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Uptake and Transport Characteristics of Chloroquine in an In-vitro Cell Culture System of the Intestinal Mucosa, Caco-2

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Abstract

The transepithelial transport and uptake of chloroquine were studied in cultured human intestinal Caco-2 cell layers, to investigate whether a specific mechanism facilitates the flux of chloroquine. Due to ionization of chloroquine at the pH of the intestinal lumen, the fraction of the neutral form, which is required for partitioning into biological membranes, is very low, while oral bioavailability has been reported to be nearly complete. Several observations, such as concentration-dependent uptake and temperature-dependent transepithelial flux, suggest the presence of carrier mediated transport. However, alternative mechanisms may be invoked to explain these observations. It is suggested that concentration dependence can originate from ion-trapping in acidic compartments of the cell or non-specific binding to cell components, while temperature-dependent transport can, at least partly, be explained by the temperature dependence of the acid dissociation constants of chloroquine. No differences were observed in the transepithelial flux of the enantiomers of chloroquine. pH-dependent uptake as well as pH-dependent transepithelial transport suggest that the translocation of chloroquine occurs according to the fraction of neutral molecules.

From the data obtained in this study, it is concluded that chloroquine crosses the gastrointestinal barrier by passive diffusion. The extensive area of the gastrointestinal tract probably compensates for the low fraction of the neutral molecule. An interesting finding of this study was the concentration-dependent increase in transepithelial electrical resistance across monolayers incubated with chloroquine at the apical side.

The antimalarial drug chloroquine is a basic compound having two sites of ionization, with reported pK_a values of 8·11 and 10·41 at 37°C, respectively (Ferrari & Cutler 1987). Therefore, the fraction of un-ionized drug in the intestinal lumen is expected to be very low. A drug usually crosses biological membranes in the un-ionized, desolvated form, unless specific transport mechanisms are involved which facilitate transmembrane transport. The availability of chloroquine after oral administration does not seem to be impeded by the low fraction of un-ionized chloroquine at the pH of the small intestine, as the bioavailability has been reported to be nearly complete (Gustafsson et al 1983). Therefore, a specific mechanism might be involved in the transepithelial translocation of chloroquine. The transport of monoprotonated chloroquine across biological membranes has been suggested (Homewood et al 1972), as well as the contribution of a carrier that transports basic amino acids (Yayon & Ginsburg 1982). Ferrari & Cutler (1990) investigated the transport of chloroquine across the plasma membrane of uninfected erythrocytes and they concluded that chloroquine is transported across these biological membranes in the un-ionized form by passive diffusion. To explain the accumulation in erythrocytes, they put forward a combination of ion-trapping and binding of chloroquine to cell components.

To investigate whether a specific mechanism facilitates the transepithelial flux of chloroquine, the uptake and transport across the intestinal epithelium was studied using the human colon carcinoma cell line, Caco-2. The Caco-2 model is well established as an in-vitro system mimicking the transport properties of the intestinal mucosa (Hidalgo et al 1989; Artursson 1990).

Materials and Methods

Materials

Chloroquine diphosphate, 2-[morpholino]ethanesulphonic acid (Mes), verapamil and NH_4Cl were obtained from Sigma (Bornem, Belgium). Hanks' balanced salt solution, Dulbecco's Modified Eagle Medium (DMEM) containing glutaMAX, *N*-[2-hydroxyethyl]piperazine-*N'*-2-ethane-sulphonic acid (HEPES), 0·05% trypsin and 0·02% EDTA in phosphate-buffered saline (PBS), non-essential amino acids (NEAA), penicillin-streptomycin (10 000 int. units mL^{-1} and 10 mg mL^{-1}, respectively) and foetal bovine serum were from Gibco (N.V. Life Technologies, Merelbeke, Belgium). CH_3CN, $HClO_4$ (60%) and NaH_2PO_4 were from BDH (Poole, UK), Merck (Darmstadt, Germany) and UCB (Leuven, Belgium), respectively. All chemicals were used as received. The synthesis of the enantiomers of chloroquine diphosphate was described previously (Augustijns & Verbeke 1992).

Cell culture

The Caco-2 cell line was generously provided by Dr. Y. Schneider (Laboratory of Cellular Biochemistry, Université Catholique de Louvain, Belgium). Cells were used between passage 110 and 130. The Caco-2 cells were maintained in high glucose (4·5 gL^{-1}) DMEM containing glutaMAX, 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 1%

NEAA and 10% foetal bovine serum, and grown in tissue-culture flasks (75cm^2, Nunc, Roskilde, Denmark) at 37°C in 5% CO_2. Cells were trypsinized at a ratio 1:5 after reaching 90% of confluence using 0·05% trypsin in PBS containing 0·02% EDTA. For transport experiments, cells were seeded onto tissue-culture inserts (Anopore membranes, 25 mm, Nunc), while for uptake studies, cells were plated and grown on 6-well tissue-culture plates (Nunc). Medium was changed the day after seeding, and every other day thereafter. Apical and basolateral chamber volumes were maintained at 1·5 and 2 mL, respectively. Experiments were conducted 17 to 22 days post-seeding. Transepithelial electrical resistance (TEER) was measured using an Evom Voltohmmeter (WPI, Aston, UK). Only monolayers having TEER values above 250 Ω cm^2 were used in studies. Sodium fluorescein was used as a hydrophilic marker for cell monolayer integrity (Hurni et al 1993). Typical sodium fluorescein flux values were below 0·5% cm^{-2} h^{-1}.

Transepithelial transport

For the determination of the transepithelial flux of chloroquine across Caco-2 cells, the polarized monolayers were preincubated with transepithelial transport medium [Hanks' balanced salt solution, supplemented with 10 mM HEPES (transport medium pH 7·2) or 10 mM Mes (transport medium pH 6·0) and 25 mM glucose], and TEER values were measured to check cell monolayer integrity. The transport medium was replaced by 1·5 mL transport medium with chloroquine at the apical side, and 2 mL transport medium at the basolateral side of monolayers. To maintain sink conditions, the inserts were transferred into fresh 6-well plates after 15, 30, 45 and 60 min. The samples were directly injected into the HPLC system. All flux experiments were conducted in triplicate. The transport was expressed as the percentage of drug added to the donor compartment.

Drug accumulation

The rate of drug accumulation was measured by adding chloroquine in transport medium to the cells grown on 6-well tissue-culture plates. When NH_4Cl was included in the chloroquine solution, cells were pre-incubated in transport medium with the same concentration of NH_4Cl for 30 min. At various time points, the monolayers were quickly rinsed with ice-cold transport medium. The cells were solubilized by incubation with 3 mL Triton-X100 (0·1%) in 0·3 M NaOH. An aliquot (50 μL) was taken for the measurement of total protein according to the method of Bradford (1976), using bovine serum albumin as a standard (Bio-Rad protein assay kit). After adding 50 μL internal standard solution, 6,8-dichloro-4-(1-methyl-4-diethylaminobutyl)-aminoquinoline, 0·1 mg mL^{-1}, the remaining solution was extracted with 6 mL CH_2Cl_2. Following centrifugation, the water layer was discarded, and the organic layer was evaporated under a gentle stream of air. The residue was dissolved in 1 mL mobile phase, and an aliquot was injected into the HPLC system. Cellular accumulation of chloroquine was expressed as nmol (mg protein)$^{-1}$.

Drug efflux

Caco-2 monolayers grown in 6-well plates were incubated with 100 μM chloroquine for 60 min. After washing with ice-cold transport medium, 3 mL transport medium (37°C) was added to the monolayers. After various wash-out periods, the monolayers were processed for chloroquine remaining in the cells as described under uptake. The results are presented as the percentage of chloroquine before the wash-out period.

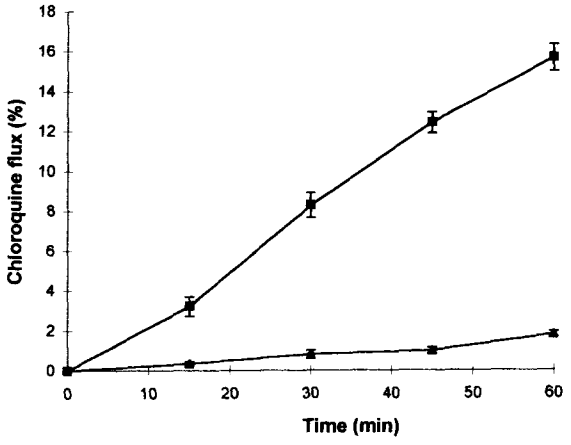

FIG. 1. Time course of apical to basolateral transport of chloroquine (100 μM) across Caco-2 monolayers following incubation at various pH values (■, pH 7·2; ▲, pH 6·0) (mean ± s.d.; n = 3).

Octanol-transport medium partitioning

Chloroquine phosphate was dissolved in transport medium to give a final concentration of 100 μM. pH was adjusted to 7·2. Three millilitres of the solution and 3 mL *n*-octanol were shaken for 2 h at various temperatures. After separation of the two phases, the drug concentration in the aqueous phase was determined using HPLC.

HPLC analysis of chloroquine

The HPLC system consisted of a Waters 600 controller, a Waters 717 plus auto-injector and a Waters UV-detector (343 nm). The column was a Novapak C-18 (Waters, 4 μm) under radial compression. The mobile phase consisted of 220 mL acetonitrile and 600 mL sodium phosphate buffer (0·04 M, pH 3·6) containing 8 mL $HClO_4$ (60%) as a counter ion for chloroquine. Chromatograms were recorded using the MAXIMA 820 computer program (Waters). Calibration graphs for chloroquine were constructed using standards treated in the same way (with or without internal standard).

Results and Discussion

The effect of changing the pH of the apical medium from 7·2 to 6·0 on the transepithelial flux of chloroquine is shown in Fig. 1. At 37°C, the transport was linear with time of incubation, and the apical to basolateral flux was 10 fold higher at pH 7·2 than at pH 6·0. The decrease in transport at pH 6·0 compared with pH 7·2 suggests that transcellular transport is the main mode of transport. It has been shown previously that paracellular transport across Caco-2 monolayers, as monitored by the flux of mannitol, is independent of apical pH (Gochoco et al 1994). Therefore, the

Table 1. Concentration dependence of chloroquine transport and uptake using Caco-2 monolayers incubated with chloroquine for 60 min at 37°C, pH 7·2.

Chloroquine (mM)	Transport (nmol)	Uptake (nmol (mg protein)$^{-1}$)
0·01	–	8·0 ± 0·6
0·02	1·8 ± 0·6	–
0·1	16·5 ± 3·9	46·3 ± 2·5
0·5	86·2 ± 8·1	–
1	173 ± 44	145 ± 7

Mean ± s.d., n = 3.

pH effect on transport observed in this study is expected to be due to partitioning into cells, which is required by the transcellular pathway. The amount transported at pH 7·2 was a linear function of the concentration of chloroquine (Table 1), illustrating that transepithelial flux is independent of concentration. The absence of any concentration–dependence of transport over the concentration range tested suggests that no specific mechanism is involved.

When the time course of transport was studied for 60 min at 37 and 9°C, transepithelial flux decreased from 15·7 ± 0·7% to 1·1 ± 0·2%, clearly proving temperature dependence of chloroquine flux across Caco-2 monolayers. Ferrari & Cutler (1987) reported that the acid dissociation constants of chloroquine are temperature dependent, and decreased by approximately one unit with increasing temperature over the range 0–37°C. Therefore, we studied the possible contribution of temperature on partitioning into membranes by measuring the octanol/transport medium partitioning at various temperatures. The data in Table 2 indicate that there is a strong decrease of partitioning into the organic layer when the temperature was decreased below 20°C. At pH 7·2, the fraction of the neutral form of chloroquine, which is required for partitioning into membranes, is thus higher at a higher temperature. It is therefore not unexpected that small changes in pK_a values due to temperature variations result in a very significant change in chloroquine uptake and transepithelial transport.

Increasing concentrations of phosphate (0–10 mM) as a possible counter-ion for chloroquine did not result in enhanced partitioning in octanol, suggesting that ion-pair formation cannot be invoked as an alternative absorption mechanism (data not shown).

As specific carrier systems exhibit stereoselective properties, the apical to basolateral flux of chloroquine enantiomers was investigated by incubating Caco-2 monolayers with a concentration of 100 μM (*R*)-, (*S*)- or racemic chloroquine. No significant differences were observed when the transepithelial flux was measured for 60 min at 37°C (data not shown).

Another mechanism that might affect the transepithelial transport of chloroquine across Caco-2 monolayers is an efflux mechanism that has recently been described in the apical membrane of these cells. This efflux mechanism is thought to be related to P-glycoprotein (Hunter et al 1993). It has been shown that the rapid efflux of chloroquine from chloroquine-resistant *Plasmodium falciparum* strains is phenotypically similar to the efflux observed in mammalian cells with multidrug resistance (Krogstad et al 1992). This observation suggests that chloroquine is a substrate for this type of carrier, and that the transepithelial flux of chloroquine might be affected by this phenomenon. Verapamil, a drug known to reverse multidrug resistance, failed to influence the transepithelial flux of 100 μM chloroquine when coadministered at 100 μM (13·6 ± 1 vs 11·8 ± 1·3% for control conditions, $P > 0·05$), suggesting that the transport of chloroquine (100 μM) is not affected by this carrier. However, the concentration of chloroquine used in this study may have been too high to observe any effect of this carrier.

The time-dependent uptake of chloroquine by Caco-2 cells grown on culture dishes was studied at various concentrations (10 μM, 100 μM, 1 mM) for up to 1 h. Although the number of concentrations tested was limited, the data indicate that there is a disproportional increase in the accumulation of chloroquine (Table 1). Although this concentration–dependent uptake could, along with the temperature–dependent transport, be evocative of the presence of a specific mechanism involved in the transport of chloroquine, non-specific factors can be invoked as alternative explanations. The concentration dependence can, for example, originate from the saturation of non-specific binding

Table 2. Temperature–dependent recovery of chloroquine (100 μM) in the aqueous layer (pH 7·2) after equilibration with an equal volume of octanol for 2 h.

Temperature (°C)	Chloroquine in water layer (%)
5	44·2 ± 1·5
10	30·4 ± 4·2
15	23·4 ± 0·7
20	14·8 ± 0·5
30	13·2 ± 0·7
37	12·2 ± 0·1

Mean ± s.d., n = 3.

Table 3. Effect of NH_4Cl on the uptake of chloroquine by Caco-2 cells. Cells were incubated with 50 μM chloroquine for 60 min (pH 7·2, 37°C). Four monolayers were used in each experiment. The chloroquine uptake in the absence of NH_4Cl was defined as 100%.

Concentration NH_4Cl(mM)	% (s.d.)
0 (control)	100 (5·8)
2	75·4 (7·1)
10	48·3 (6·5)
50	33·1 (2·7)

Table 4. Uptake of chloroquine (100 μM) by Caco-2 monolayers (60 min at 37°C) at various pH values of the incubation medium.

pH	Uptake (nmol (mg protein)$^{-1}$)
6	2·6 ± 0·3
6·5	9·6 ± 1·0
7	33 ± 1·2
7·5	59 ± 2·0
8	77 ± 2·0

Mean ± s.d., n = 3.

Table 5. Concentration dependence of TEER values after 1 h apical incubation with chloroquine.

Chloroquine (mM)	TEER (% of reference)
0	100 ± 10
0·02	96 ± 9
0·1	125 ± 12
0·5	203 ± 18
1	259 ± 19
2	268 ± 33
5	347 ± 24

Mean ± s.d., n = 3.

sites on the cell membrane, or from ion-trapping in acidic compartments of the cell. Onyeji et al (1992) studied the transport of chloroquine in the rat submaxillary gland and suggested that the concentration-dependent uptake could be explained by the lysosomatropic properties of chloroquine. As a basic drug, it causes an increase in the pH of the lysosomes, thus reducing the pH gradient as a driving force. NH_4Cl is a weak base that has been reported to increase the pH of intracellular cell compartments (Krogstad et al 1985); therefore, the effect of NH_4Cl was studied on chloroquine uptake in Caco-2 cells. The results in Table 3 clearly show a decrease in chloroquine uptake with increasing concentration of NH_4Cl, suggesting the entrapment of chloroquine in acidic cell compartments in Caco-2 cells.

When the efflux of chloroquine after incubation for 1 h with 100 μM was measured, 86·8 ± 2·9% remained associated with the cells after 180 min, confirming the very strong cellular binding or trapping in intracellular cell compartments.

The influence of pH of the extracellular medium on the uptake of chloroquine is shown in Table 4, clearly showing an increase in chloroquine translocation in response to a higher fraction of neutral molecules.

No metabolites that could be traced with the analytical method (desethylchloroquine and bisdesethylchloroquine) could be observed in this study, suggesting that transepithelial transport is not affected by metabolism.

Exposure of Caco-2 monolayers to increasing concentrations of chloroquine resulted in an immediate, concentration-dependent increase in TEER values (Table 5), suggesting a specific interaction of chloroquine with the Caco-2 monolayers. The mechanism underlying this observation remains to be elucidated and is presently under investigation.

Cellular uptake did not correlate with transepithelial transport, as illustrated by the fact that uptake was concentration dependent, while transport was not. These data illustrate that uptake experiments are not very representative of absorption studies.

No conclusive evidence for a specific mechanism is observed in this study, pointing to passive diffusion of the neutral molecule through the gastrointestinal barrier.

The results also show that the active transport component can not always be quantitatively determined by comparing transport rates at 37 and 4°C. The influence of temperature on the physicochemical factors should be taken into account to prevent false conclusions on active transport.

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