidation is repeated endlessly and is called 'futile cycling' (25). The superoxide produced during this 'futile cycling' is also toxic but can be eliminated by superoxide dismutase and catalase. The second way to metabolize the toxic free radical anion is an other one-electron transfer step to the more toxic nitrosoderivate that leads to DNA damage (26).

**Figure 1.** Metabolism of a nitroimidazole (NI) by an oxygen-insensitive nitroreductase. R represents the electron donor, e represents an electron.
Figure 2. Metabolism of a nitroimidazole (NI) by an oxygen-sensitive nitroreductase. R represents the electron donor, e represents an electron. SOD represents superoxide dismutase.

The genetic background of nitroimidazole resistance in *H. pylori* has recently been elucidated by Goodwin et al (24). They showed that null-mutations in the *rdxA* gene result in the resistant phenotype and that strains with a defect *rdxA* gene had no survival disadvantage as compared to isogenic susceptible strains (24). Such null-mutations were present in all of the few clinical isolates tested so far (24,27). It was, however, suggested that more clinical isolates should be studied before mutations in this gene can be accepted as the most important mechanism of nitroimidazole resistance (28). One study using a mouse model confirmed the clinical data and showed that after the induction of nitroimidazole resistance by exposure to metronidazole the majority of resistant isolates showed null-mutations in the *rdxA* gene while none of the susceptible isolates did (29). Interestingly, however, two phenotypically resistant strains still had the 'wild-type' *rdxA* gene. In these strains mutations in genes regulating the expression of the *rdxA* gene could be involved, as well as other mechanisms of resistance. Several of such mechanisms of resistance have been hypothesized: increased DNA-repair (30), down
regulation of the activity of nitroreductases (19,23,31), increased catalase and superoxide dismutase activity (25), and nitroimidazole efflux mechanisms (29). So far, however, none of these mechanisms has been substantiated.

The way a nitroimidazole is metabolized in *H. pylori* has important implications. Although the knock-out of the most important enzyme (encoded by *rdxA*) results in resistance, the activity of the other enzymes, as well as the ability of the strain to neutralize the toxic metabolites and to repair DNA damage may result in 'background' variation in susceptibility. It is, therefore, not unexpected that large variations in the minimal inhibitory concentration (MIC) are seen both among susceptible and resistant isolates (32,33,34). Furthermore, considerable variations in MIC can be observed when one isolate is tested several times (34,35). This is, apparently, the result of variations in expression of the different nitroreductases caused by differences in environmental conditions or by the bacterial growth phase and viability. Besides, this 'background' nitroreductase activity explains that the addition of a nitroimidazole to a treatment regimen may theoretically increase the efficacy of the regimen in resistant strains (36).

As metabolites of nitroimidazoles are mutagenic (17,18,24) the use of a nitroimidazole results in an increased mutation frequency in all genes, including the *rdxA* gene (29). This causes an rapid induction of nitroimidazole resistant mutants. Moreover, in the presence of a nitroimidazole the resistant mutants have a survival advantage over the susceptible strains. A nitroimidazole, therefore, both induces the mutation leading to nitroimidazole resistance and selects these mutants. It is, therefore, not unexpected that nitroimidazole resistance in *H. pylori* is so rapidly observed after the use of a nitroimidazole containing treatment.

Finally, it was shown that the absence of functional *rdxA* encoded nitroreductase causes no survival disadvantage of the micro-organism in the absence of this antibiotic (24). Resistant mutants may, therefore, persist for decades, often in co-existence with susceptible organisms (37).

**Determination of nitroimidazole resistance.**

Susceptibility testing to a nitroimidazole is considered difficult by many investigators (36-40). In daily practice several tests are used (agar dilution, disk diffusion, and E-test) and these tests are poorly standardized (28). Agar dilution is accepted as the gold standard for susceptibility testing and several studies reported that it was highly reproducible (41-43). It is, however, laborious and time consuming. Today, the E-test is
used most frequently as a substitute (44) as it is easier to perform and has a good reproducibility (34,35,43). Finally, disk diffusion is a cheap and easy way to perform a susceptibility test, but no MIC value is produced. Although disk diffusion was used most frequently in the past it has been substituted by the E-test by most investigators now (44). Several studies comparing the different techniques have been published, but results are conflicting (34,39-41,43,45-53). This could be expected, as \textit{H. pylori} is a very slowly growing microorganism that needs specific growth conditions and thus methodological problems are easily encountered. For example, the choice of the medium (54-56), the age of the colonies (57), the incubation period (53,58), and the inoculum size (57) all may influence the outcome of the susceptibility test. Moreover, as mentioned above, the metabolism of a nitroimidazole in \textit{H. pylori} by several nitroreductases may result in large variation in MIC when isolates are tested several times, although strains usually remain classified as either resistant or susceptible (34). Finally, subpopulations of both resistant and susceptible bacteria are frequently found in one patient. Examining only one biopsy specimen may lead to sampling error and this may limit the utility of susceptibility testing (59). We performed a study in a cohort of 259 patients. In each patient biopsy specimens were taken for \textit{H. pylori} culture and subsequent susceptibility testing from both the antrum and corpus of the stomach. When only antral biopsy specimens were taken into account the prevalence of nitroimidazole resistance was 17%. When both antral and corpus biopsies were considered the prevalence increased to 24% (unpublished observations).

In our laboratory, without any special facilities, culture is performed with high accuracy both in the pre and post treatment situation (60,61). Susceptibility testing to nitroimidazoles is performed with the E-test on a medium containing Columbia agar (40 g/l) and sheepblood (5%), and no antibiotics. Plates are incubated at 36°C under microaerophilic conditions (5% oxygen, 85% nitrogen and 10% carbon dioxide) using a system of automatic jar evacuation and are examined after two and three days. Using this technique, E-test results are reproducible, comparable to agar dilution and disk-diffusion (34), and most important, significantly related to treatment efficacy (62-64).

**Epidemiology of nitroimidazole resistance.**

The presence of nitroimidazole resistance in \textit{H. pylori} is usually associated with previous use of nitroimidazoles. In developing countries the prevalence of resistance is high, probably as a consequence of the frequent use of metronidazole for infections with
protozoa (28,38,65). Subjects originating from these countries are, therefore, commonly infected with resistant strains (66). Data from the western world suggest that the prevalence of resistance is higher in southern Europe and the USA as compared to that in the northern parts of Europe and the UK (28). This is in concurrence with resistance rates in other micro-organisms and the general use of antibiotics in these countries (28,67). Furthermore, the prevalence of resistance is higher in females then in males, probably due to the use of these drugs for gynecological infections (65,68,69).

Several studies from the Western world showed that during the last years the prevalence of nitroimidazole resistance has been rising and that nowadays 10-50% of the strains are resistant to these drugs (70-76). Whether this increase represents a true increase is still uncertain as most studies suffer from methodological problems. Most of these studies were performed retrospectively and selection bias is, therefore, easily introduced. For example, the number of endoscopies has increased and indications for H. pylori testing have changed. This has led to an increased number of patients with functional dyspepsia tested. It has been suggested that the prevalence of nitroimidazole resistance is higher in these patients than in peptic ulcer patients (77). Thus this change in the population tested may have caused the increase in nitroimidazole resistance prevalence and it does not necessarily reflect an increase in the population. On the other hand, our own study was corrected for this possible bias but nevertheless showed a significant increase in nitroimidazole resistance in our region of the Netherlands. In our opinion, previous exposure to nitroimidazoles must have been the most important factor determining the observed increase, although this could not be substantiated (76).

Whatever the cause, nitroimidazole resistance is more frequently encountered in clinical practice then it was only a decade ago. This high prevalence of resistance urges an adjustment in empiric therapy meaning that either the use of a nitroimidazole is avoided or is compensated for by the other drugs of the combination therapy.

With the increased use of antibiotics, combined resistance of H. pylori to macrolides and nitroimidazoles and probably also to amoxicilline (78) and tetracycline (79), will become a major challenge for clinicians. Routine culture and subsequent susceptibility testing will then become warranted (28).

Clinical significance of nitroimidazole resistance.

Numerous studies have shown that nitroimidazole resistance reduces the efficacy of a nitroimidazole-containing treatment regimen, and these studies have been recently
summarized in a meta-analysis (80). It was shown that the extent to which nitroimidazole resistance reduces the efficacy of these regimens depends on the other components of the regimen and the duration of the treatment. The impact of nitroimidazole resistance on clarithromycin containing regimens is less then on amoxicillin containing regimens. The same is true for tetracycline as compared to amoxicillin. The efficacy of quadruple therapy (protonpump inhibitor and a bismuth compound) is less influenced by nitroimidazole resistance then the efficacy of standard bismuth based triple therapy. In the same way the addition of a protonpump inhibitor increased the efficacy of the combination of clarithromycin and metronidazole, especially in resistant strains (81). Possibly, but data are limited, the efficacy of a regimen containing ranitidine bismuth citrate, clarithromycin and metronidazole is also largely independent of nitroimidazole susceptibility (64,80). With respect to duration, the longer the treatment regimen the less the influence of nitroimidazole resistance.

Whether or not a nitroimidazole still has an effect on nitroimidazole resistant strains in a nitroimidazole-containing regimen is unsettled. As stated above, in vitro data show that a nitroimidazole is still reduced to active compounds in resistant strains and this may be of clinical significance (36). Studies comparing the efficacy of a nitroimidazole-containing regimen to that of the same regimen without the nitroimidazole in resistant strains, however, are sparse. One study using ranitidine bismuth citrate and clarithromycin suggested no additional effect of the nitroimidazole in resistant strains but the dual therapy was already effective on its own (82). When data from different clinical trials are compared a higher efficacy of bismuth based triple therapy as compared to the same therapy but without the nitroimidazole in nitroimidazole resistant strains is suggested (83). One study suggested that increasing the dose of a nitroimidazole in resistant strains increases efficacy (84). Controlled trials, however, are needed to resolve this issue.

As nitroimidazole resistance decreases efficacy, a nitroimidazole should be avoided if resistance is (likely to be) present. When this is not possible the regimen should include highly effective other components, and probably the combination of both an acid suppressant and a bismuth compound (quadruple therapy or ranitidine bismuth citrate). Tetracycline and clarithromycin are preferable to amoxicillin as the second antibiotic. It is suggested that clarithromycin should also be avoided, as resistance to this drug is easily introduced after treatment failure, resulting in a combined resistance (85).
The rapid emergence of resistance after treatment failure, possibly related to the mutagenic properties of nitroimidazoles, has been confirmed by several clinical studies (62,64,68,83,85-88). Although it has been suggested that certain compounds, for example bismuth compounds (89,90), may prevent the development of resistance, it is more likely that this is not related to specific properties of these compounds but related to the overall ability of the regimen to kill resistant mutants (80,91). Highly effective regimens are, therefore, the most important factor to prevent the emergence of resistance. This is in concurrence with observations on drug therapy in other infectious diseases (92,93).

Finally, Several authors have pointed out that other heterocyclic drugs, such as furazolidone and nitazoxanide, could be used as substitutes for nitroimidazoles (94-98). These drugs have a similar mechanism of action as nitroimidazoles. Their affinity for electrons, however, is so high that they are always reduced to active metabolites in *H. pylori* and resistance to these drugs has not been detected so far. Although some small clinical trials with these drugs show that they are effective (94-98) side effects are frequently encountered (98). The use of these drugs in clinical practice should, therefore, probably be reserved for multiple resistant strains in which other therapies have failed.

**Summary and general recommendations.**

Although a nitroimidazole is a very effective drug for the treatment of *H. pylori* infection, its usefulness is hampered by the rapid induction of resistance that is mostly related to null mutations in the *rdxA* gene encoding for an oxygen-insensitive NADPH nitroreductase. In these resistant strains nitroimidazoles are insufficiently activated to toxic metabolites that cause DNA damage. These resistant mutants may persist for decades.

Nitroimidazoles are being frequently used, not only for *H. pylori* eradication but also for protozoic and anaerobic infections. Nitroimidazole resistance is, therefore, frequently encountered and the prevalence is rising. Susceptibility testing to these drugs is not difficult, but may sometimes give inconsistent results. This is related to the metabolism of a nitroimidazole in *H. pylori* by several enzymes and the slow growth of the microorganism under specific growth conditions. Furthermore, co-infections with resistant and susceptible bacteria are frequently encountered and may result in sampling error.
Nitroimidazole resistance of *H. pylori* is associated with treatment failure although eradication of a resistant strain is still possible using a nitroimidazole containing regimen. The other components of the regimen are then the most important factors determining the efficacy of the regimen. Whether the addition of a nitroimidazole to a treatment regimen for the eradication of a resistant strain is still effective remains unresolved. If possible, a nitroimidazole containing regimen should be avoided when resistance is present. If this is not possible a regimen with highly effective other components should be used. Furazolidone and nitazoxanide are drugs with a similar mode of action as nitroimidazoles but resistance of *H. pylori* to these drugs has not been observed. They are, therefore, interesting substitutes for nitroimidazoles.

References.


15. Hazell SL. Will *Helicobacter pylori* be the next organism for which we will have exhausted our treatment options? Eur J Clin Microbiol Infect Dis 1999; 18:83-6.


