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In-vitro transfer of nitazoxanide across the intestinal epithelial barrier

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Abstract

Nitazoxanide is a thiazolide compound that exhibits antimicrobial properties against helminths, protozoa, anaerobic bacteria and also *Helicobacter pylori*. The mucosal diffusion of this new drug has not been studied. The aim of this study was to examine the transport of radiolabelled nitazoxanide across the epithelial barrier according to the mode (mucosal or serosal) of drug administration. HT29-19A intestinal epithelial cells, grown as monolayers on microporous filters, were used as an epithelial model. In a short-term (100 min) transport study, the apical to basal and the basal to apical transport of nitazoxanide across the monolayers was studied in an Ussing chamber. In a long-term (24 h) study, the transport of the drug and its intracellular accumulation were studied in filter-grown epithelial monolayers kept in culture plates. In the short-term transport study, both the apical and basal fluxes achieved a steady state after 70 min and there was no significant difference between the apical to basal (3991 ± 1001 ng h^{-1} cm^{-2}) and the basal to apical (4246 ± 856 ng h^{-1} cm^{-2}) nitazoxanide fluxes. In the long-term transport study, after apical or basal drug application, a gradual increase in the drug concentration in the opposite compartment was noted, which reached similar values for apical and basal fluxes (2497 ± 125 and 2309 ± 81 ng mL^{-1}, respectively) after 24 h. Moreover, a rapid, although transitory, intracellular accumulation of nitazoxanide was observed at 10 min after apical (299 ± 25 ng/10^6 cells) and basal (124 ± 10 ng/10^6 cells) drug application, but decreased thereafter. There is an important transepithelial transport of nitazoxanide across the digestive epithelial monolayer with a rapid intracellular accumulation of the drug. No difference between the apical to basal and basal to apical fluxes of the drug was observed, suggesting that both topical and systemic modes of action of this antibiotic are successful.

Introduction

Nitazoxanide is a thiazolide compound that possesses antimicrobial properties against helminths, protozoa and anaerobic bacteria (Dubreuil et al 1996; McVay & Rolfe 2000; Gilles & Hoffman 2002). It also exhibits good antimicrobial activity against *Helicobacter pylori* in-vitro (Megraud et al 1998; Yamamoto et al 1999). Its efficacy in the eradication of *H. pylori* was demonstrated in a pilot study (Megraud et al 1998) and is now being confirmed in larger clinical trials. *H. pylori* is located on the gastric epithelium surface, in the mucus layer, and is thus not easily accessible to drugs. The question of whether bacterial eradication is achieved by the topical or systemic effect of antibiotics in the gastric mucosa is still under debate. For amoxycillin and clarithromycin, two antibiotics commonly used in the eradication treatment, the systemic effect seems to be the most important. Furthermore, the capacity of clarithromycin to accumulate in the epithelial cells makes it particularly useful in the eradication of *H. pylori* (T. Matysiak-Budnik et al, unpublished observations).

The pharmacokinetics and metabolism of nitazoxanide have been studied in humans (Broekhuysen et al 2000). However, no studies have been performed on the mode of absorption of this drug using an epithelial barrier model that allows the transepithelial transport of the drug to be followed. Indeed, it is essential to get an insight into the mucosal diffusion of such a new drug. Our aim was to study the absorption of nitazoxanide across the epithelial barrier according to the mode (mucosal or serosal) of drug administration, with special emphasis on its intracellular absorption. We used

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HT29-19A epithelial cells, grown as monolayers on microporous filters and presenting tight junctions, as a model of digestive epithelium. The kinetics of the transfer was performed with radiolabelled nitazoxanide, which allows a precise evaluation of drug concentration.

Materials and Methods

Culture of the intestinal cell line HT29-19A

The human differentiated colon carcinoma cell line HT29-19A, widely used in the functional studies of the epithelial barrier, was used as an epithelial model. The cells were cultured, passed and seeded as described previously (Matysiak-Budnik et al 2001). They were studied after reaching confluency and forming a monolayer, with apical and basolateral domains separated by tight junctions, presenting stable values of electrical resistance.

Nitazoxanide

Labelled ^{14}C-nitazoxanide (MW 307.3; specific activity 12.0 mCi mM^{-1}) was provided by Romark Laboratories.

Short-term transport of nitazoxanide in Ussing chamber

The study in the Ussing chamber lasted 100 min, a time period during which the viability of the tissue was preserved, and which allowed the early transport of nitazoxanide to be studied.

Each side of the exposed monolayer (surface area 0.150 cm^2) was bathed with Ringer solution, oxygenated and thermostatted at 37°C for the entire duration of the experiment. The electrical parameters, potential difference (PD) and electrical resistance (R), confirming the integrity of the epithelium, were recorded for 2 h at 30-min intervals. ^{14}C-Nitazoxanide was introduced into the apical (mucosal) or basal (serosal) compartment of the Ussing chamber (final volume 1500 μL) at a final concentration of 10 μg mL^{-1} (specific activity 1.443 kBq μg^{-1}). This concentration was chosen as it corresponds to the minimal inhibitory concentration values with respect to *H. pylori* strains tested in-vitro (Megraud et al 1998), and also to the plasma concentrations of tizoxanide (= desacetyl-nitazoxanide, the only measurable metabolite of nitazoxanide) found after injection of a single dose of 500 mg of nitazoxanide in humans (Stockis et al 1996). The concentration of nitazoxanide on the opposite side was evaluated by sampling the opposite compartment at 30-min intervals. At each time point, 800 μL of the receiver compartment was sampled and replaced by an equivalent volume of Ringer solution. The concentration of labelled nitazoxanide was measured by beta scintillation counting. Unidirectional fluxes of nitazoxanide (JNitazox), apical to basal or basal to apical, were calculated using the following equation: JNitazox = $\Delta Q/dt \times 1/A$, where ΔQ represents the amount of nitazoxanide accumulated in the opposite compartment during the time interval dt, and A is the exposed area of tissue. The fluxes are expressed as ng h^{-1} cm^{-2}. The values of electrical resistance of the monolayers were recorded and used in the analysis of a possible correlation between this resistance and nitazoxanide fluxes.

Long-term transport of nitazoxanide across filter-grown intestinal monolayers

This part of the study was performed in order to evaluate the long-term (up to 24 h) transport of nitazoxanide across the epithelium and particularly its intracellular accumulation. Since cell viability in Ussing chambers cannot be maintained for more than 2–3 h, this part of the study was carried out in culture plates using Falcon inserts. The inserts bearing the cells were bathed in the cell culture medium and kept at 37°C for the entire duration of the experiment, which maintained the viability of the tissue. ^{14}C-Nitazoxanide was added at a final concentration of 10 μg mL^{-1} to the basal (volume 1500 μL) or apical (volume 500 μL) compartment of the Falcon insert (surface 0.9 cm^2) bearing the cell monolayers grown on the microporous filters. The apical and basal compartments were collected at 10, 30, 60 and 360 min, and at 10 and 24 h, starting from the introduction of the drug. For each time point, six separate filter-grown monolayers were studied. At the same time points, the filter-grown cells (one filter bearing approx. 10^6 cells) were cut out of the inserts, washed with Ringer solution in order to remove the extracellular antibiotic, and placed into 1 M nitric acid for cell lysis and release of intracellular nitazoxanide. After a 24-h incubation, the lysed cells were centrifuged for 3 min at 3000 rev min^{-1}, and the supernatant obtained was used to measure the radioactivity. Radioactivity in all compartments (apical, basal and intracellular) was analysed by liquid scintillation photometry.

Statistical analysis

Data were analysed by using the SAS package (SAS Institute, Cary, NC, USA). The results are expressed as means ± s.d. Comparison of means was performed by analysis of variance and the differences were considered significant for $P < 0.05$.

Results

Effect of nitazoxanide on the epithelial barrier integrity measured in Ussing chamber

Nitazoxanide applied on the apical or basal side of the epithelial monolayer at the final concentration of 10 μg mL^{-1} did not affect the integrity of the epithelial barrier as attested by the electrical resistance, which did not differ between the nitazoxanide-exposed (2 h) and control cells (R = 109 ± 38 and 99 ± 34 Ω cm^{-2}, respectively).

Short-term transport of nitazoxanide across HT29-19A cell monolayers

The time-course of the nitazoxanide fluxes is presented in Figure 1. The apical to basal fluxes achieved a steady state

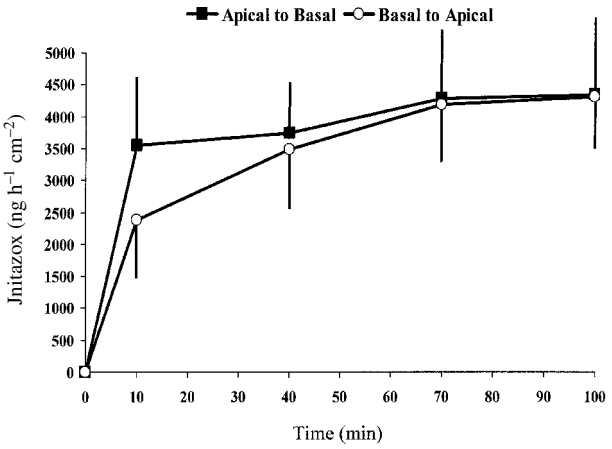

Figure 1 Time course of the short-term apical to basal (n = 11) and basal to apical (n = 10) nitazoxanide fluxes (Jnitazox) across HT29-19A cell monolayers measured in an Ussing chamber. There was no significant difference between the apical to basal and basal to apical fluxes. For both fluxes, a steady state was achieved after 70 min.

after 70 min, and were 3550±1100, 3736±961, 4286±2058 and 4350±2000 ng h^{-1} cm^{-2} after 10, 40, 70 and 100 min, respectively. Similarly, the basal to apical fluxes of the drug reached a steady state after 70 min and were 2379±1079, 3477±873, 4181±810 and 4311±1000 ng h^{-1} cm^{-2} after 10, 40, 70 and 100 min, respectively.

Steady-state fluxes (from 70 to 100 min) showed no significant difference between the overall apical to basal (3991±1001 ng h^{-1} cm^{-2}) and the basal to apical (4246±856 ng h^{-1} cm^{-2}) nitazoxanide fluxes across the epithelial monolayer.

Analysis of the correlation between the nitazoxanide fluxes and electrical resistance, measured in Ussing chamber

This analysis was performed by correlating the end-point (70 and 100 min) fluxes of nitazoxanide (basal to apical and apical to basal) across the monolayer with the electrical resistance of this monolayer. The mean R for apical to basal fluxes was 109.8±38 Ω cm^{-2} and that of basal to apical fluxes was 88.4±34 Ω cm^{-2}. An inverse correlation was found between the nitazoxanide fluxes (basal and apical) across the epithelial monolayer and the electrical resistance of this monolayer ($P < 0.05$, r = 0.4492), suggesting that the drug could cross the epithelium, at least in part, via the paracellular route.

Long-term apical to basal and basal to apical transport of nitazoxanide across HT29-19A cell monolayers

The transport of nitazoxanide, applied apically or basally, across the epithelial layer, as a function of time is presented in Figure 2. At each time point, the results are presented as mean±s.d. of six separate measurements (six separate filter-grown monolayers).

After apical application at a final concentration of 10 μg mL^{-1}, there was a gradual increase in the drug concentration in the basal compartment, which reached values of 355±138 ng mL^{-1} after 10 min, 463±176 ngmL^{-1} after 1 h, 2011±211 ng mL^{-1} after 6 h, 2329±189ng mL^{-1} after 10 h, and 2497±125 ng mL^{-1} after 24 h. Similarly, after basal administration of nitazoxanide, a gradual increase in the drug concentration in the apical compartment was observed (410±17 ng mL^{-1} after 10 min, 1202±67 ng mL^{-1} after 1 h, 1119±167 ng mL^{-1} after 6 h, 1245±287 ng mL^{-1} after 10 h, and 2309±81 ng mL^{-1} after 24 h) (Figure 2A). Although a higher drug concentration was observed in the apical than in the basal compartment at up to 2 h ($P <$ 0.01), and at 6 and 10 h this concentration was higher in the basal than in the apical compartment ($P < 0.01$), after 24 h a similar drug concentration, corresponding to about 20% of the initial dose, was observed in both compartments. Moreover, a rapid intracellular accumulation of nitazoxanide, which was found inside the cells at just 10 min after apical (299±25 ng/10^6 cells) and basal (124±10 ng/10^6 cells) drug application, was noted. This intracellular accumulation was very transitory and it

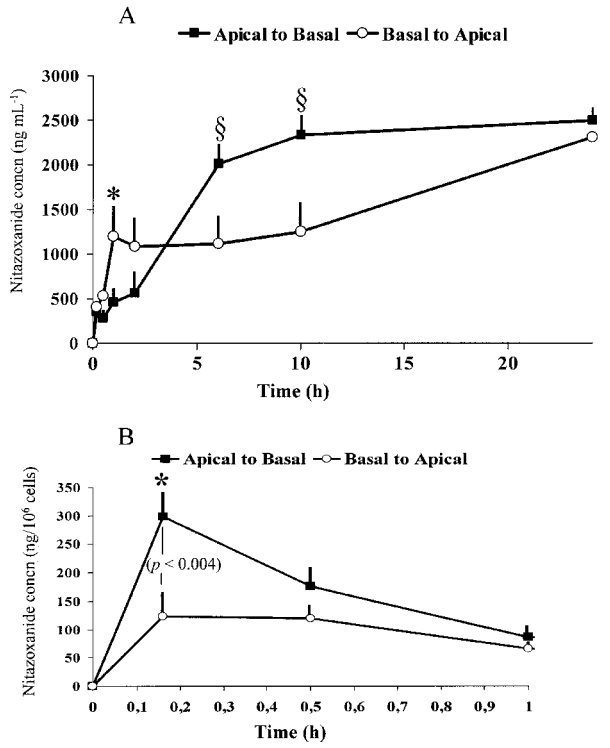

Figure 2 Time course of the long-term apical to basal (n = 6) and basal to apical (n = 6) nitazoxanide fluxes across the filter-grown HT29-19A cell monolayers. A. Apical to basal or basal to apical accumulation of nitazoxanide. A progressive increase in the drug concentration in the basal and apical compartments was observed. B. Early (1 h) intracellular accumulation of nitazoxanide after apical and basal drug application. This accumulation was significantly more important after apical than after basal drug application. A rapid (up to 1 h) decrease in the intratissular nitazoxanide concentration was observed. *Significantly greater than basal concentration ($P < 0.01$), §Significantly greater than apical concentration ($P < 0.01$).

decreased rapidly; a negligible quantity of nitazoxanide (30–40 ng/10^6 of cells) was found inside the cells after 2 h (Figure 2B).

Epithelial permeability of HT29-19A cell monolayer to nitazoxanide

The permeability of the monolayer to nitazoxanide is a property of the epithelial layer independent of the initial drug concentration. It can be calculated on the basis of the fluxes of nitazoxanide across the HT29-19A monolayer and its initial concentration in a donor compartment, according to the following formula (Heyman et al 1980): P = JNitazox (ms)/C, where P is the permeability (cm h^{-1}), JNitazox (ms) is the mucosal to serosal nitazoxanide flux ($\mu g\ h^{-1}\ cm^{-2}$), and C is the initial drug concentration in the donor compartment ($\mu g/cm^3$).

We have calculated the apical to basal permeability of the HT29-19A monolayer to nitazoxanide and compared it with the permeability of this cell layer to other molecules studied in our laboratory using the same system (T. Matysiak-Budnik et al, unpublished results). The results indicate that the permeability of HT29-19A monolayer to nitazoxanide is particularly high ($P = 0.4$ cm h^{-1}), much higher than the permeability of this monolayer to other antibiotics such as amoxycillin ($P = 7.2 \times 10^{-3}$ cm h^{-1}) and clarithromycin ($P = 8.8 \times 10^{-3}$ cm h^{-1}), or that of a reference permeability marker, mannitol ($P = 6.4 \times 10^{-5}$ cm h^{-1}).

Discussion

In order to study the transepithelial absorption of nitazoxanide, we used the HT29-19A intestinal epithelial cell line as an epithelial model. This is a well-established model of the digestive epithelium comprised of polarized cells developing tight junctions that are required for studying transepithelial transport. This model has been widely used in studies of absorption of different permeability markers (Heyman et al 1994; Matysiak-Budnik et al 1998, 2001; Madara & Stafford 1989) and drugs (Dantzig & Bergin 1988, 1990) across the epithelial barrier, thus allowing comparison of the results obtained. In our hands, it has also turned out to be a useful model to study the transepithelial absorption of different antibiotics (T. Matysiak-Budnik et al, unpublished observations). Since the target for nitazoxanide, *H. pylori*, is situated in the stomach, two modes of action of nitazoxanide are plausible, topical action and systemic action, the latter implying the initial intestinal absorption of nitazoxanide. This study has shown that there is an important transepithelial transport of nitazoxanide across the digestive epithelial monolayer. There is no significant difference between the overall apical to basal (mucosal to serosal) and basal to apical (serosal to mucosal) fluxes of the drug, suggesting that both topical and systemic modes of action of this antibiotic may be successful. The intracellular drug accumulation seems to be more important after apical than after basal drug application. This could suggest the presence of a specific transporter situated on the apical cell membrane, but this aspect was not specifically addressed in this study and there is no data in the literature indicating the existence of such a transporter. The entry of nitazoxanide into cells is most probably owing to the diffusion of this small molecule (MW 307.3), presenting a neutral electrical charge, across the cellular membrane. The intracellular accumulation is very transitory and decreases rapidly during the first 2 h, which indicates that the efflux of nitazoxanide from the cells is more important that the influx of the drug into the cells. It also suggests that there is a transcellular passage of nitazoxanide across the epithelium. A rapid decrease of the intracellular concentration of nitazoxanide could be related to the active efflux of the drug from the cells by the efflux molecules. Indeed, HT29-19A cells are known to possess the capacity to express the multidrug resistance protein 1, already implicated in an increased efflux activity of these cells (Kok et al 2000). Even though the intracellular accumulation of nitazoxanide found in our study was not very high, the capacity of nitazoxanide to accumulate inside the cells is very important because it indicates that the antibiotic could be potentially effective against intracellular pathogens. Indeed, this finding is in agreement with clinical data showing good activity of nitazoxanide against intracellular forms of *Cryptosporidium parvum* (Gargala et al 2000). It is also of interest for *H. pylori* because a small proportion of bacteria can be internalized by epithelial cells (Evans et al 1992) and these intracellular bacteria could be responsible for the failure of eradication therapy.

The existence of an inverse correlation between the nitazoxanide fluxes, both apical and basal, and the electrical resistance of the epithelium (which is an index of the epithelial integrity) could suggest that at least some fraction of nitazoxanide passes the epithelium through the paracellular pathway. This could be supported by the fact that nitazoxanide is a small molecule that can easily cross the tight junctions. However, a study of the parallel transfer of nitazoxanide and a reference marker of a paracellular route would be necessary to confirm this hypothesis. Finally, it is interesting to note that the fluxes of nitazoxanide across the epithelial monolayer are about 10-times (24-h fluxes) to 30-times (100-min fluxes) higher than the corresponding fluxes of two other antibiotics used in the eradication of *H. pylori*, amoxycillin and clarithromycin, using the same methodology (T. Matysiak-Budnik et al, unpublished observations). Similarly, the permeability of the HT29-19A monolayer to nitazoxanide is particularly high, about 100-times higher that its permeability to amoxycillin or clarithromycin, and about 10 000-times higher than that to mannitol. Although these in-vitro results may not reflect the fate of nitazoxanide in-vivo, they suggest a very good intratissular penetration of nitazoxanide in-vivo.

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