Gemfibrozil Enhances the Listeriacidal Effects of Fluoroquinolone Antibiotics in J774 Macrophages

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Summary

J774 macrophage–like cells express organic anion transporters that promote the efflux of fluoroquinolone antibiotics such as norfloxacin (NFX) from these cells. Gemfibrozil (GFZ) blocks organic anion transport in J774 cells, thereby facilitating the intracellular accumulation of NFX (Cao, C., H.C. Neu, and S.C. Silverstein. 1991. *J. Cell Biol.* 115:467*a* [Abstr.]). To determine whether GFZ enhances the efficacy of fluoroquinolone antibiotics against intracellular bacterial pathogens, J774 cells were infected with *Listeria monocytogenes* and incubated in medium containing a fluoroquinolone antibiotic in the presence or absence of GFZ. Intracellular growth of *L. monocytogenes* was evaluated by lysing J774 cells and assaying for colony-forming units of *Listeria*. GFZ intensified the bacteriostatic effect of 4 μg/ml NFX and rendered 8 μg/ml bactericidal for *L. monocytogenes*. GFZ had a similar potentiating effect when used in combination with 2 μg/ml ciprofloxacin (CFX). CFX plus GFZ was bactericidal for intracellular *L. monocytogenes*. Treatment of J774 cells with NFX plus GFZ markedly reduced the cytotoxic effect of the bacteria on these cells. Over 55% of cells treated with 8 μg/ml NFX alone were dead 16 h after infection, whereas only 5% of cells treated with 8 μg/ml NFX plus GFZ were dead at 16 h. Similarly, GFZ potentiated the ability of 2 μg/ml to protect J774 cells against the cytocidal effect of *Listeria*. NFX in combination with GFZ limited cell-to-cell spread of *L. monocytogenes*. In antibiotic-free medium, >99% of J774 cells contained intracellular *L. monocytogenes* at 14 h after infection. NFX alone in the medium did not change this outcome. However, 4 μg/ml NFX plus GFZ decreased bacterial spread by approximately 40% at 24 h postinfection, and 8 μg/ml NFX plus GFZ prevented all spread beyond the initially infected cell population. These results suggest that GFZ could be used clinically to enhance the efficacy of fluoroquinolone and of other anionic antibiotics against bacteria that grow and/or reside within macrophages and/or other cells.

Bacteria that grow intracellularly, such as *Legionella*, *Mycobacteria*, *Salmonella*, and *Listeria*, are difficult to eradicate with antimicrobial agents. There are many reasons for the resistance of these organisms to antimicrobial agents, one of which is lack of drug availability within the animal cells in which the bacteria grow. Some antibiotics penetrate animal cells poorly. Other agents are substrates for membrane transporters that promote drug efflux from cells. Steinberg et al. (1) described a transport activity in mouse macrophages and cells of the J774 macrophage–like cell line that promotes secretion of anionic organic compounds from these cells. Cao et al. (2) showed that membrane-permeant organic anions, such as the fluoroquinolone antibiotics norfloxacin (NFX)[1] and temofloxacin (3), are substrates for organic anion transporters in J774 cells and that inhibitors of these transporters promote retention of fluoroquinolone antibiotics in these cells. Carlier et al. (4) reported that fluoroquinolone antibiotics accumulate in the cytoplasmic matrix of J774 cells.

While these studies showed that inhibitors of organic anion transport increased the retention of fluoroquinolone antibiotics in the cytoplasm of macrophages, they did not address the question of whether the resulting increase in intracellular concentration of these antibiotics had any effect upon their efficacy against bacterial pathogens. Therefore, we sought to test the hypothesis that by increasing the intracellular concentration of fluoroquinolone antibiotics, organic anion transport inhibitors would increase the antimicrobial efficacy of these drugs.

A major impediment to testing this hypothesis was our finding that prolonged treatment (>8 h) of J774 cells with previously identified inhibitors of organic anion transport,

[1] *Abbreviations used in this paper:* BHI, brain heart infusion; CFX, ciprofloxacin; GFZ, gemfibrozil; MBC, minimal bactericidal concentration; NFX, norfloxacin.

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such as probenecid or sulfinpyrazone, at concentrations sufficient to block transport in these cells was toxic for the cells. A way to surmount this difficulty became apparent when Cao et al. (5) discovered that gemfibrozil (GFZ), an agent used to lower plasma lipoproteins, is nearly as effective as probenecid or sulfinpyrazone as an inhibitor of organic anion transport in J774 cells, but at a 10–30-fold lower concentration (i.e., 0.2–0.5 mM). Most importantly, at 0.2–0.5 mM, GFZ lacks the toxicity associated with probenecid or sufinpyrazone. Cells incubated in the presence of GFZ for days to weeks showed no loss of viability or decrease in growth rate. Thus, GFZ made it possible to test the hypothesis that by enhancing intracellular accumulation of fluoroquinolone antibiotics, inhibitors of organic anion transport would increase the efficacy of these antibiotics against bacterial pathogens. We selected *Listeria monocytogenes* as the microbial pathogen with which to examine this hypothesis because it grows readily in J774 cells (6), because it grows within the cytoplasmic matrix, the same cellular compartment in which fluoroquinolone antibiotics are located in these cells (4), and because it spreads efficiently from cell to cell without passing through the extracellular medium (6). A preliminary report of these studies has been published (7).

Materials and Methods

Bacterial Strains and Growth Conditions. *L. monocytogenes*, serotype 1 (strain 10403S), provided by Dr. D. Portnoy (University of Pennsylvania, Philadelphia, PA), was grown to stationary phase (10^9 CFU/ml) in 1 ml of brain heart infusion (BHI) broth (Difco Laboratories, Inc., Detroit, MI) overnight at 37°C in a shaking water bath. Bacteria were sedimented in a microfuge (USA Scientific Plastics, Ocala, FL) for 1 min at 8,000 rpm. The supernatant was removed and the bacteria were resuspended in 1 ml PBS (Dulbecco's phosphate-buffered solution without divalent cations) at pH 7.4, centrifuged again at 8,000 rpm, and resuspended in 1 ml of DMEM (Life Technologies, Inc., Grand Island, NY) containing 5% heat-inactivated (HI) (56°C for 30 min) FCS. Stock cultures were kept as suspensions of cells at −20°C in Todd Hewitt Broth (BBL Microbiological Systems, Becton Dickinson & Co., Cockeysville, MD) containing 5% sheep blood and 5% glycerol.

J774 Cells and Growth Medium. J774 macrophage–like cells were grown at 37°C in spinner culture in DMEM supplemented with 10% HI-FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin, centrifuged for 5 min at 1,000 rpm, and resuspended in DMEM containing 5% HI-FCS, but without antibiotics, at 10^6 cells/5 ml. 12, 12 × 1-mm sterile round coverslips (Fischer Scientific Co./Erie Scientific Co., Portsmouth, NH) were placed in each 60-mm tissue culture dish. 10^6 J774 cells in 5 ml of antibiotic-free medium were added and incubated overnight in a CO_2 incubator at 37°C. These coverslip cultures of adherent J774 cells were used for experiments with *L. monocytogenes*.

Infection and Intracellular Growth of L. monocytogenes in J774 Cells. Infection and assessment of intracellular growth of *L. monocytogenes* in J774 cells were done essentially as described by Portnoy et al. (8). *Listeria* grown overnight in BHI broth were resuspended in DMEM-5% HI-FCS without antibiotics at 2 × 10^5 CFU/ml. 5 ml of this bacterial suspension was added to each 60-mm tissue culture dish containing J774 cells on coverslips and 5 ml of antibiotic-free DMEM-5% HI-FCS. This yielded a ratio of *Listeria* to J774 cells of ~1:1.

The J774 cells were incubated with *L. monocytogenes* at 37°C for 1 h. The coverslips then were removed, placed in fresh 60-mm dishes containing 10 ml of DMEM-5%HI-FCS (supplemented with gentamicin [5 μg/ml] to kill extracellular *L. monocytogenes*), and incubated in this medium at 37°C for an additional 1 h. GFZ, a fluoroquinolone antibiotic, or both drugs then were added as indicated. Three coverslips bearing *Listeria*-infected J774 cells were harvested from each culture dish at the times indicated (zero time is addition of *L. monocytogenes* to the J774 cells). The number of bacteria in the J774 cells on each coverslip was determined by depositing each coverslip into 5 or 10 ml of sterile distilled water in a 15-ml conical tube (Falcon Labware, Oxnard, CA), vortexing the tube for 15 s to lyse the J774 cells, diluting the *Listeria*-containing solution in sterile water, and plating appropriate samples on 10-cm petri dishes containing 20 ml of Lennox L Base Broth (LB) agar. The LB agar plates were incubated at 37°C overnight, the bacterial colonies counted, and the number of bacteria in the J774 cells on each coverslip was calculated from the number of colonies on the agar. Each data point represents the average of the number of bacterial colonies recovered from three coverslip cultures. All experiments reported were repeated on three or more occasions and yielded similar results on each occasion.

Effects of GFZ and Fluoroquinolone Antibiotics on Extracellular Listeria Growth. GFZ and/or NFX were added, where indicated, to 8-ml samples of BHI broth containing 5 × 10^7 CFU/ml of *L. monocytogenes*, and the samples were incubated at 37°C. At the indicated times, a 1-ml aliquot was removed from each tube and its OD at 600 nm was assayed in a spectrophotometer (Spektralphotometer model PM 6; Zeiss, Germany). Bacterial growth was plotted as change in OD at 600 nm. Turbidometric analyses of extracellular bacterial growth also were performed by incubating *Listeria* in DMEM-5% HI-FCS without phenol red.

Susceptibility of L. monocytogenes to Killing by Quinolone Antibiotics Extracellularly. *L. monocytogenes* grown in overnight culture in BHI was resuspended in DMEM-5% HI-FCS at 5 × 10^5 CFU/ml. 1 ml of this bacterial suspension was added to 1 ml of DMEM-5% HI-FCS containing varying concentrations of NFX or CFX as indicated. The samples were incubated 18–20 h at 37°C. The minimum concentration of drug required to inhibit *Listeria* growth after the defined incubation period (MIC) was determined by visual inspection of the medium for turbidity. The MIC was 4 μg/ml for NFX and 1–2 μg/ml for CFX.

To identify the minimum concentration of drug required to achieve killing of the bacteria, *Listeria*-containing samples, prepared as described above in DMEM-5% HI-FCS containing varying concentrations of fluoroquinolone antibiotics, were incubated at 37°C for 18–20 h, at which time 10-μl aliquots of each sample were plated onto LB agar. The LB agar plates were incubated at 37°C overnight. The minimal bactericidal concentration (MBC) of the antibiotic was the dose at which there was a 99.9% reduction in CFU. The MBC was 32 μg/ml for NFX and 16 μg/ml for CFX.

Viability of Listeria-infected J774 Cells. For each determination, three coverslip cultures of *Listeria*-infected J774 cells were treated as indicated; at the appropriate time each coverslip was placed in a well of a four-well plate (4 Well Multidish; Nunclon-Intermediate, Denmark) containing 0.4% trypan blue in 400 μl of PBS. Each coverslip was immediately examined by phase contrast microscopy using a microscope with an ocular that defines a 1-mm^2 field. The total number of J774 cells/mm^2 and the number of J774 cells stained with trypan blue were counted in 5–10 1-mm^2 areas per coverslip, and the percent of viable cells was calculated. Viability of uninfected J774 cells was ~98%.

Visualization of Listeria monocytogenes in J774 Cells. As described

above, coverslips (12 × 1 mm) bearing confluent monolayers of J774 cells were incubated with *Listeria* at a ratio of 1 or 5 CFU per J774 cell. At the indicated times after infection, three coverslips were harvested for each experimental condition, stained (Diff-Quik Stain Set; Baxter Scientific Products, Dade Division, Miami, FL), and examined by light microscopy. 25 high-power fields were examined per coverslip, and the percentage of J774 cells containing one or more *Listeria* was evaluated.

Results

Cao et al. (2) reported that inhibitors of organic anion transport facilitate accumulation of fluoroquinolone antibiotics by J774 cells. In those studies, Cao et al. (2) used probenecid or sulfinpyrazone to block organic anion transport. Preliminary studies showed that treatment of J774 cells for >8 h with these drugs at concentrations that maximally block organic anion secretion reduced the viability of the J774 cells. Recently, Cao et al. (5) reported that GFZ, an agent that lowers plasma lipoproteins, inhibits organic anion transport in J774 cells. GFZ enhanced the intracellular accumulation of radiolabeled NFX at an ED_{50} of 15 μM, which is 30-fold lower than the ED_{50} of probenecid or sulfinpyrazone required to achieve a similar effect. At 0.2–0.5 mM, GFZ maximally promoted intracellular accumulation of NFX, but had no deleterious effect on viability or growth of J774 cells (5). Therefore, we used 0.2 mM GFZ to inhibit antibiotic efflux in all experiments reported here.

GFZ Potentiates the Inhibitory Effect of Fluoroquinolones on Intracellular Growth of L. monocytogenes. J774 cells on coverslips were incubated with *L. monocytogenes* at a ratio of 1 CFU *Listeria* per J774 cell for 1 h at 37°C, at which time gentamicin was added to the medium to eliminate extracellular bacteria. Previous work by Portnoy et al. (8) confirmed that under these conditions gentamicin does not affect intracellular growth of *Listeria*. 2 h after infection, J774 cells were treated with 0.2 mM GFZ alone, 2–8 μg/ml NFX alone, or 0.2 mM GFZ in combination with NFX at the concentrations indicated. Intracellular growth of *Listeria* was monitored at 2, 6, and 16 h.

NFX alone, at concentrations of 2, 4, or 8 μg/ml, had little effect on the rate of growth of *L. monocytogenes* intracellularly during the first 6 h postinfection, but did reduce the maximal growth of *L. monocytogenes* at 16 h by ~0.5 log (e.g., from 10^6 to 5 × 10^5 CFU). GFZ significantly enhanced the efficacy of NFX against intracellular *L. monocytogenes* at all doses of NFX tested (see Fig. 1, *A* and *B*; 2 μg/ml NFX; data not shown). The most pronounced effects were observed in cells treated with 4 or 8 μg/ml NFX plus GFZ. In combination with GFZ, 4 μg/ml NFX slowed *Listeria* growth at 6 h and blocked further bacterial growth between 6 and 16 h (Fig. 1 *A*), while GFZ rendered 8 μg/ml NFX bactericidal for intracellular *Listeria* (Fig. 1 *B*). J774 cells treated with GFZ plus 8 μg/ml NFX contained 500–1,000-fold fewer *Listeria* at 16 h than cells treated with either drug alone. Most importantly, cells treated with this combination of drugs contained 20-fold fewer *Listeria* at 16 h than at 2 h (Fig. 1 *B*), indicative of killing of the bacterial inoculum. Control experiments showed that GFZ alone at concentrations of 0.2 or 0.4 mM had no effect on the rate or extent of *L. monocytogenes* growth in J774 cells (Fig. 1 *C*).

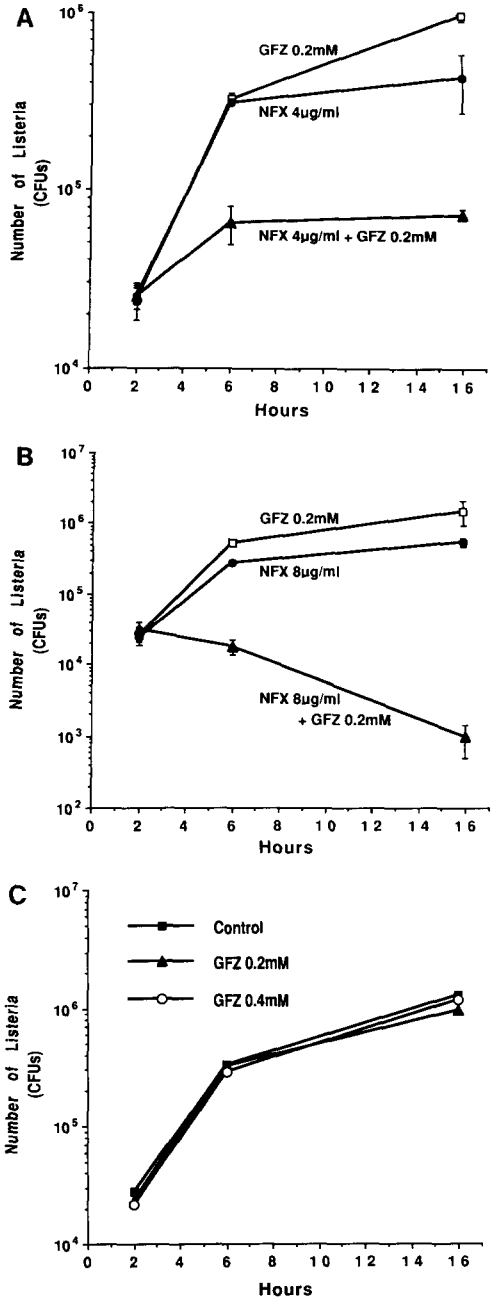

Figure 1. GFZ increases the efficacy of NFX against intracellular *L. monocytogenes* but does not itself affect intracellular growth of the bacteria in J774 cells. J774 cells on coverslips were incubated with *Listeria* at 1 CFU per J774 cell for 2 h, as described in Materials and Methods and in the text. At 2 h, GFZ (0.2 or 0.4 mM), NFX (4 or 8 μg/ml), or GFZ plus NFX were added to the medium. Control cultures contained no drugs. Intracellular growth was monitored over 16 h as described in Materials and Methods. 4 μg/ml NFX (*A*), 8 μg/ml NFX (*B*), or 0.2–0.4 mM GFZ vs. no drug (*C*). These experiments are representative of three or more experiments, all yielding similar results.

To determine the amount of NFX alone required to kill

the same number of intracellular *Listeria* as the combination of 8 μg/ml NFX and GFZ, we incubated infected J774 cells with medium containing 16, 32, or 64 μg/ml NFX for 16 h and monitored bacterial growth. A concentration of 32 μg/ml of NFX was necessary to achieve the 500–1,000-fold reduction in *Listeria* at 16 h seen with 8 μg/ml NFX in combination with GFZ (Fig. 2). Thus, GFZ enhanced the efficacy of NFX fourfold.

To establish that GFZ potentiates the intracellular effect of fluoroquinolones other than NFX, we studied the effect of ciprofloxacin (CFX) on intracellular growth of *L. monocytogenes*. Used alone, 1–2 μg/ml CFX decreased the maximal intracellular growth of *Listeria* at 16 h by ~0.5 log (e.g., from 10^6 to 5 × 10^5 CFU). GFZ markedly intensified the inhibitory effect of 1–2 μg/ml CFX on intracellular *Listeria* (Fig. 3, *A* and *B*). In J774 cells treated with 1 μg/ml CFX plus GFZ, *Listeria* growth was slowed between 2 and 6 h and further growth was prevented between 6 and 16 h (Fig. 3 *A*). GFZ transformed 2 μg/ml CFX into a bactericidal agent for *Listeria* in J774 cells (Fig. 3 *B*). J774 cells incubated with GFZ plus 2 μg/ml CFX contained ~500–1,000-fold fewer *Listeria* at 16 h than cells treated with either drug alone. In addition, cells treated with this drug combination contained 20-fold fewer *Listeria* at 16 h than at 2 h, demonstrating killing of 95% of the initial bacterial inoculum. In the absence of GFZ, >8 μg/ml CFX was required to produce a bactericidal effect on intracellular *L. monocytogenes* that was similar in magnitude to the combination of 2 μg/ml CFX plus GFZ (Fig. 4). Thus, as observed with NFX, GFZ potentiates the inhibitory effect of CFX on intracellular growth of *L. monocytogenes*.

GFZ Does Not Potentiate the Effect of NFX on Extracellular Growth of L. monocytogenes. To determine whether GFZ affects extracellular growth of *Listeria*, the growth of *Listeria* in BHI broth containing 0.1–0.8 mM GFZ was monitored. There was no significant difference in the rate or extent of *Listeria* growth in control medium vs. medium containing 0.1–0.4 mM GFZ. GFZ at 0.8 mM minimally reduced both

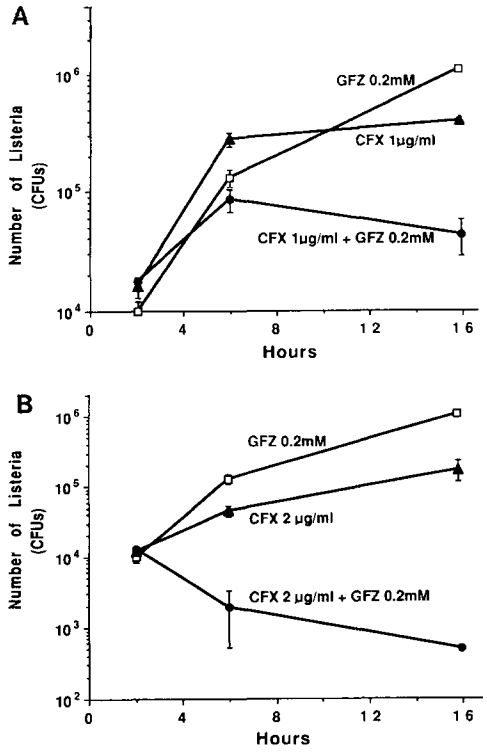

Figure 3. GFZ enhances the efficacy of CFX against intracellular *L. monocytogenes*. J774 cells on coverslips were incubated with *Listeria* at 1 CFU per J774 cell in the presence of GFZ (0.2 mM) alone, CFX (1 or 2 μg/ml) alone, or GFZ plus CFX. Intracellular growth of *Listeria* was monitored as described in Fig. 1 and Materials and Methods. 1 μg/ml CFX (*A*) or 2 μg/ml CFX (*B*). These experiments are representative of three or more experiments, all yielding similar results.

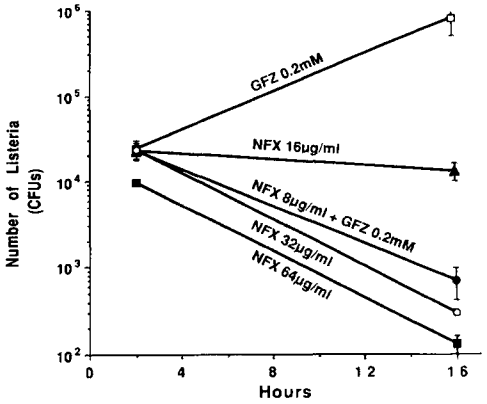

Figure 2. NFX (8 μg/ml) plus GFZ (0.2 mM) is as effective in killing *Listeria* intracellularly as NFX (32 μg/ml) alone. Monolayers of J774 cells on coverslips were incubated with *Listeria* at 1 CFU per J774 cell. At 2 h, fresh medium was added containing GFZ (0.2 mM) alone, NFX (16, 32, or 64 μg/ml) alone, or NFX (8 μg/ml) plus GFZ (0.2 mM) as indicated. Intracellular bacterial growth was monitored as described Materials and Methods. This experiment is representative of three experiments, all yielding similar results.

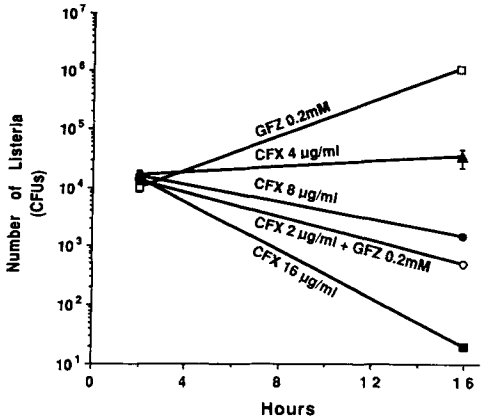

Figure 4. CFX (2 μg/ml) plus GFZ (0.2 mM) is more effective in killing *Listeria* intracellularly than CFX (8 μg/ml) alone. Monolayers of J774 cells on coverslips were incubated with *Listeria* at 1 CFU per J774 cell. At 2 h, fresh medium was added containing GFZ (0.2 mM) alone, CFX (4, 8, or 16 μg/ml) alone, or CFX (2 μg/ml) plus GFZ (0.2 mM) as indicated. Intracellular bacterial growth was monitored as described in Materials and Methods. This experiment is representative of two experiments, both of which gave similar results.

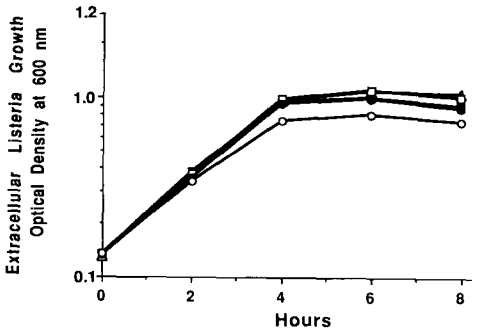

Figure 5. Extracellular growth of *L. monocytogenes* in medium containing GFZ. Growth of *Listeria* in BHI broth at 37°C was monitored by measuring the OD of the medium at 600 nm as described in Materials and Methods. 0.1 mM GFZ (open triangles), 0.2 mM GFZ (□), 0.4 mM GFZ (●), 0.8 mM GFZ (○), and control without GFZ (■). This experiment is representative of three or more experiments, yielding similar results.

the rate and extent of *Listeria* growth (Fig. 5). Thus, the capacity of 0.2 mM GFZ to potentiate the effect of NFX on intracellular growth of *L. monocytogenes* cannot be ascribed to an inhibitory effect of GFZ on *L. monocytogenes* growth. Similar experiments were performed to determine whether GFZ potentiates the effect of NFX on extracellular growth of *Listeria*. Regardless of the concentration of NFX used, GFZ had no effect on the rate or final yield of *Listeria* (Fig. 6, *A* and *B*).

The effects of GFZ and NFX, alone or in combination, on extracellular *Listeria* growth in DMEM-5% HI-FCS also were examined. As noted with BHI broth, GFZ had no effect on *Listeria* growth and did not potentiate the effect of NFX on extracellular growth of the bacteria (data not shown).

These findings demonstrate that GFZ potentiates the effect of NFX and CFX only on intracellular *Listeria*, suggesting that this effect is due to the ability of GFZ to block organic anion transport in J774 cells, thereby increasing intracellular accumulation of the fluoroquinolones.

GFZ plus NFX or CFX Prevents the Cytotoxic Effect of L. monocytogenes on J774 Cells. To evaluate whether the combination of GFZ and NFX or CFX protects J774 cells from the cytocidal effect of *L. monocytogenes*, we examined the effect of these drugs on the viability of *Listeria*-infected J774 cells. After a 2-h incubation with *Listeria* at 37°C, 98% of J774 cells excluded trypan blue. This is the same percentage viability observed with uninfected J774 cells. In contrast, at 16 h after infection with 1 CFU *Listeria* per J774 cell, only 42% of J774 cells remained viable. Cell viability decreased to 24% by 24 h after infection. NFX (2, 4, or 8 μg/ml) alone did not significantly alter this outcome (Fig. 7). GFZ added to 2 μg/ml NFX minimally prevented the decrease in cell viability observed in *Listeria*-infected J774 cells at 24 h. However, GFZ in addition to 4 or 8 μg/ml NFX had a significant cytoprotective effect on *Listeria*-infected J774 cells. At least 75% of J774 cells incubated with this drug combination re-

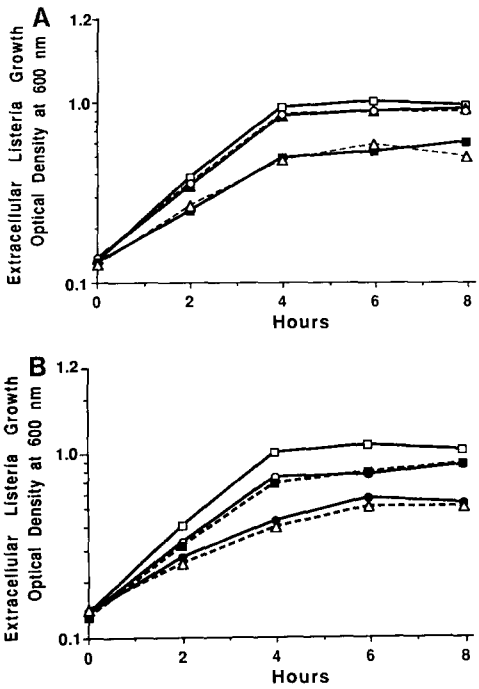

Figure 6. GFZ does not enhance the effect of NFX on extracellular growth of *L. monocytogenes*. BHI broth containing 5 × 10^7 CFU/ml of *Listeria* was incubated at 37°C with varying concentrations of NFX in the presence or absence of GFZ (0.2 mM). *Listeria* growth was monitored by measuring the OD of the medium at 600 nm as described in Fig. 5 and Materials and Methods. This experiment is representative of three or more experiments yielding similar results. (*A*) 2 μg/ml NFX (▲), 2 μg/ml NFX and 0.2 mM GFZ (--○--), 8 μg/ml NFX (■), 8 μg/ml NFX and 0.2 mM GFZ (--△--), and control in drug-free medium (□). (*B*) 4 μg/ml NFX (○), 4 μg/ml NFX and 0.2 mM GFZ (--■--), 16 μg/ml NFX (●), 16 μg/ml NFX and 0.2 mM GFZ (--△--), and control (□).

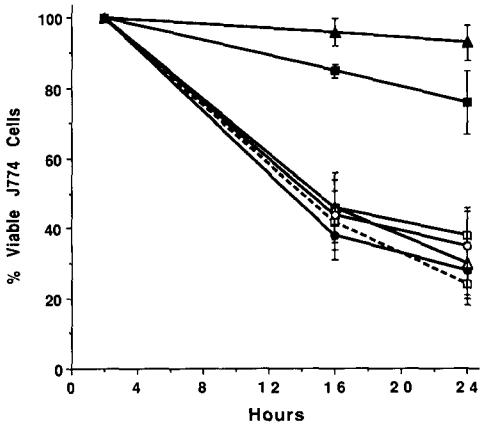

Figure 7. GFZ enhances the capacity of NFX to protect J774 cells against the cytotoxic effect of *Listeria* infection. J774 cells on coverslips were incubated with *Listeria* at a ratio of 1:1 for 2 h at 37°C, at which time drugs were added to the medium as indicated. Coverslip cultures were harvested and stained with trypan blue at 2, 16, and 24 h as described in Materials and Methods, and the percentage of cells stained with trypan blue was calculated. This experiment is representative of four experiments, yielding similar results. 2 μg/ml NFX (●), 2 μg/ml NFX and 0.2 mM GFZ (□), 4 μg/ml NFX (△), 4 μg/ml NFX and 0.2 mM GFZ (■), 8 μg/ml NFX (○), 8 μg/ml NFX and 0.2 mM GFZ (▲), and control with 0.2 mM GFZ (--□--).

mained viable at 16 and 24 h, a greater than threefold reduction in the number of dead J774 cells compared with cultures incubated with GFZ or NFX alone. In the absence of GFZ, the minimum concentration of NFX in the medium that protected J774 cells against the cytocidal effect of intracellular *L. monocytogenes* was 32 μg/ml (Fig. 8).

To determine whether the percentage of trypan blue–stained cells was increasing because the total number of cells on each coverslip was diminishing, the total number of cells remaining on each coverslip was evaluated by phase contrast microscopy. The total number of J774 cells/mm^2, counted at 2, 16, and 24 h after infection, was constant, regardless of the drug combination present in the medium (data not shown). Thus, *Listeria*-infected J774 cells were not released from the coverslips.

To determine the length of time J774 cells can be infected with *L. monocytogenes* without sustaining lethal injury, we added 8 μg/ml NFX plus GFZ at varying times after infection. We found that cells treated with these drugs >3 h postinfection were not rescued from the cytocidal effects of *Listeria* (data not shown).

Thus, 4 and 8 μg/ml NFX in the presence of GFZ, drug combinations that significantly inhibit intracellular bacterial growth in the first 6 h after infection (Fig. 1, *A* and *B*), also provide protection against the cytocidal effect of *Listeria*. These drugs must be given within 3 h after infection to exert this protective effect.

Similar experiments were performed with CFX and GFZ. J774 cells were incubated with *L. monocytogenes* at a 1:1 ratio and 1–32 μg/ml CFX alone, or 1 or 2 μg/ml CFX plus GFZ were added to individual culture dishes. The viability of the J774 cells at 2 and 16 h postinfection was monitored.

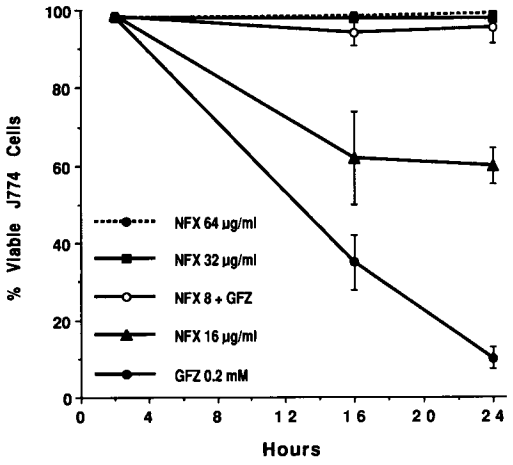

Figure 8. NFX (8 μg/ml) plus GFZ (0.2 mM) is as effective in protecting J774 cells against the cytotoxic effect of *Listeria* infection as NFX (32 μg/ml) alone. J774 cells on coverslips were incubated with *Listeria* at a ratio of 1:1 for 2 h at 37°C, at which time NFX (16, 32, and 64 μg/ml) alone, NFX (8 μg/ml) plus GFZ (0.2 mM), or GFZ (0.2 mM) alone were added to the medium as indicated. Coverslip cultures were harvested and stained with trypan blue at 2, 16, and 24 h after infection as described in Fig. 7 and Materials and Methods, and the percentage of cells stained with trypan blue was calculated. This experiment is representative of three experiments yielding similar results.

2 h after infection, ≥98% of J774 cells were viable. GFZ plus 1 μg/ml CFX had a pronounced cytoprotective effect, yielding 67% viable cells at 16 h vs. 19% viable cells treated with 1 μg/ml CFX alone (Table 1). 97% of cells treated with GFZ and 2 μg/ml CFX were viable at 16 h, indicating almost complete protection from the cytocidal effect of *Listeria*. 8 μg/ml CFX alone was required to provide a comparable cytoprotective effect (Table 1). 16–32 μg/ml CFX yielded the same percent viability of J774 cells at 16 h after infection as 8 μg/ml CFX (data not shown). Thus, as with NFX, GFZ increased the potency of CFX fourfold, and together with CFX protected J774 cells against the cytocidal effects of *L. monocytogenes*.

GFZ plus NFX Blocked Spread of L. monocytogenes from Cell to Cell. To determine whether NFX plus GFZ limited cell-to-cell transmission of *L. monocytogenes*, J774 cells were incubated with *Listeria* at 1 or 5 CFU per cell. By 2 h after infection, 20% of cells incubated with 1 CFU per cell and 40% of cells incubated with 5 CFU per cell contained at least one bacterium (Table 2). Addition of 0.2 mM GFZ or 4 or 8 μg/ml NFX alone to the medium at 2 h had no effect on cell-to-cell spread of *Listeria*. >99% of the macrophages under these experimental conditions were infected with bacteria by 14 h. In contrast, at both multiplicities of infection, 4 μg/ml NFX plus GFZ reduced bacterial spread to neighboring cells by ~40% at 24 h compared with use of either

Table 1. *Percentage of Viable J774 Cells after Treatment with CFX in the Presence or Absence of GFZ*

Drugs	Dose	Percent viable J774 cells* 2 h	16 h
GFZ	0.2 mM	98.0 ± 0.55‡	35.0 ± 7.2
CFX	1 μg/ml	98.5 ± 0.92	19.0 ± 4.6
CFX + GFZ	1 μg/ml + 0.2 mM	98.1 ± 0.61	67.0 ± 12.5
CFX	2 μg/ml	98.7 ± 0.84	49.0 ± 5.8
CFX + GFZ	2 μg/ml + 0.2 mM	98.3 ± 0.95	97.0 ± 1.5
CFX	4 μg/ml	98.0 ± 1.1	61.0 ± 7.4
CFX	8 μg/ml	98.3 ± 0.9	95.1 ± 2.1

GFZ potentiates the capacity of CFX to protect J774 cells against the cytotoxic effect of *Listeria* infection. J774 cells on coverslips were incubated with *Listeria* at a ratio of 1:1 for 2 h at 37°C, at which time CFX (1–8 μg/ml) alone or CFX (1 or 2 μg/ml) in the presence of GFZ (0.2 mM) were added to the medium as indicated. Coverslip cultures were harvested and stained with trypan blue at 2 and 16 h after infection, as described in Fig. 7 and Materials and Methods, and the percentage of cells stained with trypan blue was calculated. The results reported in this table are representative of experiments repeated on two or more occasions, all of which yielded similar results.

* Infection ratio (*Listeria* CFU/J774 cells) is 1:1.

‡ Mean percent J774 cell viability ± SD.

Table 2. *GFZ Enhances the Ability of NFX to Reduce Cell-to-Cell Spread of L. monocytogenes*

Drugs	Dose	*Listeria* CFU/ J774 cells	Percent J774 cells infected with *Listeria monocytogenes* 2 h	14 h	24 h
GFZ	0.2 mM	1:1	21.0 ± 9.1*	>99	>99
NFX	8 μg/ml	1:1	20.5 ± 6.2	>99	>99
NFX + GFZ	4 μg/ml + 0.2 mM	1:1	20.0 ± 8.5	54.6 ± 10.7	57.0 ± 13.2
NFX + GFZ	8 μg/ml + 0.2 mM	1:1	21.0 ± 6.4	22.0 ± 8.1	29.0 ± 9.0
GFZ	0.2 mM	5:1	39.5 ± 8.1	>99	>99
NFX	4 μg/ml	5:1	42.5 ± 10.2	>99	>99
NFX	8 μg/ml	5:1	40.0 ± 7.3	>99	>99
NFX + GFZ	4 μg/ml + 0.2 mM	5:1	39.5 ± 5.1	56.0 ± 9.0	66.0 ± 10.7
NFX + GFZ	8 μg/ml + 0.2 mM	5:1	40.0 ± 6.2	41.0 ± 7.2	31.0 ± 10.1

Monolayers of J774 cells on coverslips were infected with *Listeria* at a ratio of one or five CFU per J774 cell. The infected cells were incubated at 37°C, harvested at 2, 14, and 24 h after infection, Gram-stained, and examined by light microscopy. 25 fields (× 400) were viewed per coverslip. The total number of cells and the percentage of J774 cells containing visible intracellular organisms were calculated from the average of the 75 microscopic fields examined from the three coverslips, as described in Materials and Methods. The results reported in this table are of a representative experiment, which was repeated on occasions with similar results on each occasion.

* Mean percent J774 cell infection with *L. monocytogenes* ± SD.

drug alone (Table 2). A more significant effect was seen with 8 μg/ml NFX plus GFZ, which prevented all bacterial movement from cell to cell beyond the initially infected cell population. These data confirm that spread of the infection was restricted by this drug combination, even at a high initial burden of bacteria.

To evaluate whether NFX plus GFZ could rescue infected J774 cells from the cytocidal effect of *Listeria*, we examined the viability of these cells at 16 and 24 h after infection at a multiplicity of 5 CFU *Listeria* per J774 cell. While 40% of the cells treated with 8 μg/ml NFX plus 0.2 mM GFZ were infected at 2 h (Table 2), only 19% of these cells stained with trypan blue at 16 and 24 h (data not shown). These findings suggest that ~50% of the initially infected J774 cells were protected from the cytocidal effects of *L. monocytogenes*. These results demonstrate that NFX supplemented with GFZ limits spread of *Listeria* from cell to cell and rescues a significant percentage of *Listeria*-infected J774 cells from the cytocidal effects of this bacterium.

Discussion

These studies demonstrate that GFZ enhanced by fourfold the capacity of two fluoroquinolones, NFX and CFX, to kill intracellular *L. monocytogenes*, and to protect *Listeria*-infected J774 cells from the cytocidal effects of this bacterium. GFZ also potentiated the capacity of NFX to block cell-to-cell spread of *Listeria*, thereby restricting the extent of infection. Control experiments showed that GFZ alone had no effect on growth of *Listeria* extra- or intracellularly, and that it did not potentiate the efficacy of fluoroquinolone antibiotics against *Listeria* growing extracellularly. The concentrations of fluoroquinolone antibiotics studied were selected on the basis of the minimal inhibitory concentrations of these drugs against extracellular *L. monocytogenes*.

Carlier et al. (4, 9, 10) have studied the accumulation of fluoroquinolone antibiotics by J774 cells. They report that these cells concentrate CFX several-fold above its concentration in the medium. They obtained similar results for several other fluoroquinolones (ofloxacin, pefloxacin, lomefloxacin, fleroxacin, and sparfloxacin) (4, 9, 10). In contrast, we have found that at steady state the intracellular concentration of NFX in J774 cells is approximately the same as that in the medium (2, 3). We cannot explain the difference in our findings and those of Carlier et al. (4, 9, 10). However, it is important to note that there is excellent correspondence between the concentration of NFX needed to kill *L. monocytogenes* extracellularly, and our estimate of the intracellular Listeriacidal concentrations of this drug (see below).

Carlier et al. (4) used cell fractionation methods to determine the intracellular location of fluoroquinolone antibiotics. They reported that >80% of pefloxacin, lomefloxacin, and fleroxacin in J774 cells resides in the soluble cytoplasm; a small percentage cosediments with endosomes and lysosomes. Like Carlier et al. (4, 9, 10), we have used cell fractionation to determine the distribution of radiolabeled NFX in J774 cells. Cells incubated in medium containing 0.2 mM GFZ and radiolabeled NFX in J774 cells. Cells incubated in medium containing 0.2 mM GFZ and radiolabeled NFX accumulated three- to fourfold more NFX than cells incubated in medium containing NFX alone (3, 5). The distribution of NFX among intracellular compartments was similar whether or not the

cells had been treated with GFZ. In both cases, >80% of cell-associated radiolabeled antibiotic was recovered in the soluble cytoplasmic fraction, and a small but measurable percentage was recovered in the lysosomal and endosomal fractions.

L. monocytogenes grows in the cytoplasmic matrix of J774 cells (6), the same cellular compartment in which >80% of intracellular fluoroquinolone resides. Thus, the concentration of fluoroquinolone antibiotic experienced by these bacteria intracellularly should be approximately the same as that measured radiochemically in the cells' cytoplasm. These studies demonstrate that this occurs. There is excellent agreement between the concentration of NFX required to kill extra- and intracellular *Listeria*. The MBC of NFX for extracellular *Listeria* was 32 μg/ml. In the absence of GFZ, the minimum concentration of NFX in the medium that protected J774 cells against the cytocidal effect of *Listeria* growing intracellularly (Fig. 8) and that killed intracellular *Listeria* (Fig. 2) was 32 μg/ml. In the presence of GFZ, the minimal concentration of this antibiotic that protected J774 cells against the cytocidal effect of *Listeria* (Figs. 7 and 8) and that killed intracellular *Listeria* (Fig. 1 *B*) was 8 μg/ml. As noted above, GFZ (0.2 mM) increased the intracellular accumulation of NFX approximately fourfold. Thus, the NFX concentration in the cytoplasm of J774 cells incubated in medium containing 8 μg/ml NFX plus 0.2 mM GFZ was ~32 μg/ml. This latter value agrees precisely with the MBC of NFX for both extracellular and intracellular *L. monocytogenes*, and with the minimal concentration of this antibiotic required to protect J774 cells against the cytocidal effect of *L. monocytogenes*. Together, these findings indicate that *Listeria* can be used as a sensitive measure of the concentration of an antibiotic in the cytoplasm of macrophages, and probably of other cells as well.

Similar results were obtained with CFX. The concentration of CFX required to protect J774 cells against the cytocidal effect of *Listeria* was reduced fourfold by addition of GFZ to the medium (Table 1). The MBC of CFX for *Listeria* growing extracellularly was 16 μg/ml. In the absence of GFZ, 8–16 μg/ml CFX killed *Listeria* growing intracellularly (Fig. 4). In the presence of GFZ, 2 μg/ml CFX exerted a similar bactericidal effect on intracellular bacteria (Fig. 3 *B*). Assuming intra- and extracellular *Listeria* are equally sensitive to CFX, an assumption that is supported by our findings with NFX, these results suggest that GFZ causes at least a fourfold increase in the accumulation of CFX by J774 cells. These results also suggest that *L. monocytogenes* can be used to measure the concentration of antibiotics in the cytoplasm.

We have studied the mechanism by which GFZ promotes the intracellular retention of fluoroquinolone antibiotics. Our results show that GFZ blocks the efflux of anionic organic compounds from J774 cells, presumably by inhibiting organic anion transporters in the cells' membranes.

Although NFX is negatively charged at neutral pH, it enters and exits J774 cells via diffusion across the cells' plasma membrane (2, 3). Efflux of NFX from these cells is speeded by the organic anion transporters in their membranes. By blocking these transporters with GFZ, efflux of organic anions is slowed, thereby allowing retention of a higher NFX concentration in the cells' cytoplasm than in the medium. That these antibiotics are more concentrated within the cytoplasm of GFZ or probenecid-treated J774 cells than in the surrounding medium has been demonstrated functionally by measuring their effects on intracellular *Listeria* (Figs. 1, *A* and *B*, and 3, *A* and *B*; reference 2), and directly using radiolabeled NFX (2, 5).

The present studies show that GFZ markedly increases the capacity of fluoroquinolone antibiotics to block the growth, intercellular spread, and cytocidal effect of an intracellular pathogen. The concentration of NFX required to achieve these effects is well above the plasma level that can be achieved safely in humans (11). However, the concentration of CFX (2 μg/ml) that was effective is within the range of plasma levels achieved in humans treated with this drug (12). Further work is warranted to determine whether GFZ enhances accumulation of fluoroquinolone antibiotics by human monocytes and macrophages, and whether it increases the efficacy of these antibiotics against intracellular bacterial pathogens in vivo.

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