In vitro transfer of clarithromycin and amoxicillin across the epithelial barrier: effect of Helicobacter pylori

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The mode of absorption of amoxicillin and clarithromycin, two antibiotics used in the treatment of Helicobacter pylori infection, has not been completely elucidated. The aims of this study were to investigate the passage of these antibiotics across normal and infected epithelium and to measure their accumulation in HT29-19A or Caco2 epithelial cell monolayers. In non-infected cultures, basal-to-apical fluxes were significantly higher than apical-to-basal fluxes for both antibiotics, but this difference disappeared in monolayers infected with H. pylori. In 24 h studies, clarithromycin, but not amoxicillin, showed rapid intracellular accumulation. No difference was found between the transepithelial passage of amoxicillin across the HT29-19A and Caco2 monolayers.

Keywords: Helicobacter pylori, amoxicillin, clarithromycin, Ussing chamber, absorption

Introduction

Amoxicillin and clarithromycin are two antibiotics widely used as antibacterials that have been used to treat Helicobacter pylori infection. Resistance in H. pylori to amoxicillin has rarely been reported, whereas resistance to clarithromycin is increasing steadily.1 Treatments with each one of these drugs when used as a single antibiotic therapy led to eradication of H. pylori in only 10–40% of infected patients, and when associated with a proton pump inhibitor, in 20–80% of patients.2,3 The relatively low efficacy of amoxicillin and clarithromycin in vivo may be due to insufficient local drug concentrations at the locus of infection after oral administration.4,5

H. pylori is located at the surface of the gastric epithelium, in the mucus layer, and thus is not easily accessible to drugs. The question of whether bacterial eradication is achieved by the topical or systemic effect of amoxicillin in the gastric mucosa remains the subject of discussion. For a long time, the main mode of action of amoxicillin has been considered to be topical since, in healthy volunteers, amoxicillin has been detected in the gastric mucosa after oral administration.5 However, after intravenous (iv) administration, amoxicillin was found in gastric juice7 and led to eradication of H. pylori,8,9 suggesting that eradication of the bacteria may be achieved by a systemic action of amoxicillin.

In contrast to amoxicillin, which is present mainly in the extracellular space, it has been reported that clarithromycin may accumulate in the cells, suggesting a mainly systemic effect.10 After iv injection, the plasma concentration of clarithromycin decreases immediately, and after that it increases slowly with time.11 Based on this observation, it has been hypothesized that the drug accumulates in the cells and is subsequently released gradually into the extracellular fluid.12 Although the absorption of amoxicillin and clarithromycin has been studied in vitro13,14 and in vivo,15 there is no direct evidence supporting this hypothesis. For this reason, we have undertaken studies to measure the transepithelial passage of both drugs.

Employing an epithelial barrier model, the absorption of amoxicillin and clarithromycin was investigated as a function of the mode (mucosal or serosal) of their administration, as
well as their intracellular absorption. The effects of the presence of *H. pylori* on the absorption of the antibiotics by HT29-19A epithelial cells grown as monolayers on microporous filters and presenting tight junctions were measured using radiotracers.

**Materials and methods**

*Culture of the intestinal cell lines HT29-19A and Caco2*

The intestinal cell line HT29-19A is a clone derived from the human colonic adenocarcinoma cell line HT29, which forms tight monolayers of differentiated cells, widely used in functional studies of the epithelial barrier.16 These cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Life Technologies) containing 10% heat-inactivated fetal calf serum (Gibco BRL, Life Technologies), 4 mM glutamine and 50 μg/mL gentamicin, in a humidified atmosphere of 5% CO₂/95% air. In addition, the Caco2 intestinal cell line was used for amoxicillin study because these cells are known to express a specific transport carrier for β-lactam antibiotics (PepT1),17 which is not constitutively expressed in HT29-19A cells.18 These cells were cultured in DMEM supplemented with 20% heat-inactivated fetal calf serum, 4 mmol/L glutamine and 1% non-essential amino acids.

For experimental purposes, HT29-19A cells were seeded at a density of 0.6 × 10⁶ per cm² on polyethylene terephthalate filters with a pore diameter of 0.4 μm and a surface area of 0.9 cm² (Falcon; Becton Dickinson Labware, NJ, USA). Caco2 cells were seeded at a density of 0.6 × 10⁶ per cm² on Transwell polycarbonate insert filters with a pore diameter of 0.4 μm and a surface area of 1 cm² (Costar, Corning, NY, USA).

Upon reaching confluence, both HT29-19A and Caco2 cells progressively formed confluent monolayers with apical and basolateral domains separated by tight junctions. They were studied after 21 days of culture, when the electrical resistance, checked on the filter-grown monolayers mounted in an Ussing chamber, reached stable values of ~150 Ω/cm².

**Bacterial strain**

An *H. pylori* strain isolated from a patient with duodenal ulcer and subcultured minimally (less than five times) was used in this work. Bacteria were grown on Vitox chocolate agar (Oxoid, Dardilly, France) under micro-aerobic conditions (CampyGen in jar) for 24–36 h at 37°C. A bacterial suspension, containing from 6 × 10⁷ to 9 × 10⁷ cfu/mL, was prepared and used to infect the epithelial cells on the apical side. After 24 h incubation, the cell monolayers were washed with phosphate-buffered saline in order to remove planktonic (not attached) bacteria, and the filter-grown monolayers (with attached bacteria) were placed between two half chambers of an Ussing chamber to study the fluxes of the antibiotics.

**Antibiotics**

Radiolabelled [¹⁴C]clarithromycin (specific activity: 56.6 mCi/mM; radiochemical purity: ≥99%, labelled with ¹⁴C in 6-O-methyl position) was provided by Abbott Laboratories (Abbott Park, IL, USA) and [³H]amoxicillin (specific activity: 43.7 mCi/mM; radiochemical purity: 96.4%, labelled with ³H in position 3 of the phenol moiety) was provided by SmithKline Beecham (King of Prussia, PA, USA).

**Short-term absorption of the antibiotics in Ussing chamber (Figure 1a)**

The Ussing chamber system has already been used to study antibiotic transfer across the epithelium in vitro.13,19 We used this system to study short-term absorption of the antibiotics across the HT29-19A epithelial monolayer. The study in the Ussing chamber lasted 1 h 40 min, a period of time during which the viability of the tissue is preserved, allowing the study of the early absorption of the antibiotics. Each side of the exposed monolayer (surface area 0.150 cm²) was bathed with 1.5 mL of Ringer solution, and oxygenated at 37°C for the whole duration of the experiment. The electrical parameters, potential difference (PD) and electrical resistance (R), attesting the integrity of the epithelium, were checked for 2 h at 30 min intervals. Administration of [³H]amoxicillin and [¹⁴C]clarithromycin started after a 30 min period of equilibration of the system. Both antibiotics were introduced together into the apical (mucosal) or basal (serosal) compartment of the Ussing chamber at a final concentration of 10 mg/L each. Transport of the antibiotics was evaluated by sampling the opposite compartment at 30 min intervals. At each time point, a 500 μL sample was obtained from a receiver compartment and was replaced with an equivalent volume of Ringer solution.

The concentration of radiolabelled amoxicillin and clarithromycin was measured on 500 μL samples by β scintillation counting. Unidirectional fluxes of amoxicillin (Jₐₐₘox) and clarithromycin (Jₐₐₗₗₐₗₐₗₐₗₐₗ), apical-to-basal or basal-to-apical, were calculated using the following equation:

\[
J_{ₐₐₘox} (or Jₐₐₗₐₗₐₗₐₗₐₗₐₗ) = \frac{ΔQ}{dr·1/A}
\]

where  ΔQ represents the amount of amoxicillin or clarithromycin accumulated in the opposite compartment during the time interval dr, and A the exposed area of tissue. The fluxes of both antibiotics are expressed in ng/h/cm².

The same experiment was carried out with non-infected (n = 20) and *H. pylori*-infected (n = 10) cell monolayers.
Absorption of clarithromycin and amoxicillin

Long-term absorption of the antibiotics across filter-grown intestinal monolayers (Figure 1b)

This part of the study was carried out in order to evaluate the long-term (up to 24 h) absorption of antibiotics across the epithelium, and particularly their intracellular accumulation. Since the cell viability in Ussing chambers cannot be maintained for more than 2–3 h, these experiments were carried out using the cell monolayers grown on Falcon inserts. The inserts bearing the cells were bathed in the cell culture medium and kept at 37°C under an air 95%/5% CO₂ atmosphere, which maintained the viability of the tissue for the whole duration of the experiment. Antibiotic, [14C]clarithromycin or [3H]amoxicillin, was added separately, at final concentrations of 10 mg/L, to the basal (serosal) or apical (mucosal) compartment of the insert (surface 0.9 cm²) bearing the cell monolayers grown on the microporous filters. The apical and basal compartments were sampled at 10, 30, 60 and 360 min and at 10 and 24 h, starting from introduction of the antibiotic. For each time point, six separate filter-grown monolayers were studied. For each time point, the filter-grown cells (one filter bearing ≈10⁶ cells) were cut out of the inserts, washed four times with Ringer solution in order to remove extracellular antibiotic, and placed into 1 M nitric acid in order to obtain the cell lysis and liberation of intracellular drug. After a 24 h incubation, the lysed cells were centrifuged for 3 min at 3000 rpm, and 500 µL of supernatant was used to measure the radioactivity.

Additionally, the same 24 h study was carried out using Caco2 cells with only amoxicillin. This cell line has been described as bearing the specific oligopeptide transporter PepT1, which can be used by this antibiotic to cross the epithelium.

HPLC chromatographic analysis of [14C]clarithromycin or its metabolites during transport across the HT29-19A cell monolayers

While amoxicillin is excreted mainly in an intact form, clarithromycin is known to undergo substantial metabolism. Therefore, this part of the study was carried out in order to determine whether clarithromycin is metabolized during its transepithelial passage across the HT29-19A intestinal cell monolayer. HPLC chromatographic methods have been used widely for the determination of macrolide antibiotics in different biological samples. We used a gel exclusion HPLC method with in-line detection of radioactivity to analyse clarithromycin and its metabolites. To this end, [14C]clarithromycin was added at a concentration of 10 mg/L to the basal or apical compartment of filter-grown HT29-19A monolayers. After 8 h incubation, samples from the media in the apical and basal compartments were collected and assayed. The 500 µL samples were concentrated using the Speed-vac system by evaporating the excess of water during centrifugation at low temperature in the Speed Vac Plus apparatus (Savant Instruments, Farmingdale, NY, USA). The resulting volume of 200 µL was injected into a steric exclusion HPLC column (Superdex Peptide PE 7.5/300). The column was eluted at 30°C for 45 min with 0.1% trifluoro-
acetic acid in 30% acetonitrile at a flow of 0.3 mL/min with detection of eluting compounds using a radiochemical detector (Berthold HPLC radioactivity monitor LB 506 6-1).

**Analysis of antimicrobial activity of clarithromycin after its passage across the HT29-19A intestinal cell monolayers**

This experiment was carried out in order to verify whether clarithromycin antimicrobial activity is preserved after passage across the epithelial monolayer. Antimicrobial activity in the culture media containing [14C]clarithromycin was measured before and after its apical–basal passage across the HT29-19A cell monolayer according to a standard procedure. Briefly, *Micrococcus luteus*, inoculated on Mueller–Hinton agar (Bio-Rad, Marne la Coquette, France), was used as the test organism. The culture media were tested directly and after dilution and the results were compared with a standard curve made with concentrations of clarithromycin (0.015–2 mg/L). Readings were made after 24 h incubation at 30°C in air.

**Statistical analysis**

Data were analysed using the SAS package (SAS Institute, Cary, NC, USA). The results are expressed as means ± S.D. Comparison of means was carried out by analysis of variance and by non-parametric tests (Wilcoxon two-sample test). Differences were considered significant for *P* < 0.05.

**Results**

**Effect of amoxicillin and clarithromycin on the epithelial barrier integrity**

Amoxicillin and clarithromycin applied together on the apical or basal side of the epithelial monolayer at a final concentration of 10 mg/L each did not affect the integrity of the epithelial barrier as assessed by its electrical resistance, which did not differ between the amoxicillin + clarithromycin exposed cells (*n* = 8) and control cells (*n* = 8), whose resistances were 150 ± 20 and 135 ± 15 Ω/cm², respectively.

**Short-term absorption of amoxicillin and clarithromycin across the control and *H. pylori*-infected HT29-19A cell monolayers**

**Time course of antibiotic absorption across control monolayers.** Clarithromycin and amoxicillin fluxes (means ± s.d.) as a function of time are shown in Figure 2. The apical-to-basal fluxes of amoxicillin rapidly achieved a steady state, and were 50 ± 40, 57 ± 35, 63 ± 27 and 86 ± 49 ng/h·cm² at 10, 40, 70 and 100 min, respectively. The clarithromycin fluxes increased throughout the duration of the experiment and were 14 ± 24, 33 ± 50, 56 ± 65 and 127 ± 110 ng/h·cm² at 10, 40, 70 and 100 min, respectively.

Similar profiles were observed for the basal-to-apical fluxes, with a steady state rapidly achieved by amoxicillin, and a progressive increase in the flux of clarithromycin. Although clarithromycin seemed to display a different profile from amoxicillin, there was no significant difference between the amoxicillin and clarithromycin fluxes at any time point.

**Dependence of end-point antibiotic fluxes on the side of drug application and effects of the presence or absence of *H. pylori*.** This analysis was carried out by comparing the mean fluxes at 70 and 100 min. Values of 72 ± 35 and 115 ± 84 ng/h·cm² were measured for apical-to-basal and basal-to-apical amoxicillin fluxes, respectively. They were not significantly different from those determined for clarithromycin (88 ± 83 and 160 ± 107 ng/h·cm², respectively). For both ant-
Absorption of clarithromycin and amoxicillin

biotics, the basal-to-apical fluxes were higher than the apical-to-basal ones (P < 0.04 for amoxicillin and P < 0.02 for clarithromycin) (Figure 3).

The presence of \( H. \text{pylori} \) did not modify the apical-to-basal fluxes of both antibiotics. The values measured for \( H. \text{pylori} \)-infected cells were 93 ± 76 ng/h/cm² for amoxicillin, and 72 ± 46 ng/h/cm² for clarithromycin. The basal-to-apical fluxes (57 ± 12 ng/h/cm² for amoxicillin, and 104 ± 105 ng/h/cm² for clarithromycin) were similar to the apical-to-basal values, at variance with control monolayers, which showed differences between the fluxes in both directions, suggesting that \( H. \text{pylori} \) decreased the basal-to-apical fluxes of both antibiotics (Figure 3).

**Figure 3.** End-point (70 and 100 min) apical-to-basal and basal-to-apical fluxes of amoxicillin and clarithromycin across normal and \( H. \text{pylori} \)-infected HT29-19A monolayers measured in an Ussing chamber (mean ± S.D.). *Significantly different from apical-to-basal fluxes, P < 0.02.

**Figure 4.** Long-term basal-to-apical (serosal-to-mucosal) transport of clarithromycin and amoxicillin across HT29-19A cell monolayers in cell culture inserts (mean ± S.D.). For all time points starting from 5 h, the values for clarithromycin were significantly higher than those for amoxicillin (P < 0.02).

**Long-term basal-to-apical absorption of clarithromycin and amoxicillin across HT29-19A cell monolayers**

**Clarithromycin.** The basal-to-apical transport of clarithromycin across the epithelial layer as a function of time is shown in Figure 4. At each time point, the results are presented as means ± S.D. of six independent measurements. Up to 60 min, very little clarithromycin was found in the apical compartment; its concentration was 64 ± 5 ng/mL at 120 min and increased gradually to a value of 686 ± 29 ng/mL after 24 h. In contrast, 10 min after the basal application of clarithromycin, the drug was detected inside the cells at a concentration of 70 ± 10 ng/10⁶ cells. The intracellular concentration of clarithromycin increased rapidly to values of 141 ± 7, 237 ± 22 and 408 ± 35 ng/10⁶ cells after 30, 60 and 120 min, respectively, and reached a concentration of 571 ± 55 ng/10⁶ cells after 24 h. The data showed that basally applied clarithromycin was first captured by the epithelial cells and subsequently released to the apical compartment.

**Amoxicillin.** Figure 4 shows that after basal application of 10 mg/L amoxicillin, only a small quantity of antibiotic (13 ± 1 ng/mL) was found in the apical compartment after 60 min. The concentration of antibiotic in this compartment increased gradually, reaching 56 ± 4 ng/mL at 6 h, 88 ± 9 ng/mL at 10 h and 224 ± 7 ng/mL at 24 h. Virtually no amoxicillin (<7 ng/mL) was found inside the cells during 24 h, indicating that amoxicillin did not accumulate in the cells, and suggesting the presence of a paracellular transport pathway for the transepithelial passage of this antibiotic, while not excluding a minor transcellular pathway.

Altogether, the concentration of clarithromycin in the apical compartment was higher than that of amoxicillin (P < 0.02 for all time point values starting from 5 h).
Long-term absorption of amoxicillin across Caco2 cell monolayers

The time course of the basal-to-apical and apical-to-basal amoxicillin fluxes across the Caco2 cell monolayers was similar to that obtained with HT29-19A cell monolayers. No intracellular accumulation of antibiotic was observed, and the concentrations of amoxicillin at different time points in either compartment after its application on the opposite side were not significantly different from those observed with HT29-19A cell monolayers. In addition, the transport of amoxicillin across the Caco2 monolayers showed no significant differences between apical or basal drug application (Figure 5).

Analysis of \([^{14}\text{C}]\) clarithromycin metabolites

The HPLC chromatogram of clarithromycin after transepithelial passage across an HT29-19A monolayer was slightly different from that of the parent clarithromycin, with elution times of 26.62 and 28.13 min for the parent clarithromycin and its metabolite after passage across the monolayer, respectively (Figure 6). No evidence of other degradation products was found after transepithelial transport. This small difference in the elution times between the two molecules suggests that both compounds have a similar molecular weight. This result, together with the result of the microbiological assay (see below), strongly suggests that the compound found in the receiver compartment correspond to 14-hydroxy-clarithromycin, the principal biologically active metabolite of clarithromycin.

Antimicrobial activity of clarithromycin after its passage across HT29-19A cell monolayers

The antimicrobial activity of the medium in the apical compartment (where clarithromycin was added) was equivalent to 0.25 mg/L pure clarithromycin (reference) activity, whereas the antimicrobial activity of the medium in the basal compartment (where the antibiotic was recovered), was equivalent to 0.5 mg/L reference activity. These results indicate that there was no loss of antimicrobial activity during the transepithelial passage of clarithromycin.

Discussion

Amoxicillin and clarithromycin are antibiotics that are used widely in the treatment of \(H. pylori\) infection. In the present study, using an \textit{in vitro} epithelial barrier model, it has been demonstrated that these two antibiotics had different modes of transport across the epithelium. Clarithromycin was initially taken up by the epithelial cells, and then gradually released into the opposite compartment of the epithelium as its 14-hydroxy metabolite. In contrast, amoxicillin did not accumulate in the cells and crossed the epithelium either via a paracellular pathway, across the intercellular tight junctions or via a transcellular pathway. This study also showed that the basal-to-apical passage of amoxicillin and clarithromycin is larger than the apical-to-basal passage across the epithelium.

The presence of bacteria seemed to decrease the basal-to-apical fluxes of both antibiotics but had no significant influence on their apical-to-basal fluxes. There was no significant difference between short-term (100 min) transepithelial fluxes of both antibiotics, but clarithromycin presented much better long-term (24 h) transepithelial absorption than amoxicillin.

The fact that the cells concentrated clarithromycin but not amoxicillin was already known, but the mode of absorption of these two antibiotics across the epithelial barrier had not been established. Amoxicillin, like all \(\beta\)-lactam antibiotics, uses the apically located, energy-dependent dipeptide
carrier system to cross the intestinal epithelium along the transepithelial pathway. This system is present in human enterocytes and has been found in some intestinal cell lines, such as Caco2 and also HT29 cells,17,24 but is reported to be absent in the HT29-19A cells used in this study.18,25 For this reason, transport of amoxicillin across the latter cells was compared with that of Caco2 cells, known to express the transporter. The results with the HT29-19A monolayer suggested that an amoxicillin transporter may be absent in this cell line since no antibiotic was found inside the cells. On the other hand, the possibility cannot be excluded that the drug crosses the epithelium, in part at least, through the tight intercellular junctions, as the lower molecular weight of amoxicillin (mol. wt 365) relative to that of clarithromycin (mol. wt 747) may favour a paracellular diffusion of the former across the epithelium. Moreover, as a zwitterion, it is easier for amoxicillin, compared with negatively charged clarithromycin, to cross negatively charged tight junctions. Similar absorption profiles were observed with the Caco2 cells to those seen with HT29-19A cells, despite the well-documented presence of a specific PepT1 transporter in this cell line.17,26,27 No intracellular antibiotic was found and there was no significant difference between the apical-to-basal and basal-to-apical amoxicillin fluxes across the Caco2 monolayer. These observations suggested that either the specific PepT1 transporter was not active, or that its presence did not modify significantly the kinetics of transepithelial transport of amoxicillin.

It was assumed that amoxicillin passed through the epithelium in a non-metabolized form as it has been demonstrated in humans that >90% of amoxicillin is excreted intact.28 In contrast, clarithromycin is known to undergo a rapid biotransformation leading to the production of the microbiologically active metabolite 14-hydroxy-clarithromycin.11 We have shown that transepithelial passage of clarithromycin slightly modified its HPLC profile, suggesting the metabolism of the parent compound. In all probability, the metabolite corresponded to its hydroxy-metabolite because it was confirmed that it preserved the antimicrobial activity of the parent compound.

Clarithromycin is known to concentrate in cells such as macrophages, polymorphonuclear leucocytes and lymphocytes, and has antimicrobial activity both extra- and intracellularly.29 In contrast, amoxicillin has been shown to act mainly in the extracellular milieu. These two different behaviours may explain the better efficacy of clarithromycin in the eradication of H. pylori when each one of these drugs is used as the only antibiotic. For clarithromycin, the mode of transepithelial transport may serve to maintain a constant level of the drug on the apical side of the gastric mucosa owing to its slow release from the gastric cells where it had been concentrated. In contrast, for amoxicillin, diffusion to the apical side of the gastric epithelium would more closely follow serum concentrations. The improved eradication results obtained by administering a continuous infusion of amoxicillin would further support such an explanation. It has been hypothesized that eradication failure could also be due to the persistence of a few intracellular H. pylori.30 The intracellular penetration of clarithromycin, in contrast to amoxicillin, may allow it to kill the few intracellular bacteria present.

In conclusion, in this study, we have shown that amoxicillin and clarithromycin have different modes of transepithelial absorption. The higher passage rate of clarithromycin and its capacity to accumulate inside the epithelial cells may explain, in part, its better efficacy when used alone, in the eradication of H. pylori.

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References


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