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Ciprofloxacin permeability and its active secretion through rat small intestine in vitro

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Abstract

The biopharmaceutical aspect of the fluoroquinolones–metal cations interaction, which reduces antibacterial agents bioavailability and the mechanism of the fluoroquinolone intestinal efflux are still poorly understood. The purpose of this work was to gain further insights into these two issues by measuring the permeability of ciprofloxacin through the rat small intestine in side-by-side diffusion chambers using different incubation media and transport inhibitors. The permeability of ciprofloxacin from the mucosal to the serosal side was low. It was not influenced by the different concentrations of Ca^{2+} and Mg^{2+} in the donor solution. The active efflux of ciprofloxacin was the highest in the region of the rat small intestine excised proximal to the ileo-caecal junction or when the pH value of the incubation saline was slightly acidic. Thus ciprofloxacin appears to be transported in its cationic or in its zwitterionic form. The efflux was not inhibited by verapamil, benzbromarone or quinidine, which were added to the mucosal side of the intestinal tissue. It was however inhibited by quinidine added to the serosal side. The active secretion is therefore most probably a consequence of the organic cation transporter 1 activity at the basolateral membrane of enterocytes.

Keywords: Fluoroquinolones; Ciprofloxacin; Side-by-side diffusion chambers; Metal cations; Efflux proteins; Intestinal elimination

1. Introduction

Ciprofloxacin is an antibacterial chemotherapeutic from a group of fluoroquinolones. The decrease of the bioavailability of most fluoroquinolones due to interactions with drugs or food containing metal ions is known for a long time (Höffken et al., 1985; Lomaestro and Bailie, 1995). A marked rank order correlation between the extent of fluoroquinolone coordination with various metal cations and the reduction in fluoroquinolone oral bioavailability was observed. Thus, the reduction of bioavailability is somehow related to the formation of complexes between fluoroquinolones and metal ions (Wallis et al., 1996). The complexed species is different from the uncomplexed drug in size, geometry and charge. These factors could hinder the passive diffusion of fluoroquinolones through the cell membranes as well as through the tight junctions between adjacent cells of the intestinal epithelium (Wallis et al., 1996; Turel, 2002).

Fluoroquinolones are also subject to intestinal secretion (Griffiths et al., 1994; Cavet et al., 1997; Dautrey et al., 1999a; Lowes and Simmons, 2002; Volpe, 2004). The intestinal secretion of most fluoroquinolones is mediated by efflux proteins Pgp (P-glycoprotein) and MRP2 (multidrug resistance-associated protein 2). There is at least one more secretory pathway—the "ciprofloxacin-sensitive pathway" as named by Lowes and Simmons who believe, that ciprofloxacin (unlike other fluoroquinolones) is not a substrate for Pgp and MRP2 (Lowes and Simmons, 2002). The cross-inhibition of the intestinal secretion between ciprofloxacin and other fluoroquinolones indicates that the secretory pathway of ciprofloxacin is also involved in the intestinal secretion of other fluoroquinolones (Griffiths et al., 1994; Lowes and Simmons, 2002). This unidentified secretory system is most likely composed of two efflux proteins: one based on the basolateral side of the epithelial cells mediating the ciprofloxacin transport into the epithelial cells, and a second one located in the apical membrane of epithelial cells mediating the secretion of ciprofloxacin from the citosol to the gut lumen (Griffiths et al., 1994; Cavet et al., 1997).

On the other hand, Rodríguez-Ibáñez et al. have more recently concluded that Pgp and MRP2 might be involved in the efflux

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of ciprofloxacin. This conclusion was based on the Caco-2 inhibition studies with 50 μM ciprofloxacin as a substrate in the presence of verapamil and cyclosporine A as Pgp inhibitors, quinidine as a Pgp/organic-cation-transporter 1 inhibitor and *p*-aminohipuric acid as a MRP2 substrate. However, their results were not statistically significant and could not be confirmed by a rat in situ perfusion method using 50 μM ciprofloxacin and the same inhibitors. This discrepancy was explained as a consequence of a higher expression level of efflux proteins in cultured cells than in the rat intestine (Rodríguez-Ibáñez et al., 2003).

In this work we want to evaluate the influence of Ca^{2+} and Mg^{2+} in the incubation saline on the permeability of ciprofloxacin through the rat intestine in side-by-side diffusion chambers. We will also attempt to establish whether ciprofloxacin is a substrate for Pgp and/or MRP2 in the rat small intestine. Furthermore, we will examine some properties of the active intestinal secretion of ciprofloxacin.

2. Materials and methods

2.1. Materials

Ciprofloxacin and fluorescein sodium were purchased from Fluka (Deisenhofen, Germany). Benzbromarone, verapamil and quinidine were supplied by Sigma Aldrich Chemie (Steinheim, Germany).

2.2. "In vitro" intestinal permeability studies

Rat jejunum from male Wistar rats (250–320 g) was obtained, prepared and mounted in Easy Mount side-by-side diffusion chambers (Physiologic Instruments, San Diego, CA, USA) as previously described (Žakelj et al., 2004). The experiments conform to the law for the protection of animals (Republic of Slovenia) and are registered at the Veterinary Administration of the Republic of Slovenia. For the studies of regional differences in the ciprofloxacin efflux three regions of the rat small intestine were defined as follows:

- *upper*: 15 cm of small intestine proximal to the pyloric sphincter;
- *mid*: up to 15 cm of small intestine were taken in the middle between the pyloric sphincter and the ileo-caecal junction;
- *low*: 15 cm of small intestine proximal to the ileo-caecal junction.

Standard or modified Ringer buffer with 10 mM D-glucose or 10 mM mannitol on serosal and mucosal side of the tissue, respectively, was used as an incubation saline. It was previously shown that the use of a modified Ringer buffer without Ca^{2+} and Mg^{2+} on one side of the isolated tissue (mucosal or serosal) and the implication of phosphate-free incubation saline (necessary for testing the influence of increased concentrations of Ca^{2+} and Mg^{2+}) does not impair the viability or the integrity of the isolated intestinal tissue (Žakelj et al., 2004). The tissue was kept at 37 °C during the experiments and the pH of the incubation saline was 7.5. Different pH values of the Ringer buffer were achieved by changing the amount of $H_2PO_4^-$, HPO_4^{2-}, HCO_3^- and $p(CO_2)$. Incubation salines were oxygenated and circulated by bubbling with carbogen (95% O_2 and 5% CO_2 for pH 6.5–7.5 or 98% O_2 and 2% CO_2 for pH 6.0 and 8.0). The osmolarity of all modified incubation salines was adjusted by NaCl.

After the tissue segments were placed into the diffusion chambers, 25 min was allowed for equilibration. At the start of the experiment the appropriate side of the diffusion chamber was emptied and a freshly prepared donor solution was added. The donor solution was Ringer buffer (the same pH as the acceptor solution) containing 50 μM ciprofloxacin unless noted differently. 10 μM of internal paracellular permeability standard fluorescein was added in some cases. Samples of 250 μL were withdrawn from the acceptor compartment every 25 min after the start of the experiments. This volume was replaced by the appropriate acceptor solution.

The tissue viability and integrity were controlled throughout the experiments by monitoring the trans-tissue potential difference, the short circuit current and the trans-tissue electrical resistance with a multi channel voltage–current clamp (model VCC MC8, Physiologic Instruments) as described previously (Žakelj et al., 2004). The trans-tissue potential difference is a highly reliable parameter for the determination of the tissue viability (Söderholm et al., 1998; Polentarutti et al., 1999). At the end of the experiments, the trans-tissue potential difference after the addition of D-glucose to the mucosal compartment (final concentration was 25 mM) was also measured. Based on experience with our system, we can say with confidence that the absolute values of the trans-tissue potential difference after the addition of D-glucose higher than 1.0 mV indicate reliably, that the viability of the rat intestinal mucosa has not been affected to such an extent that it could influence the permeability of tested substances. This includes normal paracellular permeability and normal function of intestinal active transport systems.

2.3. Analytical procedures and data analysis

The concentrations of fluorescein were measured by fluorescence (λ_{EX} = 485 nm, λ_{EM} = 535 nm) on 96-well plates by a microtiter reader (Tecan, Salzburg, Austria). Ciprofloxacin concentrations were measured by HPLC (Agilent 1100 series); UV detection at 278 nm after separation on a 25 mM C18 Chromolith Flash column with 11.5% of acetonitrile and 88.5% of phosphate buffer (pH 3.9). The retention time was 1.0 min; the flow rate was 3 mL/min.

The P_{app} values of the investigated substances were calculated by the following equation:

$$P_{app}\,(\mathrm{cm/s}) = \frac{\mathrm{d}Q}{\mathrm{d}t}\frac{1}{AC_0} \quad (1)$$

where $\mathrm{d}Q/\mathrm{d}t$ is the steady-state appearance rate of fluorescein on the acceptor side of the tissue, A the exposed tissue area and C_0 is the initial concentration of the investigated substance in the donor compartment.

SPSS 12.0.1 for Windows was used for statistical evaluations. Data in the text, table and figures are presented as mean ± standard error of the mean.

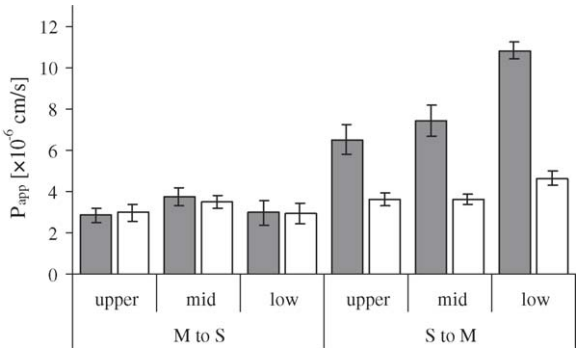

Fig. 1. The permeability coefficients of ciprofloxacin (filled columns) and of the paracellular permeability marker fluorescein (empty columns) for three regions of the rat small intestine in both directions (M to S and S to M). Each value shown is an average of at least three measurements.

3. Results and discussion

The bioavailability of a drug can be hindered by its slow dissolution rate, by its low permeability through the intestinal mucosa, or by its poor solubility at the site of absorption (Amidon et al., 1995; Rinaki et al., 2003).

Wallis et al. (1996) have observed a decrease of norfloxacin bioavailability, after administration directly to the stomach of dogs in a form of an acidic solution containing different metal cations. This study clearly showed the interaction between an already dissolved fluoroquinolone and metal cations. Therefore one can assume, that the presence of metal cations does not hinder the bioavailability of fluoroquinolones by altering the rate of their dissolution in the stomach.

A significant decrease in the value of the drug permeability through the intestinal mucosa, might explain the fluoroquinolone–metal cation interaction. A decrease in the permeability of highly permeable drugs usually does not alter the rate or the extent of drug absorption. On the other hand the bioavailability of "low permeability drugs" is highly dependent on their permeability (Amidon et al., 1988). Fig. 1 shows that the M to S (mucosal to serosal) permeability of ciprofloxacin is comparable to that of fluorescein (a low permeability paracellular marker) in all tested regions of the rat small intestine. This indicates that ciprofloxacin should be classified as a "low permeability drug". Afterwards we have measured the M to S P_{app} of ciprofloxacin in the presence and absence of Ca^{2+} and Mg^{2+} ions on the mucosal (donor) side while Ringer buffer with normal concentration of Ca^{2+} and Mg^{2+} was used in both cases for the acceptor solution on the serosal side. The obtained P_{app} values were not influenced by the presence of Ca^{2+} and Mg^{2+} ions. The P_{app} of ciprofloxacin permeating through the rat jejunum from the mucosal Ca^{2+}- and Mg^{2+}-free donor solution to the serosal side was $(5.6 \pm 0.3) \times 10^{-6}$ cm/s. The P_{app} of ciprofloxacin was $(4.4 \pm 0.8) \times 10^{-6}$ cm/s in the presence of 1.2 mM (standard concentration in Ringer buffer) of both divalent cations in the donor solution. Furthermore, even at 10 mM concentration of Ca^{2+} and Mg^{2+} in the donor solution the P_{app} of ciprofloxacin was $(5.1 \pm 0.3) \times 10^{-6}$ cm/s. These values did not differ significantly ($P > 0.05$; pairwise comparisons by a Bonferroni post-hoc test). One can thus conclude that the decrease of the bioavailability of ciprofloxacin, which occurs when it is taken concomitantly with other drugs or food containing Ca^{2+} and/or Mg^{2+} ions, may not be a consequence of diminished intestinal permeability of ciprofloxacin.

The solubility of fluoroquinolones, which could also influence their bioavailability, is very high in acidic conditions (pION INC, 2003). This facilitates their dissolution in the stomach. However, these drugs could precipitate during the neutralisation, which occurs in the duodenum and jejunum. Wallis at al. have already speculated, that such precipitation could increase in the presence of high levels of metal cations (Wallis et al., 1996). Nevertheless, the effect of metal cations on the solubility of fluoroquinolones may be highly complex and is still unclear. Some authors have reported a decrease of fluoroquinolone solubility in the presence of metal cations (Rodríguez Cruz et al., 1999; Turel, 2002), while the others have reported the use of Al^{3+} for the solubilisation of ciprofloxacin in liquid dosage forms (Allemandi et al., 1999; Alovero et al., 2003).

In Table 1 the permeability coefficients of ciprofloxacin across the rat jejunum in both directions (M to S and S to M) without inhibitors and in the presence of benzbromarone (a MRP2 inhibitor), verapamil (a Pgp/MRP2 inhibitor) and quinidine (an OCT1 (organic cation transporter 1) inhibitor) are given. It is obvious that ciprofloxacin efflux (permeability in the S to M direction) is much more pronounced than its permeability in the M to S direction at both applied concentrations (50 and 600 μM). The permeability coefficients of ciprofloxacin measured in the presence of benzbromarone or verapamil in the M to S as well as in the S to M direction did not differ significantly from those

Table 1
The effect of benzbromarone and verapamil on the permeability of ciprofloxacin in both directions and on the viability of the segments of the rat jejunum in vitro

	C_{0CIP}[a] (μM)	P_{app} (M to S) ($\times 10^{-6}$ cm/s)	P_{app} (S to M) ($\times 10^{-6}$ cm/s)	Efflux ratio[a]	Net efflux[a]	PD_{GLU}[a] (mV)
Without inhibitors[b]	50	4.1 ± 0.9	9.9 ± 1.4	2.4	5.8	−4.8 ± 0.3
Without inhibitors	600	3.2 ± 0.1	12.2 ± 0.8	3.8	9.0	−4.8 ± 0.4
30 μM benzbromarone	50	3.2 ± 0.3	10.9 ± 1.0	3.4	7.7	−3.4 ± 0.9
200 μM verapamil	50	4.3 ± 0.7	12.1 ± 1.5	2.8	7.8	−2.7 ± 0.3
1 mM quinidine (Muc.)	50	5.0 ± 0.4	10.9 ± 2.0	2.2	6.0	−1.5 ± 0.2
1 mM quinidine (Ser.)	50	2.6 ± 0.1	3.5 ± 0.2	1.3	0.8	−1.6 ± 0.1

[a] C_{0CIP} is the initial concentration of ciprofloxacin on the donor side of the diffusion chambers. The "efflux ratio" is calculated as P_{app} (S to M)/P_{app} (M to S), while the "net efflux" is P_{app} (S to M) − P_{app} (M to S). PD_{GLU} is the trans-tissue potential difference after the addition of glucose at the end of experiment.

[b] Control experiment—only the S to M P_{app} value obtained when 1 mM quinidine was present in the serosal incubation saline differed significantly from the control values ($P > 0.05$; Dunnett's t-tests).

obtained under control conditions—without inhibitors. These results are in accordance with the conclusions of Lowes and Simmons who found (using Caco-2 cell monolayers) that Pgp and MRP2 efflux proteins are not involved in the intestinal secretion of ciprofloxacin (Lowes and Simmons, 2002), but disagree with Rodríguez-Ibáñez et al. who suggested that several efflux carriers including Pgp and MRP2 might be responsible for the intestinal secretion of ciprofloxacin (Rodríguez-Ibáñez et al., 2003). Anyhow, on the basis of the results obtained in this study we can conclude that the so-called "ciprofloxacin-sensitive pathway" (and not Pgp or MRP2 efflux proteins) is most probably responsible for the intestinal secretion of ciprofloxacin.

Our results also indicate that the rat jejunal expression level of efflux proteins involved in the intestinal secretion of ciprofloxacin is similar to that observed by the other authors in Caco-2 cell monolayers. Namely, the reported values of the ciprofloxacin efflux ratio (i.e. the secretive (S to M) versus absorptive (M to S) permeability) on Caco-2 models were 2.0 (Rodríguez-Ibáñez et al., 2003), 3.5 (Cavet et al., 1997), 4.6 (Volpe, 2004), and 5.3 (Griffiths et al., 1994) and are comparable with the ratio obtained in our experiments on rat intestine (Table 1). Therefore, we can argue the estimation of Rodríguez-Ibáñez et al. that the expression level of efflux proteins, which mediate the intestinal secretion of ciprofloxacin, is higher in cultured cells than in the rat intestine (Rodríguez-Ibáñez et al., 2003).

To determine the kinetic constants (i.e. the Michaelis–Menten constant: K_M and the maximal velocity: V_{max}) of the ciprofloxacin efflux we applied 600 μM of ciprofloxacin to the donor solution. The results in Table 1 show, that these efflux proteins are not yet saturated at 600 μM of the substrate—ciprofloxacin. Namely, the S to M permeability coefficient, the efflux ratio and the net efflux of ciprofloxacin are even higher (but not statistically significant) at 600 μM donor concentration compared to 50 μM donor concentration. Much higher donor concentration of ciprofloxacin would be necessary to achieve a saturation of the efflux proteins, but this is not possible due to its insufficient solubility at physiological pH.

In Fig. 1 one can see that there was no significant difference between the M to S permeability coefficients of ciprofloxacin and fluorescein obtained in the different regions of the rat small intestine ($P > 0.05$; one-way ANOVA followed by a Bonferroni post-hoc test) (Fig. 1). This indicates that there were no regionally dependent active processes involved in the ciprofloxacin absorption from the lumen. Thus, ciprofloxacin most probably permeates through the rat intestine in vitro in the M to S direction by the same mechanism as fluorescein: passive paracellular transport (Kristl and Tukker, 1998).

The permeability coefficients of ciprofloxacin in the S to M direction were significantly higher than corresponding permeability coefficients in the M to S direction for all three regions of the rat small intestine ($P < 0.01$; independent samples t-test) (Fig. 1). Additionally, significant differences between the S to M permeability coefficients of ciprofloxacin in the different regions of the rat small intestine were determined ($P < 0.01$; one-way ANOVA). The pairwise comparisons showed that the permeability coefficient of ciprofloxacin in the S to M direction was significantly higher in the low region of the rat small intestine comparing to the values obtained in the mid and in the upper small intestine ($P < 0.01$; Bonferroni post-hoc test). Permeability coefficient of fluorescein in the S to M direction was not regionally dependent ($P > 0.05$; one-way ANOVA followed by a Bonferroni post-hoc test). Therefore one can conclude that the expression level of the efflux proteins mediating the ciprofloxacin efflux is higher in the lower regions of the rat small intestine.

Rohwedder et al. reported that intestinal ciprofloxacin efflux is responsible for 20% of ciprofloxacin elimination in patients with normal renal function and for 32% in patients with impaired renal function (Rohwedder et al., 1990). Later, Dautrey et al. have also demonstrated the importance of intestinal ciprofloxacin efflux (and not biliary secretion) of ciprofloxacin on rats (Dautrey et al., 1999b). Compounds, which are excreted into the intestinal lumen (either directly or via bile) without prior biotransformation, are normally reabsorbed (DeSesso and Jacobson, 2001). The reabsorption of ciprofloxacin is not complete (Dautrey et al., 1999b) probably because of the fact, that the intestinal ciprofloxacin efflux is the highest at the end of the absorptive part of the intestine as it was shown by the results of this study.

The permeability of ciprofloxacin through rat jejunum was measured in modified Ringer buffers (incubation salines) with pH values ranging from 6.0 to 8.0. Fig. 2 shows the obtained permeability coefficients of ciprofloxacin in both directions and the net efflux.

The permeability of ciprofloxacin through rat jejunum was measured in modified Ringer buffers (incubation salines) with pH values ranging from 6.0 to 8.0. Fig. 2 shows the obtained permeability coefficients of ciprofloxacin in both directions and the net efflux.

It is known that uncharged species of molecules permeate biological membranes better then their charged counterparts

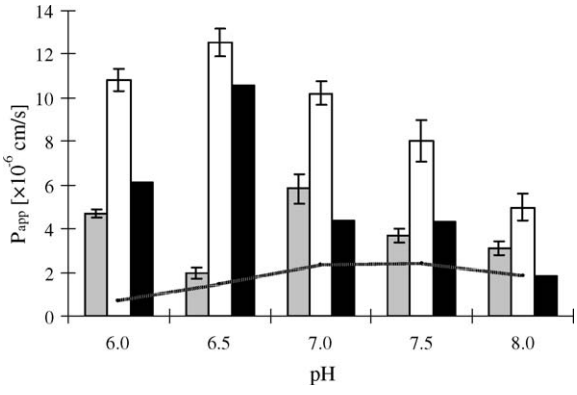

Fig. 2. Permeability coefficients of ciprofloxacin in the M to S and in the S to M direction are represented by grey and empty columns, respectively. Black columns represent the difference between the measured S to M and M to S permeability coefficients—i.e. the net efflux. The line crossing the columns represents the permeability of ciprofloxacin through an artificial lipid membrane—PAMPA (Bermejo et al., 2004). Each value shown is an average of at least four measurements.

(Högerle and Winne, 1983). Thus, the passive transcellular permeability of fluoroquinolones is expected to be the highest at pH, which results in the highest portion of their zwitterionic form (the pK_a values of ciprofloxacin are 6.16 and 8.62 for the carboxylic and the 4′N amine group, respectively; therefore its isoelectric point is at 7.39). This was confirmed by the results of Bermejo et al. who have used PAMPA (parallel artificial membrane permeability assay) to describe the pH dependence of the permeability through lipid membranes for several fluoroquinolones (Bermejo et al., 2004). Their results for the permeability of ciprofloxacin through an artificial lipid membrane are included in Fig. 2.

The M to S permeability coefficients of ciprofloxacin through the isolated rat jejunum obtained in our study are different from those obtained by PAMPA (Fig. 2). The difference is most pronounced at pH 6.0. This discrepancy could be partially explained by the findings of this study, that ciprofloxacin most probably permeates through the intestinal membrane in the M to S direction through the paracellular route, while the diffusion through the artificial lipid membranes is especially appropriate for the simulation of passive transcellular permeability. The fact that the paracellular pathway exhibits charge-selectivity may also be important. Namely, the pores in the tight junctions are predominantly cation selective (Schneeberger, 2003; Tang and Goodenough, 2003). This could explain the higher passive M to S permeability of ciprofloxacin at pH 6. The results in Fig. 2 also show that the S to M permeability through the rat jejunum is strongly pH-dependent (i.e. the highest S to M permeability and consequently the lowest M to S permeability can be observed at pH 6.5). It seems that the activity of the efflux proteins involved in the intestinal secretion of ciprofloxacin was the highest at slightly acidic pH values (pH 6.5) of the incubation saline and/or that the anionic form of ciprofloxacin is not a substrate for the efflux proteins (Fig. 2). Dautrey et al. used various competitors to demonstrate that the intestinal elimination of ciprofloxacin is mediated by organic anion and/or organic cation transport systems (Dautrey et al., 1999a). Our results thus provide further insight into ciprofloxacin transport mechanism by indicating that ciprofloxacin is actively secreted through the small intestinal mucosa in its cationic or in its zwitterionic form by (an) organic cation transport system(s) while the involvement of organic anion transport system is less likely. Dautrey et al. suggested that OCT1 could be the organic cation transport protein involved in the ciprofloxacin efflux. This suggestion was given because they have observed an inhibition of ciprofloxacin efflux in the rat in vivo model after injection of quinidine to the animals (Dautrey et al., 1999a). Our results (Table 1) confirm that quinidine inhibits the ciprofloxacin efflux. Furthermore, this inhibition can only be observed after the addition of quinidine to the serosal side of the rat intestine, which indicates, that the efflux protein crucial for the ciprofloxacin secretion is present in the basolateral membrane of enterocytes. This additionally confirms the assumption that OCT1 (an efflux protein localized at the basolateral membrane of enterocytes; Jonker et al., 2001) is important for the ciprofloxacin secretion through the rat small intestinal epithelium.

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