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# Inhibitory Effects of the Quinolone Antibiotics Trovafloxacin, Ciprofloxacin, and Levofloxacin on Osteoblastic Cells *In Vitro*

Paul D. Holtom, Steven A. Pavkovic, Peter D. Bravos, Michael J. Patzakis, Lane E. Shepherd, and Baruch Frenkel

*Departments of Orthopaedic Surgery and Medicine and Institute for Genetic Medicine, University of Southern California School of Medicine, Los Angeles, California, U.S.A.*

**Summary:** We studied the inhibitory effects of the fluoroquinolones levofloxacin, ciprofloxacin, and trovafloxacin on growth and extracellular matrix mineralization in MC3T3-E1 osteoblast-like cell cultures. Levofloxacin had the least inhibitory effect on cell growth, with a 50% inhibitory concentration of approximately 80 μg/ml at 48 and 72 hours. Ciprofloxacin had an intermediate degree of inhibition, with a 50% inhibitory concentration of 40 μg/ml at 48 and 72 hours. Trovafloxacin exerted a profound inhibitory effect on cell growth, with a 50% inhibitory concentration of 0.5 μg/ml, lower than clinically achievable serum levels. The decreased cell counts with up to 2.5 μg/ml of trovafloxacin and with up to 40 μg/ml of ciprofloxacin were not associated with decreased rates of 5-bromo-2′-deoxyuridine incorporation per cell. Alatrovafloxacin, the L-alanyl-l-alanine prodrug of trovafloxacin, exerted effects on proliferation and 5-bromo-2′-deoxyuridine incorporation similar to those of the parent compound. The quinolones evaluated also inhibited extracellular matrix mineralization by MC3T3-E1 cells. Treatment of confluent cultures with trovafloxacin, ciprofloxacin, or levofloxacin resulted in strong inhibition of calcium deposition, as determined on day 14 by alizarin red staining and biochemical analysis. The effect was apparent with 2.5-5 μg/ml of each of the three antibiotics tested and progressively increased to more than a 90% decline in the calcium/protein ratio with 20-40 μg/ml antibiotic concentration. Further *in vivo* studies are advocated to evaluate the relevance of the *in vitro* cytotoxicity reported here to bone healing in orthopaedic patients.

The quinolone antibiotics are being increasingly utilized in the treatment of osteomyelitis. The latest generation of quinolones are highly active against the common pathogens of osteomyelitis, are bactericidal, and achieve adequate bone concentrations (14). The relatively long half-lives of these agents and their high degree of oral bioavailability provide for once-a-day oral administration, which may translate into improved treatment adherence. Additionally, the latest generation of quinolones have a favorable side-effect profile and are well tolerated clinically (22).

Quinolones exert their primary antibacterial effect through the inhibition of bacterial topoisomerase II (DNA gyrase) and probably topoisomerase IV in gram-positive organisms (15). These enzymes are necessary for the induction of negative supercoiling in the tertiary structure of DNA during replication, and the binding of quinolone antibiotics to the DNA gyrase complex is fatal for dividing prokaryotic cells (9). Several investigators have reported quinolones to be cytotoxic to eukaryotic cells. Preclinical toxicological evaluations have revealed nephrotoxicity and cardiotoxicity in laboratory animals to which fluoroquinolones were administered (23,26). Perhaps the most notable example of quinolone-associated cytotoxicity is chondrotoxicity—the ability of the class as a whole to induce arthropathy in juvenile animals of many species (1). The exact mechanism whereby quinolones exert this effect remains unclear, but their ability to chelate divalent and trivalent cations seems to be involved in the development of the cartilage lesions (8). However, in addition to divalent cation chelation, other mechanisms have been suggested to mediate the toxic effects of quinolones in eukaryotic cells. Mammalian cells have type-II topoisomerases, and binding of quinolones to these enzymes has been suggested to contribute to cytotoxicity (18). In particular, substitutions introduced at positions N-1, C-7, and C-8 to improve bioavailability and broaden the antibacterial spectrum tend also to increase toxicity toward eukaryotic topoisomerase II (25). For example, cipro-

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Address correspondence and reprint requests to B. Frenkel at Institute for Genetic Medicine, University of Southern California School of Medicine, 2250 Alcazar Street, CSC/IGM240, Los Angeles, CA 90033, U.S.A. E-mail: frenkel@hsc.usc.edu

floxacin, with a cyclopropyl group at the N-1 position, strongly inhibits topoisomerase II in calf thymus DNA, with a 50% maximal effect observed at 150 μg/ml (13). Interestingly, trovafloxacin, harboring a 3-azabicyclol [3.1.0] hexane substituent at the C-7 position and exhibiting a very broad antibacterial spectrum including many anaerobic organisms, does not inhibit mammalian topoisomerase II at concentrations up to 1,000 μg/ml (10,11).

We compared the cytotoxicity of three quinolone antibiotics—trovafloxacin, ciprofloxacin, and levofloxacin—in mammalian cells *in vitro*. Because the quinolones are frequently used in the treatment of osteomyelitis and because they were implicated in delayed fracture healing in the rat (12), we employed the MC3T3-E1 murine calvaria-derived osteoblastic cells for our studies. Surprisingly, we found trovafloxacin to be more toxic than the other two quinolones tested, consistent with the idea that inhibition of eukaryotic topoisomerase II may not be the main mechanism mediating toxicity in these cells.

## MATERIALS AND METHODS

### Cell Culture and Growth Curves

MC3T3-E1 cells were maintained in alpha minimal essential medium (GIBCO BRL, Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum (Omega Scientific, Tarzana, CA, U.S.A.), and, unless otherwise stated, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. The medium was changed three times a week. Ciprofloxacin (Bayer, West Haven, CT, U.S.A.), levofloxacin (Ortho McNeill, Raritan, NJ, U.S.A.), and trovafloxacin and alatrovafloxacin (Pfizer, New York, NY, U.S.A.) were added to the tissue culture medium as indicated. The concentrations and potency of levofloxacin and trovafloxacin after serial dilution were ascertained by conducting antibiotic susceptibility tests by broth macrotube dilution with use of organisms with known minimum inhibitory concentrations. The minimum inhibitory concentrations observed with levofloxacin and trovafloxacin were 1 and 0.125 μg/ml for *Enterococcus Faecalis* and 1 and 0.5 μg/ml for *Pseudomonas Aeruginosa*, in accord with published minimum inhibitory concentrations (19). In the cell proliferation studies, cells were plated in 96-well plates at a preconfluent density of 4,000 cells/well. The cells were allowed to attach for 24 hours, treated for an additional 24-72 hours, and then trypsinized and counted with a hemocytometer. Cell counts at each antibiotic concentration and time point were compared with control values by a heteroscedastic Student's $t$ test, which allows for unequal variances (Microsoft Excel 5.0). The level of significance for the experiments was set at $p < 0.05$.

### Incorporation of 5-Bromo-2′-Deoxyuridine

DNA synthesis was assessed by measuring the incorporation of 5-bromo-2′-deoxyuridine into newly synthesized DNA with use of enzyme-linked immunosorbent assay (kit 1 647 229; Boehringer Mannheim, St. Joseph, MO, U.S.A.). Cells were plated in 96-well plates at a density of 4,000 cells/well and allowed to attach for 24 hours, after which antibiotics were added at various concentrations. Following an additional 24 hours, 5-bromo-2′-deoxyuridine was added for 20 hours. Its incorporation was determined according to the manufacturer's protocol with use of a peroxidase-conjugated anti-5-bromo-2′-deoxyuridine monoclonal antibody and tetraethylbenzidine as substrate. The contribution of nonspecific binding of 5-bromo-2′-deoxyuridine antibody was determined in parallel cultures in the absence of 5-bromo-2′-deoxyuridine, and the background value was subtracted from the total absorbance values. The net incorporation values for 5-bromo-2′-deoxyuridine were corrected for the cell number present for each antibiotic concentration following 34 hours of treatment. These counts were performed with cells plated and treated in parallel to the 5-bromo-2′-deoxyuridine-labeled wells.

### Mineralization

To assess calcium deposition, MC3T3-E1 cells were plated in 12-well tissue-culture dishes at a density of 40,000 cells per well and the serum-containing medium was supplemented with ascorbic acid (50 μg/ml) and β-glycerol phosphate (10 m$M$) starting on day 4. In these studies, the quinolone antibiotics were added postconfluently (day 5), and the cultures were harvested on day 14. For histochemical demonstration of calcium deposition, the cell layers were fixed with 70% ethanol for 1 hour and stained for 10 minutes with 40 m$M$ alizarin red (pH 4.2) (Sigma Chemical, St. Louis, MO, U.S.A.). For biochemical evaluation, the cell layers were collected and initially lysed in 150 μl of Tris buffered saline containing 0.2% Triton. Following centrifugation, an aliquot was removed for protein determination with use of the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, U.S.A.). HCl was then added to a final concentration of 0.5 $M$, and the calcium concentration was determined on the basis of the light absorbance of complexes formed with o-cresolphthalein (Sigma Procedure no. 587; Sigma Chemical).

## RESULTS

### Differential Inhibition of Cell Accumulation by Ciprofloxacin, Levofloxacin, and Trovafloxacin

We studied the effects of quinolones on MC3T3-E1 osteoblastic cells, a nontumorigenic cell line derived from newborn mouse calvaria (24). These cells undergo a reproducible differentiation process *in vitro*, with a proliferative period followed by staged development of the osteoblast phenotype, characterized by expression of bone phenotypic genes and elaboration of mineralized extracellular matrix (2). Initially, we examined the effects of ciprofloxacin, levofloxacin, and trovafloxacin on proliferation, determined by cell counts during a 3-day treatment. A dose-dependent decrease in cell number was observed with all three drugs (Fig. 1). The inhibitory effect was most prominent with trovafloxacin, with which a 50% reduction in cell number was evident at levels as low as 0.5 μg/ml at 48 and 72 hours (Fig. 1, left panel). The number of cells did not increase as a function of time with trovafloxacin concentrations of 2.5 μg/ml or greater. The effect of ciprofloxacin on cell growth was less pronounced: a concentration of 20 μg/ml was required to achieve significant inhibition of cell growth at 48 and 72 hours (Fig. 1, middle panel). The concentration resulting in 50% inhibition (IC50) was approximately 40 μg/ml at 48 and 72 hours. Levofloxacin had the least inhibitory effect on the cell counts (Fig. 1, right panel). The IC50 at 48 and 72 hours was roughly 80 μg/ml. Thus, the decreased accumulation of MC3T3-E1 cells as a function of time is strongly dependent on the spe-

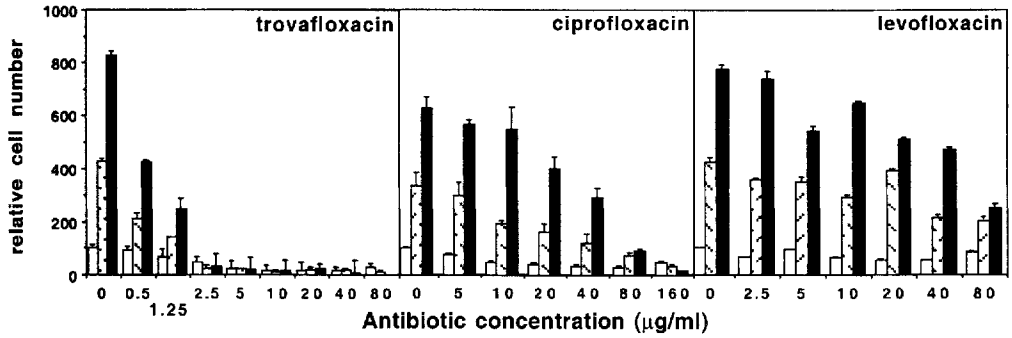


**FIG. 1.** Inhibition of MC3T3-E1 cell accumulation by trovafloxacin, ciprofloxacin, and levofloxacin. MC3T3-E1 cells were treated with trovafloxacin (left), ciprofloxacin (middle), or levofloxacin (right) at the indicated concentrations as described in the Materials and Methods section. The cells were counted following 24 (open bars), 48 (hatched bars), or 72 (solid bars) hours. The results are from one of four experiments with similar results. Each bar represents the average cell count from three to four replicate wells (±SD), with the 24-hour control value defined as 100. Similar results were obtained whether the experiments were conducted in medium containing or lacking penicillin and streptomycin. At 24 hours, statistically significant inhibition of cell accumulation was achieved only with trovafloxacin at concentrations of 2.5 μg/ml and higher ($p < 0.05$). Drug concentrations above which cell counts at 48 and 72 hours were consistently lower than the respective control levels ($p < 0.05$) were 0.5, 20, and 40 μg/ml for trovafloxacin, ciprofloxacin, and levofloxacin, respectively.

cific quinolone used, with a 40 and 160-fold greater sensitivity to trovafloxacin compared with ciprofloxacin and levofloxacin, respectively.

### Decreased Cell Accumulation in Quinolone-treated Cultures is not Accompanied by a Decline in DNA Synthesis

The decreased cell counts in the quinolone-treated cultures (Fig. 1) may have resulted from inhibition of DNA synthesis. We therefore examined the effect of trovafloxacin and ciprofloxacin on the incorporation of 5-bromo-2′-deoxyuridine into newly synthesized DNA during a 20-hour period starting 24 hours after the commencement of antibiotic treatment. Inhibition of cell accumulation by trovafloxacin at concentrations of 0.5-2.5 μg/ml (Figs. 1 and 2A, insert) was not accompanied by a parallel inhibition of 5-bromo-2′-deoxyuridine incorporation per cell. On a per well basis, 5-bromo-2′-deoxyuridine incorporation did decrease dose dependently (down to 40% of control levels at 2.5 μg/ml), but this was largely attributable to the decreased number of cells recorded in the same experiment in parallel wells (Fig. 2A, insert). Incorporation of 5-bromo-2′-deoxyuridine per cell at concentrations of 5 μg/ml and greater was apparently less than 0.04 relative units, but these data (not shown) cannot be interpreted because the low number of cells in these wells (Fig. 2A, insert) could no longer support an incorporation rate of 5-bromo-2′-deoxyuridine significantly greater than the background level of the assay (see Materials and Methods section). More importantly, the results for 0.5-2.5 μg/ml of trovafloxacin

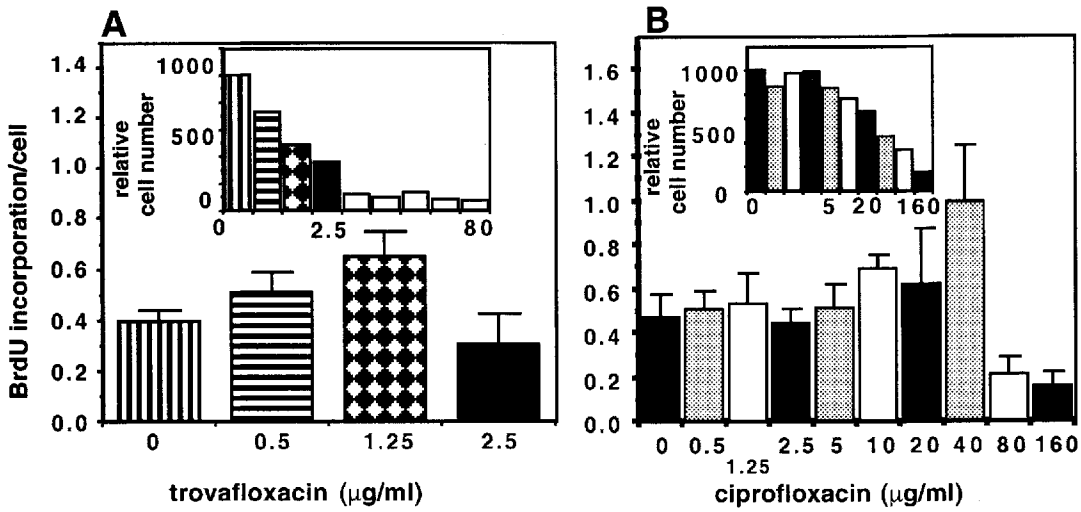


**FIG. 2.** Effect of trovafloxacin and ciprofloxacin on 5-bromo-2′-deoxyuridine (BrdU) uptake in MC3T3-E1 cells. Incorporation of 5-bromo-2′-deoxyuridine into DNA was determined as described in the Materials and Methods section in cultures treated with **A:** trovafloxacin or **B:** ciprofloxacin. The inserts show the results of cell count experiments, performed in parallel in two to three wells per antibiotic concentration. Each bar represents the mean ± SD of triplicate wells, corrected for the corresponding average cell number. Incorporation rates at 5, 10, 20, 40, and 80 μg/ml of trovafloxacin were lower than the value at 2.5 μg/ml but cannot be interpreted because of the low cell numbers in the respective wells (insert, white bars, and see text). Similar results were obtained in two additional experiments.

suggest that the inhibitory effect on cell accumulation at this concentration range is not attributable to inhibition of cell cycle progression. The possibility that trovafloxacin inhibits the increase in cell number by apoptosis remains to be explored.

Figure 2B describes the incorporation of 5-bromo-2′-deoxyuridine in MC3T3-E1 cultures treated with ciprofloxacin. Similar to our findings with trovafloxacin, ciprofloxacin concentrations, under which a significant decrease in cell number was recorded (20 and 40 μg/ml), did not inhibit incorporation of 5-bromo-2′-deoxyuridine per cell. Again, this suggests that ciprofloxacin also exerts its primary effect on MC3T3-E1 growth through a mechanism other than inhibition of cell cycle progression. In fact, ciprofloxacin at 40 μg/ml had a stimulatory effect on 5-bromo-2′-deoxyuridine incorporation. Only by 80 and 160 μg/ml did 5-bromo-2′-deoxyuridine incorporation levels drop (to 53 and 43% of the control levels, respectively), indicating inhibition of DNA synthesis at these high concentrations.

The quinolone trovafloxacin is available clinically for intravenous usage as its L-alanyl-l-alanine derivative, alatrovafloxacin, which has a higher degree of solubility at physiologic pH. We therefore performed cell count and 5-bromo-2′-deoxyuridine incorporation experiments in MC3T3-E1 cells treated with alatrovafloxacin. Alatrovafloxacin dramatically reduced the cell counts, with an IC50 of approximately 0.5 μg/ml at 48 and 72 hours (Fig. 3A). With concentrations of 1.25 μg/ml and higher, the inhibitory effect was already apparent after 24 hours of treatment. The overall inhibition by alatrovafloxacin was similar to that of trovafloxacin (Fig. 1). Also similar to trovafloxacin, the decreased number of cells in cultures treated with 0.5 and 1.25 μg/ml alatrovafloxacin was not attributable to inhibition of cell cycle progression, because these cells incorporated 5-bromo-2′-deoxyuridine at rates that are not significantly lower than those determined in nontreated cultures (Fig. 3B). Thus, the L-alanyl-l-alanine substitution in alatrovafloxacin does not influence the effect of this quinolone on osteoblast proliferation.

### Exposure of Confluent MC3T3-E1 Cultures to Quinolones Inhibits Calcium Deposition

We next examined the effect of the quinolone antibiotics on the elaboration of mineralized extracellular matrix by MC3T3-E1 cells cultured for 2 weeks in the presence of ascorbic acid and β-glycerophosphate. In the absence of quinolones, these cultures develop numerous nodules with heavy calcium-phosphate deposits, demonstrable by alizarin red (Fig. 4) and von Kossa (not shown) staining. Chronic exposure of post-confluent (day 5) cultures to quinolones attenuated calcium deposition as determined on day 14 by histochemical (Fig. 4A) and biochemical (Fig. 4B) analysis. With trovafloxacin, a significant inhibition (36% reduction in calcium accumulation compared with control) was apparent at 2.5 μg/ml, and calcium deposition was almost completely obliterated (91%) at 5 μg/ml (Fig. 4). Ciprofloxacin had a similar effect, again exhibiting strong inhibition (50% of control) at 2.5 μg/ml. Higher concentrations of ciprofloxacin resulted in progressive further inhibition but were generally more conservative than the respective concentrations of trovafloxacin. Finally, levofloxacin also inhibited the mineralization of MC3T3-E1 cultures but only at concentrations of 5 μg/ml (48% reduction in calcium deposition) or higher (Fig. 4). Thus, mineral deposition progressed normally with all of the tested quinolones at 1.25 μg/ml, but strong inhibition was observed histologically and biochemically at 2.5 (trovafloxacin and ciprofloxacin) or 5 μg/ml, with near

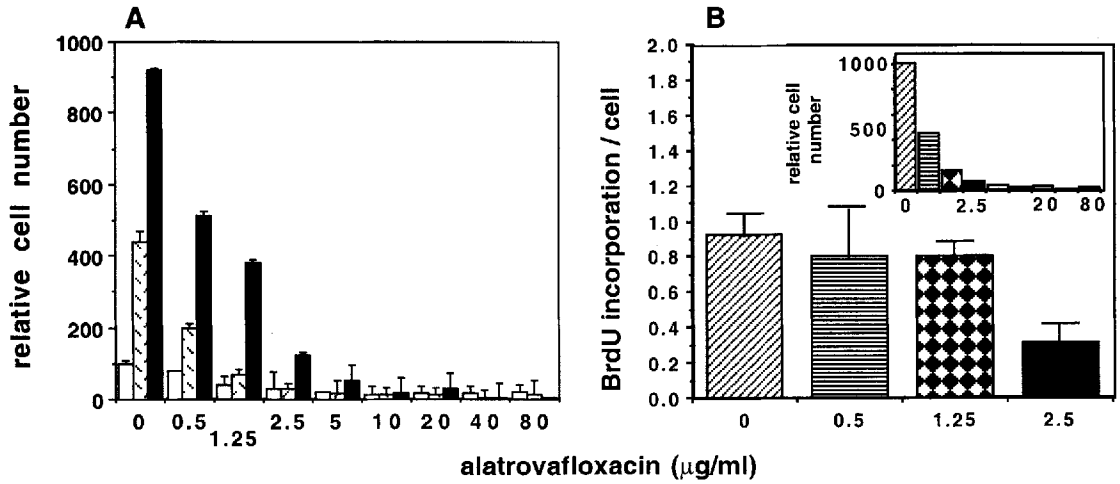


**FIG. 3.** Effect of alatrovafloxacin on cell accumulation and 5-bromo-2′-deoxyuridine (BrdU) uptake. **A:** MC3T3-E1 cell accumulation was evaluated as in Fig. 1 (mean ± SD, n = 3). Cell counts in cultures treated with 0.5 μg/ml and higher concentrations of alatrovafloxacin were significantly lower than those in the control cultures ($p < 0.05$). **B:** The effect of alatrovafloxacin on DNA synthesis was determined with use of 5-bromo-2′-deoxyuridine as in Fig. 2. One of two experiments with similar results is presented.

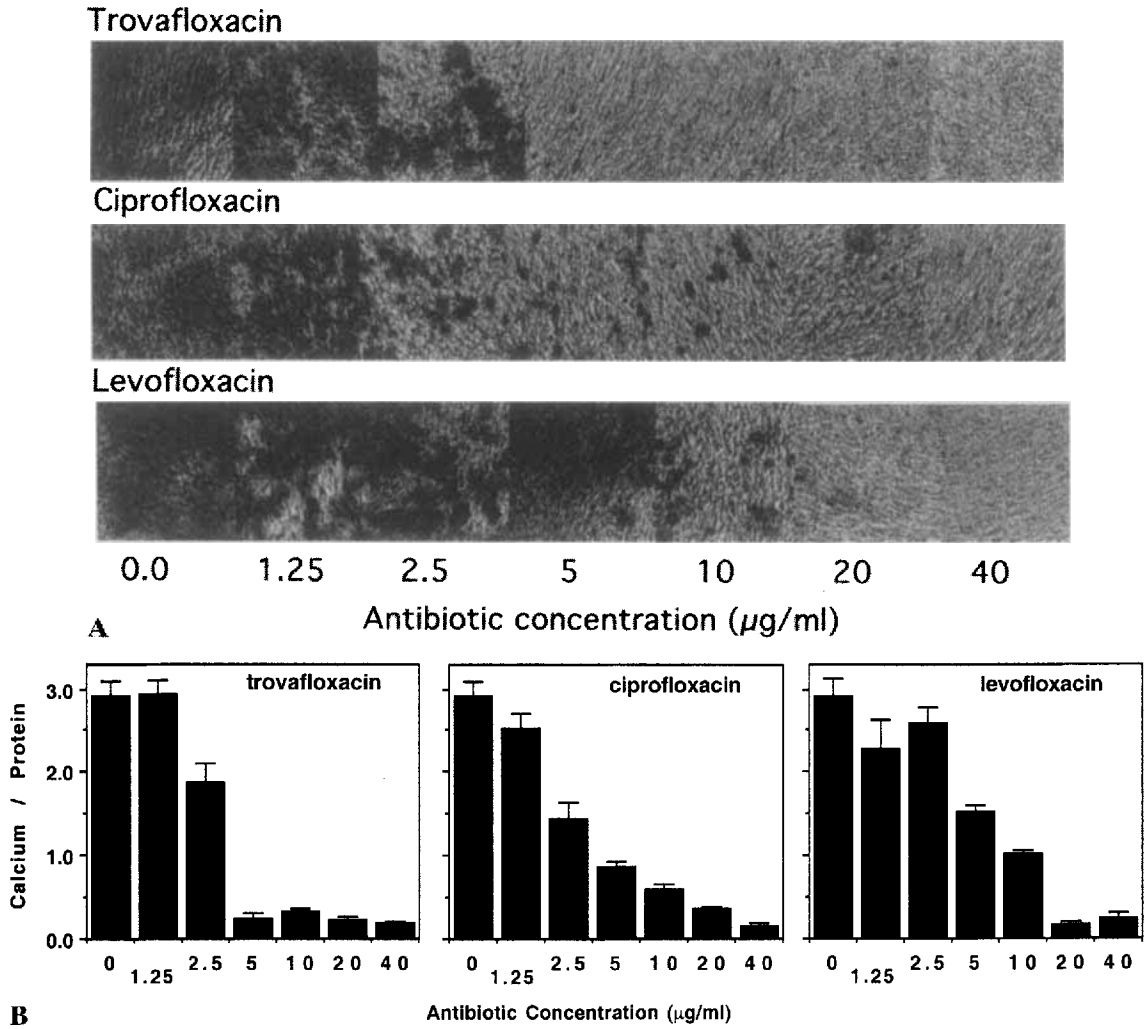


**FIG. 4.** Inhibition of extracellular matrix mineralization in MC3T3-E1 cell cultures treated with trovafloxacin, ciprofloxacin, and levofloxacin. Cells were subjected to a differentiation protocol as described in the Materials and Methods section. The cultures were treated with the indicated quinolones starting on day 5 (postconfluency). Cultures were harvested on day 14. **A:** Mineralization of the extracellular matrix is demonstrated by alizarin red staining as described in the Materials and Methods section. The quinolone concentrations are depicted in μg/ml below the micrographs. A representative field is shown for each experimental condition. Original magnification, ×100. **B:** Biochemical analysis of calcium accumulation in the cell layer. Cells were lysed in Triton followed by acid extraction and determination of the calcium content in cell layers. Calcium levels are corrected for the protein concentrations determined in the Triton lysate. Results are shown for one of three experiments with similar results (mean ± SD, n = 3).

obliteration at 40 μg/ml for all three antibiotics.

## DISCUSSION

We studied the effects of each of the quinolone antibiotics trovafloxacin, ciprofloxacin, and levofloxacin on the proliferation and mineralization of MC3T3-E1 murine osteoblastic cell culture. All three quinolones tested inhibited cell growth and mineralization in a dose-dependent manner. The most dramatic effect on cell accumulation was observed with trovafloxacin and its prodrug derivative alatrovafloxacin, with approximately 50% reductions at concentrations of only 0.5 μg/ml and virtual obliteration of the MC3T3-E1 cell cultures at 2.5 μg/ml. To our knowledge, this is the first demonstration of the toxicity of this quinolone toward osteoblastic cells. For ciprofloxacin, the inhibitory effect occurred at higher concentrations with an IC50 of approximately 40 μg/ml. These data are consistent with the recently published findings of Miclau et al. (17), who found a 27% inhibitory effect of ciprofloxacin on the growth of MG-63 human osteosarcoma cells at a concentration of 40 μg/ml at 72 hours. Prior studies by Forsgren et al. (5-7) revealed a 30-50% growth-inhibitory effect on human lymphocytes at a ciprofloxacin concentration of 20 μg/ml, with total inhibition of cell growth in the 80-160 μg/ml range, similar to our results with MC3T3-E1 osteoblastic cells. Levofloxacin was the least toxic agent in our study, with no inhibitory effect on cell number at 24 hours and a relatively mild effect at 72 hours (IC50 of ~80 μg/ml).

Because the antibacterial effect of quinolones is mediated by the targeting of prokaryotic DNA topoisomerase II (15), it seemed possible that the inhibi-

tion of eukaryotic cell proliferation would involve the corresponding eukaryotic topoisomerase. This notion is supported by our results showing that high ciprofloxacin concentrations (80 μg/ml and greater) caused a significant decrease in incorporation rates of 5-bromo-2′-deoxyuridine. These findings are consistent with those of Forsgren et al. (6), showing a decrease in [$^3$H]thymidine incorporation into human lymphocytes at 50-200 μg/ml of ciprofloxacin, and those of Hussy et al. (13), who reported a 50% inhibition of mammalian topoisomerase II by ciprofloxacin at 150 μg/ml.

Whereas high ciprofloxacin concentrations inhibited DNA synthesis, our 5-bromo-2′-deoxyuridine incorporation results with low ciprofloxacin and trovafloxacin concentrations suggest these quinolones may exert toxic effects on MC3T3-E1 cells by additional mechanisms. For example, ciprofloxacin at concentrations up to the IC50 (40 μg/ml) did not result in decreased rates of 5-bromo-2′-deoxyuridine incorporation per cell. Similarly, for trovafloxacin, 5-bromo-2′-deoxyuridine incorporation was well preserved up to 1.25 μg/ml, which is well past the point where a significant decline in cell number was observed. Therefore, it seems unlikely that inhibition of MC3T3-E1 cell accumulation by either of these quinolones is mediated exclusively by topoisomerase II, because inhibition of this enzyme would have resulted in decreased rates of 5-bromo-2′-deoxyuridine incorporation. This finding is in keeping with the work of Gootz et al. (10), who found no significant effect on eukaryotic topoisomerase II activity by trovafloxacin. The possibility of mechanisms mediating quinolone-associated cytotoxicity by means other than inhibition of nuclear DNA replication is further supported by the studies of Dalhoff and Doring (3) with prokaryotes and of Hussy et al. (13) with human lymphocytes, suggesting quinolone action at the cell membrane and mitochondria levels, respectively. It is noteworthy that Riesbeck et al. (21) found no significant effect of ciprofloxacin on mitochondrial DNA replication, protein synthesis, or oxidative phosphorylation. Nonetheless, at least two mechanisms appear to mediate the toxic effects of quinolones in mammalian cells: inhibition of DNA synthesis occurs at high drug concentrations and other, as yet unidentified, mechanisms are operative at low concentrations.

Diverse actions of quinolones on MC3T3-E1 osteoblastic cells are also suggested by comparing the effects of ciprofloxacin and levofloxacin on cell proliferation with the effects on differentiation. Ciprofloxacin started to inhibit calcium deposition in MC3T3-E1 cultures at 2.5 μg/ml, a concentration significantly lower than that affecting cell growth (20 μg/ml). Levofloxacin inhibited these cultures in a similar fashion, albeit at overall higher levels. Mineralization was affected already at 5 μg/ml, whereas several-fold higher concentrations were required to inhibit proliferation. In this respect, trovafloxacin was different from the other two quinolones, because the decrease in cell counts was apparent at concentrations (0.5 μg/ml) lower than those affecting calcium deposition (2.5 μg/ml). Thus, our data suggest that quinolones affect osteoblast function by diverse pathways and that different quinolones may have different mechanisms of action.

Previous *in vitro* studies with osteoblastic cells have demonstrated deleterious effects of nonquinolone antibiotics used in the treatment of musculoskeletal infections. However, these effects occur at concentrations far higher than those reported by Miclau et al. (17) and those used in the present study for the quinolone antibiotics. Specifically, significant inhibitory effects were observed with 200, 400, and 10,000 μg/ml for cefazolin, tobramycin, and vancomycin, respectively (4,16), compared with 0.5-5 μg/ml in the present study. Moreover, ciprofloxacin and trovafloxacin interfere with experimental fracture healing in the rat (12,20). Given the clinically achievable serum levels and the bone penetration of quinolones, it is not inconceivable that quinolones might exert an adverse effect on bone healing under certain circumstances. Serum peak levels for ciprofloxacin are approximately 2.5 μg/ml, and cortical bone concentrations are around 1.4 μg/ml (15). Thus, whereas systemic administration of ciprofloxacin is unlikely to pose a serious risk to bone healing, our results support the concerns of Miclau et al. (17) with regard to local administration of this or other quinolones. Trovafloxacin has high oral bioavailability, achieving maximal serum concentration of 3 μg/ml after a single 300-mg oral dose (27). Because concentrations as low as 0.5 μg/ml strongly inhibited osteoblast proliferation in our study, there is a clear need for further studies addressing bone healing *in vivo* following even systemic administration of trovafloxacin. In the interim, caution should be exercised.

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