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Differential uptake of grepafloxacin by human circulating blood neutrophils and those exudated into tissues

Masayuki Niwa [a,b,*], Koichi Hotta [a], Yutaka Kanamori [a], Hiroyuki Matsuno [a], Osamu Kozawa [a], Masao Hirota [c], Toshihiko Uematsu [a]

[a] *Department of Pharmacology, Gifu University School of Medicine, 40-Tsukasamachi, Gifu City 500-8705, Japan*
[b] *MEDC, Gifu University School of Medicine, 40-Tsukasamachi, Gifu City 500-8705, Japan*
[c] *Department of Drug Metabolism, Drug Safety Center, Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Tokushima 771-0130, Japan*

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Abstract

The uptake of the antimicrobial quinolone agent, grepafloxacin, both by human circulating blood neutrophils and by those exudated into tissues, was evaluated in vitro by comparing the intracellular drug concentrations. In circulating blood neutrophils, the uptake of grepafloxacin was rapid and saturable at 37 °C. The uptake of grepafloxacin into circulating blood neutrophils was reduced by lowering the environmental temperature or by the presence of metabolic inhibitors, suggesting the involvement of an active transport mechanism. Furthermore, the uptake of grepafloxacin by tissue (salivary) neutrophils was also partially temperature-dependent and was significantly greater than that by circulating blood neutrophils, i.e. exudation of neutrophils into tissue results in a markedly enhanced transport mechanism for grepafloxacin. This phenomenon may be related to the higher defense activity against infection seen in exudated tissue neutrophils.

Keywords: Fluoroquinolone; Neutrophil, human; Host defense; Grepafloxacin

1. Introduction

Fluoroquinolone derivatives are frequently used therapeutically to treat bacterial infections due to their highly potent, wide-spectrum anti-microbial activity combined with good tissue penetration (Uematsu and Nakashima, 1992; Child et al., 1995; Kozawa et al., 1996). These agents are widely acknowledged to display intracellular bioactivity against bacteria that reside and/or multiply within phagocytes (Havlichek et al., 1987; Fu et al., 1990; Nielsen et al., 1997; Wakebe and Hitsuhashi, 1992).

It has been reported that various fluoroquinolones penetrate into human neutrophils both in vitro (Pascual et al., 1989, 1997, 1999, 1999) and in vivo (Garraffo et al., 1991). Grepafloxacin is a newly developed fluoroquinolone antimicrobial agent (Imada et al., 1992) and is reported to possess a higher penetration ability into several tissues, such as liver (Sasabe et al., 1999) and lung (Cook et al., 1995), compared to other fluoroquinolones. The intraphagocytic uptake of grepafloxacin has been studied and compared with that of several other fluoroquinolones using a fluorometric method: fluoroquinolones accumulated in neutrophils to different extents, 66.2-fold greater concentration (intra- vs. extracellular) for grepafloxacin, 9.8-fold for levofloxacin and 7.6-fold for ofloxacin (Taira et al., 1993). However, the precise mechanism for the higher intracellular uptake of grepafloxacin is not yet known. Furthermore, controversial data exist concerning the presence of an active transport mechanism for fluoroquinolone uptake (Pascual et al., 1997, 1999).

Neutrophils migrate from peripheral blood into inflamed tissues to take part in the host defense reaction against infection, and in inflammatory and allergic reactions such as asthma. Recently, it has been found that many structural and functional changes take place in neutrophils in association with this migration (Niwa et al., 1996, 1997; Kanamori et al., 1997; Al-Essa et al., 1995). It is of interest to know if the cellular uptake of antimicrobial agents, such as fluoroquinolones, is modified in parallel with the structural and functional changes that occur during this migration.

* Corresponding author. Department of Pharmacology, Gifu University School of Medicine, 40-Tsukasamachi, Gifu City 500-8705, Japan. Tel.: +81-58-267-2233; fax: +81-58-267-2935.
E-mail address: mniwa@cc.gifu-u.ac.jp (M. Niwa).

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In the present study, we show firstly that grepafloxacin uptake into neutrophils involves an active process, and secondly, that grepafloxacin uptake is markedly greater in tissue neutrophils than in circulating neutrophils, which may explain, at least in part, the higher antimicrobial efficacy of grepafloxacin in tissue.

2. Materials and method

2.1. Materials

Grepafloxacin and 1-cyclopropyl-6,8-difluoro-1,4-dihydro-5-ethyl-7-(4-methyl-1-piperazinyl)-4-oxo-3-quinoline carboxylic acid (OPC-17203, Internal standard) were provided by Otsuka Pharmaceutical (Tokyo, Japan); ofloxacin and ciprofloxacin were purchased. Dextran (M.W. 208,000) and HEPES were purchased from Nacalai (Japan) and DOJIN (Japan), respectively. Other reagents used were purchased from Sigma (USA). All reagents used were endotoxin-free as determined by the limulus lysate assay, in which minimum detectable levels are 0.03 enzyme unit/ml.

2.2. Preparation of neutrophils

Human circulating blood neutrophils were isolated as previously described (Boyum, 1968) with minor changes (Niwa et al., 1995). Briefly, venous blood from healthy volunteers was collected on sodium citrate solution (3.8%), centrifuged ($110 \times g$, 10 min), and the platelet-rich plasma was discarded. The remaining part of the blood was mixed (1:1, v/v) with a solution of 3% dextran in 0.9% sodium chloride solution in a plastic syringe and fixed vertically for 20 min at 25 °C. Neutrophil-rich plasma was collected from the upper layer of the suspension and centrifuged ($250 \times g$, 10 min). The pellet was subjected to hypotonic lysis to destroy the remaining erythrocytes, centrifuged and then suspended in HBSS (Hank's Balanced Salt Solution containing 10 mM HEPES, pH 7.4). The suspension was cushioned carefully on Histopaque solution ($d = 1.077$) and centrifuged ($420 \times g$, 30 min) at 20 °C. The purified neutrophils in the pellet were resuspended in HBSS.

Human salivary neutrophils, which are considered to represent those exudated from blood to tissue, were collected from human saliva by nylon mesh filtration, followed by Histopaque centrifugation (Al-Essa et al., 1994). Briefly, intensive mouth washings with saline from each normal donor were collected through nylon mesh in 50-ml centrifuge tubes, centrifuged ($250 \times g$, 10 min) and the pellets were suspended in HBSS. Then the cell suspension was cushioned carefully on Histopaque ($d = 1.083$) and centrifuged ($420 \times g$, 30 min) at 20 °C. Pure salivary neutrophils were collected from the interface between Histopaque and HBSS, washed and resuspended again in HBSS.

The purity of each neutrophil population was greater than 95%. Cell number was counted by using a Coulter counter model ZM (Coulter, USA), and cells were diluted in HBSS to the final required concentrations and kept on ice until examined. To correct possible time-dependent changes in neutrophils, the time between the preparation of neutrophils and their use in experiments was adjusted for all experiments to be approximately 4 h.

2.3. Accumulation and determination of fluoroquinolones in neutrophils

A previously described high-performance liquid chromatography (HPLC)–fluorometric assay (Akiyama et al., 1995) was used to measure quinolone uptake by human circulating and salivary neutrophils. Neutrophils (5×10^6 cells) were incubated in HBSS containing different concentrations of fluoroquinolones (10–500 μM). After incubation for the indicated time, cells were separated from extracellular solution by centrifugation ($10{,}000 \times g$, 3 min) through a water-impermeable silicon–oil barrier (SH550/SH556, 1:4; Toray Dow Corning, Tokyo, Japan) in a microcentrifuge tube. The neutrophil pellet formed on the bottom of the microcentrifuge tubes, obtained by cutting off the portion of the microcentrifuge tube, was resuspended with methanol and agitated vigorously in a vortex shaker. The samples were then centrifuged at $21{,}600 \times g$ for 10 min, and the concentration of fluoroquinolones in the supernatant was determined by HPLC (Shimadzu, Kyoto, Japan) with a spectrofluorometer (Waters, USA). The fluorescence excitation and emission maxima of grepafloxacin in methanol are 285 and 448 nm, respectively.

The intracellular concentration of fluoroquinolones is expressed as picomoles per 10^6 neutrophils. A previously determined intracellular volume of 3.3×10^{-13} l (Simchowitz et al., 1993) was used to determine cellular-to-extracellular concentration ratios (C/E).

3. Results

3.1. Uptake of grepafloxacin by circulating blood neutrophils

The time course of grepafloxacin uptake by human circulating blood neutrophils was compared with that of ciprofloxacin and ofloxacin (Fig. 1). Grepafloxacin was rapidly taken up by neutrophils, reaching a maximum at ~5 min, irrespective of its extracellular concentration. Although the absolute intracellular amount of grepafloxacin was greater at the medial concentration of 200 μM than at 50 μM, the C/E ratio was similar at both concentrations (Fig. 1A).

Similar to grepafloxacin, ciprofloxacin (Fig. 1B) and ofloxacin (Fig. 1C) were also rapidly taken up by circulat-

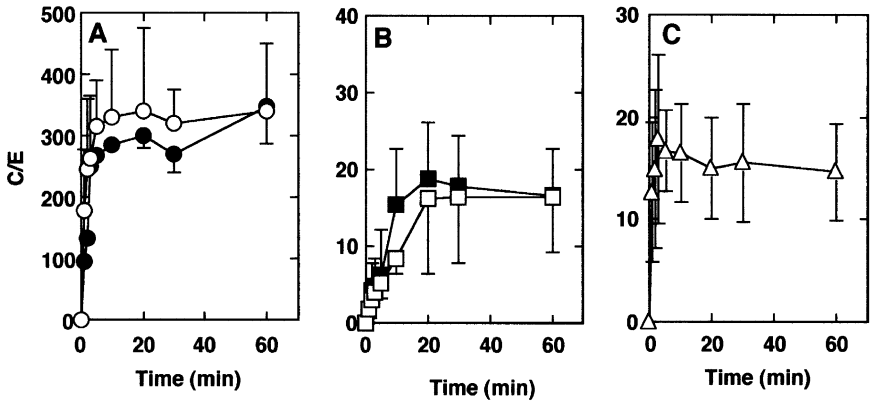

Fig. 1. Uptake of fluoroquinolones by human circulating blood neutrophils. Ratio of cellular to extracellular concentration (C/E) of fluoroquinolones. (A) grepafloxacin (○: 50 μM, ●: 200 μM); (B) ciprofloxacin (□: 50 μM, ■ 200 μM); (C) ofloxacin (△: 50 μM). Results are expressed as means ± S.D. of three to five experiments.

ing blood neutrophils, reaching a maximum at ~5 and ~10 min for ofloxacin and ciprofloxacin, respectively, within the concentration ranges tested (50 and 200 μM for ciprofloxacin; 50 μM for ofloxacin). However, the absolute amounts themselves and the C/E ratios were <1/10 less than those of grepafloxacin within the same concentration ranges.

3.2. Kinetic examination of circulating blood and tissue (salivary) neutrophils

The kinetics of the accumulation of grepafloxacin in circulating blood neutrophils were investigated by changing the concentrations of grepafloxacin in the incubation medium, to which neutrophils were exposed for 1 or 5 min. From these data, the speed at which neutrophils accumulated grepafloxacin was calculated for each extracellular concentration and plotted (Fig. 2). Results showed that the accumulation was mediated through a saturable process. By using the Eadie–Hofstee plot, K_m and V_{max} values for grepafloxacin accumulation in circulating blood neutrophils were calculated as 172.4 pmol/μl and 1528.3 pmol/min/10^6 cells, respectively.

It has been reported that tissue (salivary) neutrophils have a higher viability and exhibit a variety of functional responses when stimulated (e.g. production of active oxygen species) for a longer period than do circulating blood neutrophils (Yamamoto et al., 1991). It seems likely that the ability of tissue (salivary) neutrophils to take up drugs also differs from that of circulating blood neutrophils. To determine this possibility, the same kinetic procedure was applied to human tissue (salivary) neutrophils. Results indicate that grepafloxacin accumulation in tissue (salivary) neutrophils was significantly higher than that in circulating blood neutrophils (Fig. 2). K_m and V_{max} values for grepafloxacin accumulation in tissue (salivary) neutrophils were calculated as 542.8 pmol/μl and 5017.7 pmol/min/10^6 cells, respectively.

3.3. Effects of temperature on the uptake of grepafloxacin by circulating blood and tissue (salivary) neutrophils.

Grepafloxacin uptake by circulating blood neutrophils, determined after exposure to 50 μM grepafloxacin in the medium for 5 min, was dependent on the environmental temperature (Fig. 3). When the temperature was lowered from 37 to 25 °C, the intracellular concentration of grepafloxacin was significantly reduced, independent of its extracellular concentration. This reduction of cellular up-

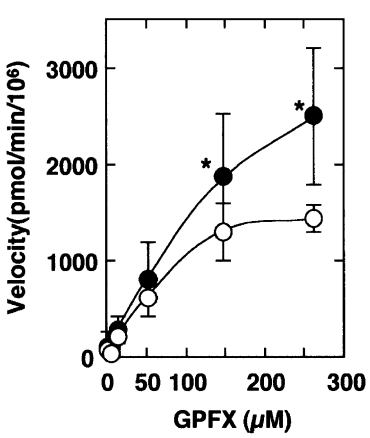

Fig. 2. Kinetics of grepafloxacin uptake by human circulating blood neutrophils and salivary neutrophils. ○: circulating blood neutrophils; ●: salivary neutrophils. Changes in velocity of cellular grepafloxacin accumulation. Each value was calculated from the following equation:

velocity (pmol/min/10^6 cells)
= (grepafloxacin accumulation at 5 min
− grepafloxacin accumulation at 1 min)/4 min.

The results are expressed as means ± S.D. of four to six independent experiments in duplicate. (*) Significant differences versus that in circulating blood neutrophils at $P < 0.05$ determined by analysis of variance (ANOVA) with Fisher's Protected Least-Significant Difference (Fisher's PLSD) test.

take was even more pronounced at 4 °C. In contrast, although grepafloxacin uptake by tissue (salivary) neutrophils was also significantly reduced when the temperature was lowered to 4 °C, the magnitude of the decrease was less than that observed in circulating blood neutrophils. Using these data, the activation energy of both types of neutrophils was calculated as described previously (Mtairag et al., 1995). Briefly, the temperature-dependent uptake of grepafloxacin was quantified by calculating the Arrhenius activating energy given in the equation: $\Delta G = -RT\ln Keq$, where ΔG is a constant (equal to 1.98), and ln Keq is the napierian logarithm of the C/E ratio at 5 min, at which time uptake reached a maximum. By transforming the energy in calories per mole to joules per mole and ln to log, the above equation becomes: $\Delta G = -1.98 \times 4.18 \times 2.3T$ logKeq. ΔG can be obtained from the slope of the curve by using the Van't Hoff plot representation of the data, finally yielding the following equation: $\log Keq = -[1/(4.18 \times 2.3 \times 1.98)]\Delta G(1/T)$. From this equation, the activation energy of circulating blood neutrophils and tissue (salivary) neutrophils was calculated as 62.4 ± 13.7 and 32.4 ± 27.3 kJ/mol, respectively. The activation energy of tissue (salivary) neutrophils was significantly lower than that of circulating blood neutrophils ($p < 0.05$, $n = 6$, calculated by Student's *t*-test).

3.4. Effect of extracellular Ca^{2+} and metabolic inhibitors on grepafloxacin uptake by circulating blood neutrophils

The effects of extracellular Ca^{2+} concentration and metabolic inhibitors on grepafloxacin uptake by circulating blood neutrophils are shown in Table 1. Grepafloxacin uptake by neutrophils was not affected by the presence or absence of extracellular Ca^{2+}. Among the inhibitors evaluated, sodium cyanide and carbonyl cyanide *m*-chlorophenyldrazone (which is also a proton ionophore) reduced the cellular uptake of grepafloxacin, while sodium fluoride and 2,4-dinitrophenol did not cause a significant reduction.

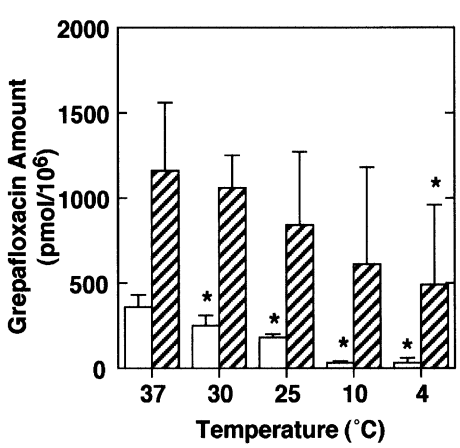

Fig. 3. Effect of temperature on grepafloxacin (50 μM) uptake for 5 min by human circulating blood and salivary neutrophils. Open column: circulating blood neutrophils; hatched column: salivary neutrophils. The results are expressed as means ± S.D. of four to six independent experiments in duplicate. (*) Significant differences versus that at 37 °C at $P < 0.05$ in each type of neutrophils determined by ANOVA with Fisher's PLSD test.

Table 1
Effect of extracellular Ca^{2+} or metabolic inhibitors on the uptake of grepafloxacin by human circulating blood neutrophils

Treatment	Concentration (mM)	Grepafloxacin amount (pmol/10^6)
$CaCl_2$	1.0	1340.35 ± 144.64
EGTA	1.0	1393.68 ± 235.17
Sodium fluoride	1.5	1229.04 ± 334.04
Sodium cyanide	1.5	1168.74 ± 133.41[a]
2,4-Dinitrophenol	1.0	1212.80 ± 143.34
CCCP	0.15	934.53 ± 77.41[a]

Cellular uptake was determined after a 5-min exposure of neutrophils to incubation medium containing 50 μM grepafloxacin and is expressed as grepafloxacin amount (pmol/10^6 cells).
Mean ± S.D., $n = 8$.
CCCP: carbonyl cyanide *m*-chlorophenyldrazone.
[a] Significant differences versus control ($CaCl_2$) at $P < 0.05$.

4. Discussion

The results of our study indicate that the uptake of grepafloxacin, a newly developed fluoroquinolone, by human neutrophils was significantly higher than that of two other fluoroquinolones that we studied, ciprofloxacin and ofloxacin. Our data suggest that the high cellular uptake of grepafloxacin involves both an active transport system and an energy-independent passive transport system. In addition, grepafloxacin uptake by neutrophils exudated into tissue was significantly greater than that by neutrophils in circulating blood.

The uptake of grepafloxacin by circulating blood neutrophils was rapid and saturable. It was markedly inhibited by lowering environmental temperature and by metabolic inhibitors (sodium cyanide and carbonyl cyanide *m*-chlorophenyldrazone), as has been described for ofloxacin (Pascual et al., 1989), moxifloxacin (Pascual et al., 1999) and BAY Y 3118 (1-cyclopropyl-7-(2,8-diazabicyclo[4.3.0] non-8-yl)-6-fluoro-8-chloro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid hydrochloride, Garcia et al., 1994). Furthermore, the activation energy for grepafloxacin was higher than that for levofloxacin, which is taken up by cells through a passive transport system (Vazifeh et al., 1999). Also, the uptake of sparfloxacin (Garcia et al., 1992) and trovafloxacin (Pascual et al., 1997) is not inhibited by either metabolic inhibitors or hypothermia. Interestingly, the uptake of trovafloxacin by neutrophils is greatly enhanced by hypothermia. While the mechanisms underlying the cellular uptake of fluoroquinolones are not yet fully understood, this evidence suggests that multiple mechanisms are involved. Furthermore, the intracellular concen-

tration of grepafloxacin is dependent on the extracellular pH, that is, the grepafloxacin concentration is high at a high pH and is low at a low pH (Taira et al., 1993). This has important implications for the uptake of grepafloxacin at sites of inflammation, where the pH is greatly reduced.

The V_{max} and K_m values of grepafloxacin uptake in circulating blood neutrophils were 1528.3 pmol/min/10^6 cells and 172.4 pmol/μl, respectively. This K_m value of grepafloxacin is about one-fourth of that of ciprofloxacin and the V_{max} is about 20 times that of ciprofloxacin (Walters et al., 1999), which indicates a higher uptake of grepafloxacin compared to ciprofloxacin by circulating blood neutrophils. Interestingly, the V_{max} and K_m values for grepafloxacin uptake by tissue (salivary) neutrophils were 5017.7 pmol/min/10^6 cells and 524.8 pmol/μl, respectively. These values indicate that tissue (salivary) neutrophils have an extremely high grepafloxacin uptake that is significantly greater than that seen in circulating blood neutrophils. To our knowledge, there have been no previous reports evaluating fluoroquinolone accumulation in neutrophils exudated into tissue. The results of the concentration-dependent grepafloxacin uptake experiments suggest that the grepafloxacin uptake mechanism in tissue (salivary) neutrophils is not identical to that in circulating neutrophils. Furthermore, in tissue (salivary) neutrophils, grepafloxacin uptake was not completely inhibited when the environmental temperature was lowered, unlike that in circulating blood neutrophils, in which a lower temperature almost completely abolished grepafloxacin uptake. The activation energy in tissue (salivary) neutrophils was significantly smaller than that in circulating blood neutrophils. These results indicate that grepafloxacin uptake may use two different mechanisms: an active transport system and an energy-*independent* passive transport system. In tissue (salivary) neutrophils, the energy-dependent active transport mechanism contributes to a much lesser extent than it does in circulating blood neutrophils. The present results suggest that tissue neutrophils efficiently take up grepafloxacin from extracellular fluid, even at very low concentrations, and concentrate it, thereby playing a significant role in the host defense against bacterial infection.

The concentration of grepafloxacin that we used in these studies may be higher than the serum concentration of grepafloxacin after clinical doses. It has been reported that the serum concentration of grepafloxacin after oral administration (400 mg) is 1.4 mg/l (3.9 μM) (Pitlick et al., 1991). Cook et al. (1995) also reported a mean serum concentration of grepafloxacin of 1.2 mg/l, but there was a significantly higher concentration of grepafloxacin in bronchial mucosa (mean ratio 3.13 compared to plasma) and in epithelial lining fluid (mean ratio 12.2 compared to plasma), giving an estimated grepafloxacin concentration in these sites of 12.2 and 47.6 μM, respectively. Thus, the concentrations we used in our studies are likely to be seen in certain tissues following clinical doses of grepafloxacin.

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