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HELICOBACTER PYLORI

Aspirin inhibits the growth of *Helicobacter pylori* and enhances its susceptibility to antimicrobial agents

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Background and aim: The role of *Helicobacter pylori* and aspirin in peptic ulcer formation and recurrence remains an important clinical topic. The interaction between aspirin and *H pylori* in vitro is also not clear. We investigated the effect of aspirin on the growth of *H pylori* and on the susceptibility of *H pylori* to antimicrobials.

Methods: Time killing studies of *H pylori* were performed with different concentrations of aspirin and salicylate. Growth of bacteria was assessed spectrophotometrically and by viable colony count. The effects of aspirin on the efficiency of colony formation and on metronidazole induced mutation to rifampicin resistance in *H pylori* were determined. Minimal inhibitory concentrations (MICs) of aspirin and metronidazole were tested by the standard agar dilution method. MICs of amoxicillin and clarithromycin were determined by the E test method.

Results: Aspirin and salicylate inhibited the growth of *H pylori* in a dose dependent manner and bactericidal activity was due to cell lysis. Aspirin 400 µg/ml caused a 2 logs decrease in colony forming units/ml at 48 hours, and suppressed the normal ability of metronidazole to induce new mutations to rifampicin. The IC₅₀ of aspirin was 512 µg/ml. Increased susceptibility of amoxicillin, clarithromycin, and metronidazole to *H pylori* was observed at 1 mM (180 µg/ml) aspirin.

Conclusions: Aspirin inhibited the growth of *H pylori*, suppressed the mutagenic effect of metronidazole, and enhanced the susceptibility of *H pylori* to antimicrobial agents. This mechanism is important in future drug development for effective clearing and overcoming resistance.

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Helicobacter *pylori* and aspirin are the two most important causative factors in the pathogenesis of peptic ulcer. However, the mechanism of interaction between *H pylori* and aspirin has not been sufficiently documented to date, either in vitro or in vivo. In one study, the number of endoscopic mucosal lesions in patients taking low dose aspirin was higher in those with *H pylori* infection than in those with no *H pylori* infection.¹ In another study, more antral ulcers occurred in *H pylori* negative than in *H pylori* positive subjects taking low dose aspirin.² It is generally recognised that *H pylori* infection does not worsen mucosal injury with aspirin or non-steroidal anti-inflammatory drug (NSAID) usage because *H pylori* infection is associated with increased mucosal levels of prostaglandins.^{2–4} Although reports from clinical studies are controversial,^{1, 2, 5} it seems that there is no meaningful difference in *H pylori* prevalence between aspirin users and non-users.^{6, 7} The background rate of infection in aspirin or NSAID users was high, ranging from 24% to 68%, but therapy with aspirin or NSAIDs does not increase susceptibility to *H pylori* infection.^{5–7}

Previous studies have reported that aspirin and other NSAIDs interfere with growth of both Gram negative and Gram positive bacteria in vitro, such as *Escherichia coli*,^{8, 9} *Candida albicans*,^{8, 9} *Staphylococcus saprophyticus*,^{8, 9} *Pseudomonas cepacia*,^{10, 11} and *Staphylococcus aureus*.¹² In some organisms, increased resistance or susceptibility to several antibiotics can be induced by growing in a subinhibitory concentration of aspirin.^{10–14} Graham and colleagues¹⁵ reported that *H pylori* was not susceptible to aspirin and other NSAIDs, such as indomethacin, ibuprofen, naproxen, or tolmetin in vivo. However, Caselli and colleagues⁵ observed an anti-*H pylori* effect of aspirin, diclofenac, and ketoprofen in vitro. Therefore, we undertook this study to evaluate the possible effect of aspirin on the growth of *H pylori* and further determined the effect of this drug on susceptibility of *H pylori* to antimicrobial agents.

MATERIAL AND METHODS

Bacterial strains

The experiments were conducted with 63 clinical isolates of *H pylori* from ethnic Chinese in Hong Kong, reference strains NCTC 11637, NCTC 12908, and isogenic metronidazole susceptible (MtzS) and resistance (MtzR) derivatives of *H pylori* 26695, containing rdxA::cam and frxA::kan single and double knockout mutations in the nitroreductase genes, and equivalent derivatives of SS1. The clinical isolates were obtained from patients undergoing upper endoscopy examination and were maintained frozen at –80°C in brain heart infusion (BHI) broth (BHI; Oxoid, Basingstoke, UK) with 20% glycerol. Endoscopic diagnosis included normal appearing mucosa in 15 patients, duodenal ulcer in 30 patients, gastric ulcer in three patients, and gastric cancer in five patients. An additional eight *H pylori* isolates from aspirin taking patients and two isolates from other NSAID taking patients were included in the study. Bacteria were re-plated on Columbia agar (Oxoid) supplemented with 7% horse blood under microaerobic conditions produced by a gas generating system (CampyGen; Oxoid) for three days. A non-pathogenic *E coli* strain (DH5a) was also included and cultured under similar conditions as described above in an aerobic ambient.

Chemicals

Aspirin (Sigma Chemical Co, St Louis, USA) was freshly dissolved in dimethylsulphoxide (DMSO; Sigma, USA) and

Abbreviations: MIC, minimal inhibitory concentration; NSAID, non-steroidal anti-inflammatory drug; MtzS, metronidazole susceptible; MtzR, metronidazole resistance; BHI, brain heart infusion; FBS, fetal bovine serum; DMSO, dimethylsulphoxide; CFU, colony forming unit; RifR, rifampicin resistance.

metronidazole (Fluka, Switzerland) was prepared in water. Sodium salicylate (Sigma Chemical Co) was dissolved in water.

Establishment of growth in broth medium

Time killing studies were performed using five different strains (NCTC 11637, NCTC 12908, 26695, one isolate from a duodenal ulcer patient, and one isolate from a gastric ulcer patient) in Brucella broth (Difco Laboratories, Detroit, Michigan, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, New York, USA). Culture media (20 ml) in a set of 10 cm petri dishes with or without test drug were inoculated with 10^8 colony forming units (CFU)/ml of *H pylori*. Dishes were placed in an anaerobic jar (Oxoid) and incubated at 37°C under microaerobic conditions, as described above, on a shaker at 90 rpm. A vehicle control of DMSO (less than 0.1%) was included in the studies.

Determination and adjustment of pH

The pH of Brucella broth supplemented with 10% FBS before inoculation with or without aspirin was measured using a digital pH meter (HI 9024; Hanna Instrument, Portugal). Growth of *H pylori* NCTC 11637 in the presence of aspirin (400 µg/ml) was compared with that in the medium with the same pH adjusted by 1 N HCl in the absence of the drug.

Growth measurement

Growth of bacteria in broth was assessed spectrophotometrically at 600 nm and by viable colony count. At 24 and 48 hours, samples were removed and serially diluted 10-fold, and colony counts were determined by plating 100 µl of each dilution on duplicate agar plates that allow optimal growth of *H pylori*. After 4–6 days of incubation under microaerobic conditions at 37°C, the plates were read.

Microscopy

Undiluted samples from the above broth medium with either aspirin or vehicle, after incubation for 24 and 48 hours, were spread onto glass slides and examined under Gram stain by light microscopy.

Determination of efficiency of plating

Strains of *H pylori* were inoculated on Columbia blood agar plates with various concentrations of aspirin (0, 100, 200 µg/ml) and metronidazole (0, 3, 8, 16, 25, 32 µg/ml), and were incubated under microaerobic conditions at 37°C for three days. Wild-type and isogenic MtzS and MtzR derivatives of strain 26695, and rdxA::cam and frxA::kan single and double knockout mutant derivatives of strains 26695 and SS1 were included in this study to test the possible effects on aspirin sensitivity of loss of nitroreductases encoded by rdxA and frxA, the activities responsible for susceptibility of *H pylori* to metronidazole.¹⁶ The efficiency of plating was the titre of CFU obtained from the plates with the test drug divided by the titre obtained from the control plates without drug.

Metronidazole induced mutation

New mutations to rifampicin resistance (RifR), which result from changes in the rpoB gene,¹⁷ were quantified as a measure of metronidazole induced mutation in *H pylori*. Bacterial cells (10^7 – 10^8) of isogenic MtzS and MtzR derivative of 26695 were spread onto Columbia agar plates containing various concentrations of metronidazole (0, 3, 8, 16, 25, 32 µg/ml) and aspirin (0, 100, 200 µg/ml). Following three days of incubation, bacterial cells were suspended in phosphate buffered saline and spread on Columbia blood agar with 5 µg/ml rifampicin, and titrated by spotting aliquots of serial 10-fold dilutions on rifampicin free medium. Each test was repeated three times and average values were reported.

MIC determination

Bacteria were prepared in BHI to yield a viable count of 3×10^8 CFU/ml (equivalent to 1 McFarland turbidity standard unit), and used as the inoculum for susceptibility testing. The minimal inhibitory concentrations (MICs) of aspirin were determined by the agar dilution method according to NCCLS document M11-A2 on 66 strains of *H pylori*,¹⁸ and the MICs of other antimicrobials (clarithromycin and amoxicillin) were determined by the E test method, according to the manufacturer's guidelines, on 24 strains (including three reference strains). MIC was recorded as the lowest concentration that inhibited visible growth of organisms and the results were determined after 72 hours of incubation at 37°C under microaerobic conditions.

For the agar dilution method, aspirin or metronidazole was diluted in distilled water and incorporated separately into Columbia blood agar. Plates contained twofold serial dilutions of drugs from 8 to 1024 µg/ml. Each bacterial suspension (1 µl) was inoculated (3×10^7 CFU/spot) on drug containing plates. Plates with no drugs were inoculated at the beginning and end of each run as controls.

To determine the possible effect of aspirin on the MICs of amoxicillin, metronidazole, and clarithromycin, susceptibility testing of these three widely used antimicrobials in the presence or absence of aspirin were performed using the E test and agar dilution methods. Aspirin at a final concentration of 1 mM (180 µg/ml) was added to Columbia blood agar to obtain aspirin containing plates. Each bacterial suspension (100 µl) was spread on plates with or without aspirin using a cotton swab. A single E test drug strip (AB Biodisk, Sweden) was applied to the surface of each dried plate. For each strain, MICs obtained in the presence or absence of aspirin were compared. *E coli* (DH5a) was included in this assay.

Statistical analysis

Time killing studies of *H pylori* were performed using five different strains. Representative data from these experiments are presented as mean (SEM). The Student's *t* test was used to compare data. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Effects of aspirin and salicylate on the growth of *H pylori*

Strains of *H pylori* were inoculated in broth medium containing various concentrations of aspirin (0, 200, 300, and 400 µg/ml) and salicylate (0, 0.56, 1.1, 1.6, and 2.2 mM). The effects on the growth of bacteria were determined spectrophotometrically as well as by a viable count method at 0, 24, and 48 hours. Similar results were obtained by performing experiments with five different strains, indicating that aspirin inhibited the growth of *H pylori* in a dose dependent manner. As determined by optical density at 600 nm, aspirin at a concentration of 100 µg/ml began to show an inhibitory effect on the growth of *H pylori* compared with the vehicle control. Concentration of aspirin of 400 µg/ml stopped increases in optical density, indicating cessation of cell growth. Further tests, based on determining the numbers of bacteria that could form colonies, showed that aspirin at a concentration of 400 µg/ml was lethal, resulting in a 1 log reduction in the numbers of CFU/ml relative to the numbers in the inoculum at 24 hours, and produced nearly a 2 log decrease at 48 hours (fig 1A). The experiment was repeated using equivalent concentration of salicylate and the results were essentially similar to those of aspirin (fig 1B). Morphological studies performed by Gram staining revealed complete lysis of cells incubated with 400 µg/ml of aspirin for 48 hours whereas no such lysis was detected in control cultures (fig 2). No significant coccoid formation was found after aspirin treatment.

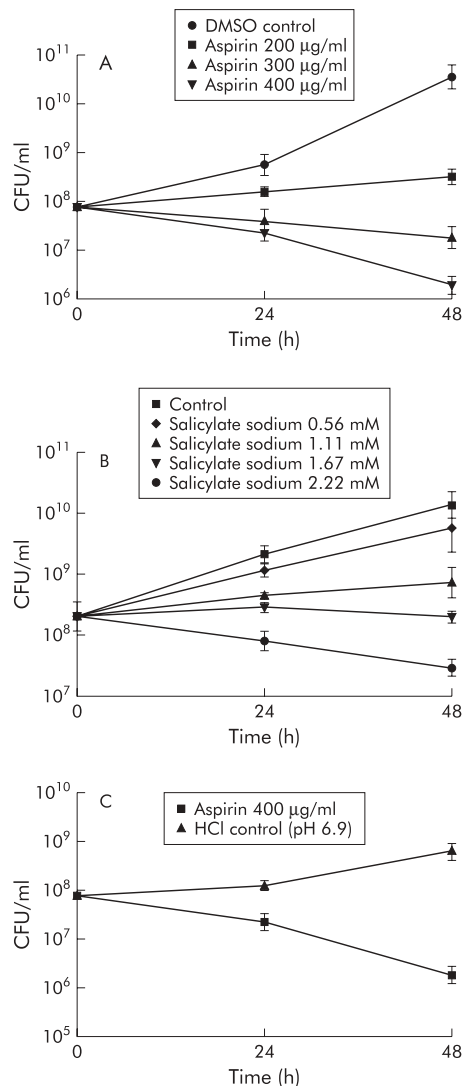


Figure 1 Inhibitory effect of aspirin and salicylate on the growth of *Helicobacter pylori*. (A) Aspirin treatment; (B) salicylate treatment. Data are representative of the average of five independent experiments using different strains. (C) Growth of *H. pylori* strain NCTC 11637 in the presence (pH dropped from 7.2 to 6.9 after aspirin) and absence (pH titrated to 6.9 by 1 N HCl) of aspirin. All results are expressed as the mean (SEM) of at least three independent experiments. DMSO, dimethylsulphoxide; CFU, colony forming unit.

Effects of decreased pH on the growth of *H. pylori*

The pH of Brucella broth supplemented with 10% FBS before inoculation was 7.26 whereas the pH of the medium after adding 400 µg/ml of aspirin was 6.90. However, growth of *H. pylori* NCTC 11637 in the presence of 400 µg/ml aspirin was 2 logs lower than that in the absence of the drug at the same pH after 48 hours of incubation (fig 1C). Thus aspirin does not exert its effect by changing the pH of the medium.

Synergism between metronidazole and aspirin

Adding aspirin to Columbia blood agar medium caused marked decreases in colony size compared with controls. No *rdxA* or *frxA* dependent differences in efficiency of plating or colony size as a function of aspirin dose were detected. The efficiency of plating decreased more dramatically with doses of 16, 25, or 32 µg/ml of metronidazole with aspirin 100 µg/ml, and especially with aspirin 200 µg/ml, compared with aspirin free plates. Thus aspirin and metronidazole were strongly synergistic with respect to inhibition of *H. pylori* colony formation.

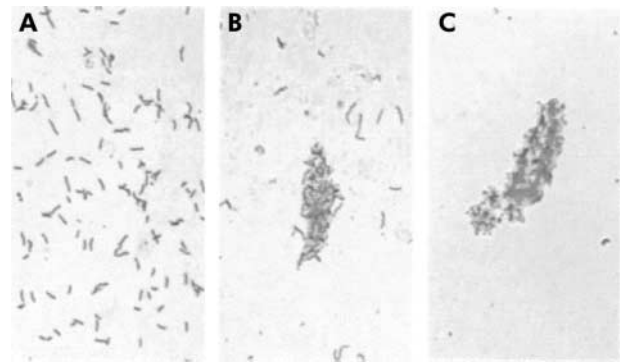


Figure 2 Gram staining of *Helicobacter pylori* strain NCTC 11637. (A) Vehicle control at 48 hours. (B) Treated with 200 µg/ml aspirin at 48 hours. (C) Treated with 400 µg/ml aspirin at 48 hours. Magnifications $\times 1000$.

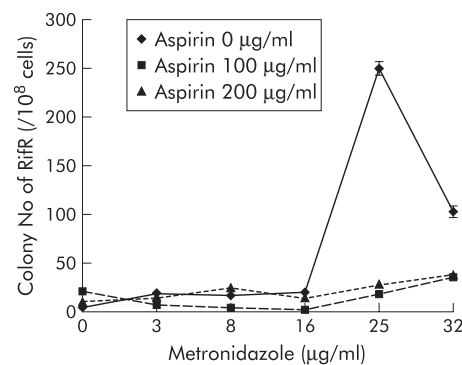


Figure 3 Inhibitory effect of aspirin on metronidazole induced mutation to rifampicin resistance (Rif^R) after three days of incubation in Columbia agar containing various concentrations of metronidazole and aspirin. All results are expressed as the mean (SEM) of three independent experiments.

Effect of aspirin on metronidazole induced mutation

Inclusion of inhibitory levels of aspirin (100 or 200 µg/ml) in blood agar medium suppressed the normal ability of metronidazole at 25 or 32 µg/ml to induce new mutations to Rif^R (fig 3).

MICs of aspirin

The MICs of aspirin for 66 *H. pylori* strains were tested. The MIC₅₀ of aspirin was 256 µg/ml, MIC₉₀ was 512 µg/ml, and the range of MIC values was 256–512 µg/ml. There were no differences regarding MICs against reference strains and clinical isolates derived from patients taking or not taking aspirin.

MICs of amoxicillin, metronidazole, and clarithromycin in the presence of aspirin

Susceptibility testing of three widely used antimicrobial agents (amoxicillin, metronidazole, and clarithromycin) in *H. pylori* eradication was performed on 24 strains in the presence of aspirin at a final concentration of 1 mM (180 µg/ml) and compared with that in the absence of aspirin. Aspirin decreased MICs in all strains (24/24, 100%) tested for amoxicillin, and 18 strains (18/24, 75%) tested for metronidazole and clarithromycin, indicating increased susceptibility of *H. pylori* to these drugs. More log₂ decreases in MICs were found for metronidazole compared with clarithromycin (3.75 (0.77) v 1.87 (0.46); $p=0.04$) (fig 4). It was difficult to determine the exact value of the log₂ decrease in MIC for amoxicillin as *H. pylori*, in the presence or absence of aspirin, was very susceptible to amoxicillin. For the six resistant strains (four resistant to metronidazole only, one resistant to both metronidazole and clarithromycin, and one resistant to clarithromycin only),

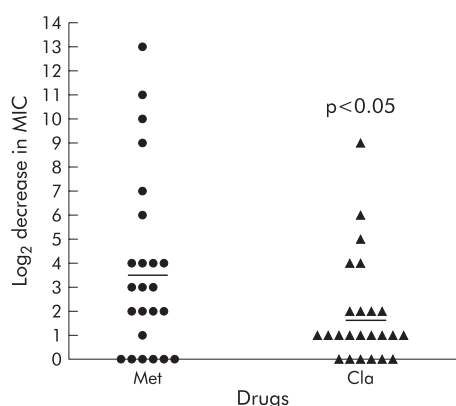


Figure 4 Log₂ decrease in the minimal inhibitory concentration (MIC) of metronidazole (Met) and clarithromycin (Cla) in the presence of aspirin in 24 *Helicobacter pylori* strains. Greater log₂ decreases in MICs were found for metronidazole compared with clarithromycin (3.75 [0.77] v. 1.87 [0.46]; $p=0.04$). Horizontal bars indicate mean log₂ decreases.

Table 1 Minimal inhibitory concentrations for the six representative strains in the presence or absence of aspirin

No of strains	Metronidazole		Clarithromycin	
	No aspirin	Aspirin	No aspirin	Aspirin
1	128	0.25	0.016	0.016
2	256	0.25	128	2
3	64	0.5	0.032	0.016
4	256	0.032	0.064	0.016
5	32	0.016	0.25	0.125
6	4	0.25	16	0.032

MICs decreased significantly in the presence of aspirin (table 1). Aspirin converted five strains from resistant to susceptible. In contrast, the MICs of these drugs increased in the presence of aspirin when *E coli* was used as the testing strain under the same conditions.

DISCUSSION

Although there is great interest in the role of *H pylori* infection and aspirin usage in peptic ulcer diseases, information on the mechanism of interaction between the two factors is lacking. *H pylori* may provide some protection against aspirin related ulcers due to its ability to stimulate mucosal synthesis of prostaglandins,²⁻⁴ or aspirin may influence the pathogenic effects of *H pylori* as a result of possible effects on this microorganism. In the present study, we found that aspirin and salicylate significantly inhibited the growth of *H pylori* in vitro, and reduced the efficiency of colony formation and colony size. Growth inhibition was found in all strains tested, including all clinical isolates, two reference strains (NCTC 11637, NCTC 12908), *H pylori* 26695 and SS1, and their isogenic MtzR derivatives (rdxA::cam and frxA::kan single and double knockout mutants). This indicates that the inhibitory effect of aspirin is universal among *H pylori*. No rdxA or frxA dependent differences in efficiency of colony formation or colony size in relation to aspirin dose were found. The fact that aspirin inhibited the growth of *H pylori* was in accordance with previous studies, which showed that aspirin could inhibit the growth of other bacterial species.⁸⁻¹²

Some have postulated that the coccoid form is a dormant stage of *H pylori* used for survival in hostile environments¹⁹⁻²¹ whereas others have argued that coccoid forms are simply

dead or dying.²² In any case, no coccoid formation was induced by the stress of aspirin treatment, not associated with the lethality of higher doses. There was a tendency for *H pylori* to form clusters when treated with aspirin. The reason for this is uncertain but may be related to changes in membrane properties (see below).

H pylori can survive in vitro in a wide pH range of 4.0–8.0.²³⁻²⁷ Aspirin (pK_a 3.5) is a weak acid,²⁸ leading to a pH drop from 7.26 to 6.90 when added to the medium at 400 µg/ml. Growth of *H pylori* was inhibited significantly in the presence of aspirin compared with controls at the same pH. This showed that the inhibitory effect of aspirin on the growth of *H pylori* was not solely due to its ability to act as a weak acid.

A plasma level of 20–100 µg/ml of aspirin is recommended for analgesia and 150–300 µg/ml for an anti-inflammatory effect.²⁸ Relatively high concentrations of aspirin are used for pain and fever, and in rheumatic fever, gout, and rheumatoid arthritis.²⁹ In our study, the inhibitory effect of aspirin on *H pylori* was observed at 100 µg/ml and the increasing susceptibility to amoxicillin, clarithromycin, and metronidazole was induced by aspirin at 1 mM (180 µg/ml). Therefore, the effective concentrations of aspirin on *H pylori* in vitro are comparable with normal plasma levels. These concentration of aspirin may be reached only transiently because it is rapidly metabolised to salicylate. We repeated the experiment using salicylate at a concentration of 0.56, 1.1, 1.6, and 2.2 mM (equivalent to aspirin 100, 200, 300, and 400 µg/ml, respectively) and the results were essentially similar (fig 1B). A plasma salicylate level of 1.4–1.8 mM is considered therapeutic and plasma levels greater than 2.2 mM are potentially toxic in chronic salicylate dosing. Thus the effect of aspirin and salicylate demonstrated in our study is achievable in vivo.

Although the mechanism by which aspirin inhibits the growth of *H pylori* and increases susceptibility to amoxicillin, clarithromycin, and metronidazole was not investigated in this study, the efficiency of plating decreased with high doses of metronidazole in the presence of aspirin. This apparent synergism between aspirin and metronidazole reflects in part the reduction in colony size to the point of invisibility but might be distinct from outright killing caused by bactericidal concentrations of metronidazole itself.¹⁶

Metronidazole at partially inhibitory concentrations causes mutation in growing *H pylori* due to conversion of this prodrug to hydroxylamine and related compounds that are both bactericidal and mutagenic. It is remarkable that aspirin treatment suppressed this normal mutagenic effect of metronidazole on *H pylori* even though it exacerbated its lethality. This implies that these two drugs act in different manners, and suggests models in which incipient mutant bacteria which have been weakened by metronidazole exposure may be killed preferentially by aspirin.

We then quantified new mutations to Rif^R in the presence or absence of aspirin, as a measure of metronidazole induced mutation in *H pylori*. The results showed clearly that inclusion of inhibitory levels of aspirin suppressed, rather than enhanced, the normal ability of metronidazole at 25 µg/ml or 32 µg/ml to induce new mutations to Rif^R. These data suggest that the inhibitory effect of aspirin on *H pylori* involves a mechanism distinct from DNA base modification and probably strand breakage also, the types of damage that underlie the mutagenic and bactericidal effects of metronidazole on this gastric pathogen.³⁰

There has been extensive study of the interaction between aspirin and various antibiotics in organisms such as *E coli*. Aspirin or salicylate increase the resistance of *E coli* to several negatively charged or neutral antibiotics, including ampicillin, tetracycline, chloramphenicol, nalidixic acid, and cephalosporins.³¹⁻³² One mechanism of this resistance entails aspirin induced downregulation of expression of the outer membrane porin OmpF,³³⁻³⁵ which serves as a channel for the entry of these antibiotics into the periplasmic space.³⁶

Salicylate decreased the amount of OmpF by two means, one dependent on and one independent of the much studied multiple antibiotic resistant gene in *E. coli*.^{34 35 37} In clinical isolates this may result from stepwise mutations that may revert when antibiotic selection pressure is removed. In addition, aspirin is a weak acid and could increase the membrane potential of species such as *E. coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*, and thereby enhance the susceptibility to drugs such as novobiocin and aminoglycoside antibiotics.^{13 14} Salicylates can also increase the uptake of divalent ions such as cadmium in *E. coli*, potentiating Cd²⁺ toxicity, independent of an increase in membrane potential.^{38 39} In this study, we observed increased susceptibility to amoxicillin, clarithromycin, and metronidazole of *H. pylori* in the presence of aspirin. Whether the above mechanisms are true for *H. pylori* remains to be determined.

Three results indicate that the increased susceptibility to amoxicillin, clarithromycin, and metronidazole caused by aspirin was not solely due to changes in pH in aspirin containing medium. Firstly, the pH of the Columbia agar medium decreased from 7.20 to 7.08 with 1 mM aspirin, and thus this pH change did not interfere significantly with the growth of *H. pylori*.⁴⁰ Secondly, the bactericidal activity of amoxicillin and clarithromycin require high growth rate and high expression of bacterial targets at neutral pH, and increased resistance to these antibiotics will be observed for the poor growth or non-dividing state of bacteria at acidic pH.^{24 41 42} Thirdly, metronidazole, a DNA targeted antibiotic, does not depend on bacterial cell division for its activity. Therefore, the activity of metronidazole is not pH dependent and remains stable.^{24 41 43} This was in accordance with our finding that the log₂ decrease in MIC of metronidazole was more frequently observed than that of amoxicillin and clarithromycin in the presence of aspirin.

In conclusion, our results provide evidence that aspirin inhibits the growth of *H. pylori* and increases the susceptibility to antimicrobial agents in vitro. How well this in vitro activity translates to in vivo effectiveness either in clearing the infection or in suppressing metronidazole induced mutation (that may be important in virulence or drug resistance) merits further analysis in clinical and experimental animal infection studies. This mechanism is therefore important in future drug development for effective clearing and overcoming resistance.

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