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Transport of gatifloxacin involves Na^+/Ca^{2+} exchange and excludes P-glycoprotein and multidrug resistance associated-proteins in primary cultured rat brain endothelial cells

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ABSTRACT

The characteristics of gatifloxacin transport across blood brain barrier were investigated using primary cultured rat brain microvessel endothelial cells (rBMECs) as an *in vitro* model. Gatifloxacin uptake by rBMECs was time-, temperature- and energy-dependent. Gatifloxacin uptake by rBMECs was not influenced by P-glycoprotein (P-GP) inhibitor cyclosporine A or multidrug resistance associated-proteins (MRPs) inhibitor probenecid. However, verapamil inhibited the uptake in a concentration-dependent manner. Transendothelial transport study showed that transport of gatifloxacin across rBMEC monolayer was bidirectional, verapamil concentration-dependently inhibited transport from the apical to basolateral side, but did not significantly affect transport from basolateral to apical side. Gatifloxacin uptake was decreased in Ca^{2+}-deprived medium but increased in Mg^{2+}-deprived medium significantly. Furthermore, organic Ca^{2+} channel blockers nifedipine and diltiazem had no effect on gatifloxacin uptake, but inorganic Ca^{2+} channel blockers Ni^{2+} and Mg^{2+} inhibited the gatifloxacin uptake. The present study suggests that gatifloxacin transport across rBMECs involves a Na^+/Ca^{2+} exchange mechanism and extracellular Ca^{2+} but not P-GP and MRPs.

1. Introduction

Gatifloxacin is one of the fluoroquinolones which have good activity against gram negative organisms. Clinical reports showed that treatment of gatifloxacin was often associated with some central nervous system (CNS) side effects, including nausea, dizziness, headache, insomnia, agitation and anxiety (Perry et al., 1999), psychosis (Satyanarayana and Campbell, 2006; Reeves, 2007) and seizures (Marinella, 2001). It was also reported that gatifloxacin showed proconvulsant and anxiogenic effects in mice (Bharal et al., 2008). Animal experiments demonstrated that the seizures were related to cerebrospinal fluid concentrations of fluoroquinolones (Delon et al., 1997; Delon et al., 1999) which indicated that CNS side effects of gatifloxacin may also result from its penetration into brain.

For optimal therapy, knowledge of factors involved in the transport of gatifloxacin in the brain should be known. Existence of blood brain barrier (BBB) is considered to be one of the main factors which limit the penetration of drugs into the brain. Transport of some fluoroquinolones across BBB has been widely studied *in vitro* and *in vivo* (de Lange et al., 2000; Liu et al., 2005). These studies showed that distribution of the fluoroquinolones into the brain was restricted. One of reasons limiting penetration of these compounds into brain is the existence of extrusion systems including P-glycoprotein (P-GP) and multidrug resistance associated-proteins (MRPs).The efflux systems transport their substrates against a concentration gradient from the brain into the plasma. Several reports showed that transport of some fluoroquinolones across BBB was mediated by P-GP or MRPs (Yamaguchi et al., 2000; Sasabe et al., 2004; González-Alvarez et al., 2005; Lowes and Simmons, 2002; Gollapudi et al., 1995). But our previous studies showed that function of P-GP was damaged in diabetic mice induced by streptozotocin (Liu et al., 2007) and gatifloxacin may enhance convulsant activity of pentylenetetrazole, but the level of gatifloxacin in brain of diabetic mice did not significantly increase (Zhu et al., 2009). This indicated that other mechanisms may be involved in gatifloxacin transport across BBB.

The aim of the study was to study characteristics of gatifloxacin transport across BBB, and to investigate whether some drug transporters and Ca^{2+} channels were involved in gatifloxacin transport across BBB, as well as the influence of extracellular calcium on gatifloxacin uptake, using the primary cultured rat brain microvessel endothelial cells (rBMECs) as an *in vitro* BBB model.

2. Materials and methods

2.1. Reagents and animals

Gatifloxacin was obtained from Nanjing Shenghe Pharmaceutical Co., (Nanjing, China). Cysclosporin A was provided by Sichuan Industrial Institute of Antibiotics (Chengdu, China). Bovine serum

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albumin (BSA) was purchased from SABC (Fraction V, USA). Verapamil, nifedipine, diltiazem and probenecid were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other agents were commercially available.

Sprague-Dawley neonate rats, 7–10 days old, were supplied by Center of Experimental Animals, China Pharmaceutical University. The studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

2.2. Methods

2.2.1. Isolation and culture of the rBMECs

The brain microvascular endothelial cell is considered to be an important component of BBB, so rBMECs were used as an *in vitro* transport model in the study. Isolation and culture of rBMECs were operated according to the methods previously described in our laboratory (Sun et al., 2006). The primary cultured rBMECs, when reached confluence, were used for experiments.

2.2.2. Uptake of gatifloxacin by rBMECs

The experiments were conducted to examine time-, concentration-, and temperature-dependent elements of gatifloxacin. Time-dependent experiment was performed at pre-designed time (5, 15, 30, 60, 90 and 120 min) using 20 μg/ml gatifloxacin. Concentration-dependent study was operated using 120 min incubation in presence of different gatifloxacin concentrations (from 10 to 100 μg/ml). The temperature-dependent study was assessed by measuring gatifloxacin uptake in presence of 20 μg/ml gatifloxacin following incubation at 37 °C and 4 °C, respectively. All the uptake studies were performed by the similar procedures according to the following description. Briefly, the cultured rBMECs were pre-incubated at 37 °C in 1 ml Hanks' balanced salt solution (HBSS) for 30 min. Then the solution was removed, and 1 ml HBSS containing gatifloxacin was added to each incubated well. At the end of incubation, 1 ml of ice-cold HBSS was added to terminate the uptake reaction, and cells were then washed 3 times with 1 ml of ice-cold HBSS. Then 0.3 ml of purified water was added in each incubated well, frozen and melted three times to break down cells. The concentration of gatifloxacin in cell samples was measured using HPLC method. Each experimental variable was represented by at least 4 replicates and each experiment repeated at least twice. Cell viability was measured using 3-(4, 5-dimethyl-2-thiazoyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay (Vellonen et al., 2004).

2.2.3. Effect of tested agents on the steady-state uptake of gatifloxacin

To study whether some drug transporters were involved in the transport of gatifloxacin across BBB, effect of P-GP and MRPs modulators, as well as, energy metabolic inhibitors on the steady-state uptake of gatifloxacin, was examined. Concisely, the cultured rBMECs were incubated with HBSS containing both gatifloxacin (20 μg/ml) and tested agents at 37 °C for 120 min, the gatifloxacin uptakes by rBMECs were measured.

2.2.4. Polarized gatifloxacin transport across rBMEC monolayers

The transendothelial transport of gatifloxacin was assessed by measuring transport from the apical to basolateral side (A–B) or from basolateral to apical (B–A) side across the rBMEC monolayers. Effect of verapamil on the transport of gatifloxacin was also evaluated. Briefly, the rBMECs were cultured in Millicell-PCF insert (1 cm diameter, 0.4 μm poresize, Millipore, USA) for 15 days after seeding. Restriction of paracellular transport of small ions was controlled by analysis of transendothelial electrical resistance (above 130 Ωcm^2). Paracellular pathway transport was assessed using 3 h permeability of fluorescein (1 μg/ml) and FITC-labeled dextran (100 μg/ml), respectively (Yang and Liu, 2008), which apparent permeability coefficients were significantly lower than Millicell-PCF membrane, less than 10% of control. At 15 min prior to the start of the experiment, the inserts were removed to empty wells and the A and B chambers were fed with pH 7.4 HBSS (37 °C).

After the pre-incubation, the solution was removed. The transport experiments in the A–B or B–A direction were initiated by adding either 0.4 ml (to the A chamber) or 0.6 ml (to B chamber) of gatifloxacin (20 μg/ml) or containing verapamil (10, 100 and 200 μM), and the other chamber was filled with drug-free HBSS. At 30, 60, 90 and 120 min incubation at 37 °C, 50 μl samples were taken from the acceptor compartment with replenishment by fresh HBSS. Each transport group consisted of 4 replicates, with each study repeated twice. The apparent permeability coefficient (P_{app}) for drug was calculated, according to the equation (Artursson, 1990).

$$P_{app} = \left(\frac{V_{acceptor}}{Area \times time}\right) \times \left(\frac{C_{acceptor}}{C_{initial,donor}}\right)$$

2.2.5. Effect of extracellular calcium and magnesium on gatifloxacin uptake

To investigate whether Ca^{2+} is involved in the uptake of gatifloxacin by rBMECs, the uptake experiments were operated in Ca^{2+}-deprived medium (Ca^{2+}-free HBSS either with or without 1 mM EGTA). The effect of extracellular Mg^{2+} on gatifloxacin by rBMECs was also investigated in presence of Mg^{2+}-depleted medium.

2.2.6. Influence of Ca^{2+} channel inhibitors on gatifloxacin uptake

The experiment was designed to investigate whether Ca^{2+} channel mediated gatifloxacin transport across BBB, gatifloxacin uptake by rBMECs at 120 min was measured in the absence or presence of inorganic Ca^{2+} channel blockers (Ni^{2+} and Mg^{2+}) and other two L-type Ca^{2+} channel antagonists (diltiazem and nifedipine).

2.2.7. Drug assays and statistical analysis

The concentrations of gatifloxacin in the rBMECs or in the Millicell acceptor compartments were measured by HPLC method (Liu et al., 2005). The sensitivity of the assay was 30 ng/ml and a good linearity was obtained from 30 to 500 ng/ml. Protein content in cultured cells was measured by the Bradford method (Bradford, 1976), using BSA as the standard. Net uptake, expressed as the ratio of drug concentration to protein concentration (ng/μg protein), was obtained by dividing the apparent amount of drug in the cells (ng/ml) by the amount of protein (μg/ml).

Analysis of variance (ANOVA) was used to test for differences among the groups. Post hoc analysis was carried out using the Duncan's multiple range test to test for significant difference among the means ($P<0.05$ or $P<0.01$ was considered as statistical significance). All results are expressed as mean ± standard deviation (S.D.).

3. Results

3.1. Gatifloxacin uptake by rBMECs

Time-, concentration- and temperature-dependent experiments were performed to assess the kinetics of gatifloxacin uptake by rBMECs. It was found that the course of the gatifloxacin uptake was time- and temperature-dependent (Fig. 1A). A plateau of accumulation was observed between 60 and 120 min. Lowering the temperature from 37 °C to 4 °C significantly decreased uptake of gatifloxacin by rBMECs, which resulted in decrease of gatifloxacin uptake by 85.0% at 120 min ($P<0.01$). Concentration-dependent study was carried out at 120 min incubation (steady-state uptake) using gatifloxacin concentrations from 10 to 100 μg/ml. The cellular accumulation of gatifloxacin increased parallel to the extracellular concentration over a wide range of concentrations (regression analysis: $r=0.999$; $P<0.001$) (Fig. 1B). Accordingly, gatifloxacin uptake by rBMECs at