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Antimicrosporidial activity of (fluoro)quinolones *in vitro* and *in vivo*

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Abstract. Microsporidia are a cause of emerging and opportunistic infections in humans and animals. Although two drugs are currently being used to treat microsporidiosis, concerns exist that albendazole is only selective for inhibiting some species of microsporidia that infect mammals, and fumagillin appears to have been found to be toxic. During a limited sequence survey of the *Vittaforma corneae* (syn. *Nosema corneum* Shadduck, Meccoli, Davis et Font, 1990) genome, a partial gene encoding for the ParC topoisomerase IV subunit was identified. The purpose of this set of studies was to determine if fluoroquinolones, which target topoisomerase IV, exert activity against *Encephalitozoon intestinalis* (syn. *Septata intestinalis* Cali, Kotler et Orenstein, 1993) and *V. corneae in vitro*, and whether these compounds could prolong survival of *V. corneae*-infected athymic mice. Fifteen fluoroquinolones were tested. Of these, norfloxacin and ofloxacin inhibited *E. intestinalis* replication by more than 70% compared with non-treated control cultures, while gatifloxacin, lomefloxacin, moxifloxacin, and nalidixic acid (sodium salt) inhibited both *E. intestinalis* and *V. corneae* by at least 60% at concentrations not toxic to the host cells. These drugs were tested *in vivo* also, where gatifloxacin, lomefloxacin, norfloxacin, and ofloxacin prolonged survival of *V. corneae*-infected athymic mice ($P < 0.05$), whereas moxifloxacin and nalidixic acid failed to prolong survival. Therefore, these results support continued studies for evaluating the efficacy of the fluoroquinolones for treating microsporidiosis and for characterizing the target(s) of these fluoroquinolones in the microsporidia.

Microsporidia are single-celled, obligately intracellular eukaryotic parasites that are recognized causes of emerging and opportunistic infections in persons with AIDS, organ transplant recipients, children, travellers, contact lens wearers, and the elderly (Bryan and Schwartz 1999, Didier 2005). Clinical symptoms associated with intestinal microsporidiosis typically include persistent diarrhoea, malabsorption, abdominal pain, and weight loss, and those associated with disseminated infections include conjunctivitis, sinusitis, myositis, pneumonia, peritonitis, hepatitis, or nephritis (Kotler and Orenstein 1998, 1999, Weber et al. 2000). Concerns for food- and water-borne transmission have resulted in the inclusion of microsporidia on the United States Environmental Protection Agency's microbial contaminant candidate list in response to the Safe Drinking Water Act (http://www.epa.gov/safewater/ccl/cclfs.html), and on the list of biodefence pathogens in Category B published by the National Institutes of Health and Centers for Disease Control and Prevention (http://www2.Niaid.Nih.Gov/Biodefense/bandc-priority.htm).

Current therapies for microsporidiosis are variably effective (Conteas et al. 2000). Albendazole, a benzimidazole that inhibits microtubule assembly, is effective against several microsporidia including the *Encephalitozoon* species, but is less effective against *Enterocytozoon bieneusi* (Blanshard et al. 1992). Fumagillin, a product of *Aspergillus fumigatus* that is well known for its effective treatment of nosematosis in honeybees (Katznelson and Jamieson 1952), also inhibits microsporidia *in vitro* (Shadduck 1980, Didier 1997) and is used topically to effectively treat ocular microsporidia infections (Friedberg and Ritterband 1999). Fumagillin appears to be effective against *E. bieneusi* infections in humans (Molina et al. 2002), but concerns exist about fumagillin's toxicity when used systemically in humans, which has generated interest in finding effective, but less toxic fumagillin analogues for treating microsporidiosis.

During studies to identify, clone, and express microsporidian methionine aminopeptidase 2, the putative drug target of fumagillin, a gene encoding topoisomerase IV was identified in the human microsporidian, *Vittaforma corneae* (Mittleider et al. 2002). Interestingly, topoisomerase IV has only been identified in prokaryotes. The genome of *E. cuniculi* includes genes for DNA topoisomerases I, II, and III, but no gene for DNA topoisomerase IV has been identified (Katinka et al. 2001). Of practical interest is that quinolones target

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the type II topoisomerases, DNA gyrase and topoisomerase IV (Hooper 2000, Andersson and MacGowan 2003, Hawkey 2003).

The purpose of this study was to test the *in vitro* and *in vivo* antimicrosporidial activities of a panel of fluoroquinolones. The *in vitro* screening assay utilized *Encephalitozoon intestinalis* and *V. corneae*. *E. bieneusi* is the most common microsporidian that infects humans but it cannot be grown in long-term culture and does not infect small laboratory animals. *E. intestinalis* was used in the *in vitro* screening assay because it is the second-most common microsporidian infecting humans and can be grown in long-term tissue. The human microsporidian isolate of *V. corneae* was used in the *in vitro* and *in vivo* models because it was found to encode a gene for topoisomerase IV, which is a target of the fluoroquinolones, can be grown in tissue culture, is most closely related to *E. bieneusi* based on a comparison of ribosomal gene sequences, and causes lethal infections in athymic mice (Didier et al. 1994, Baker et al. 1995, Mittleider et al. 2002). In addition, *V. corneae* can serve as a surrogate for *E. bieneusi* in drug test models because both organisms replicate in the host cell cytoplasm and both organisms are relatively less sensitive to albendazole while *Encephalitozoon* species are relatively sensitive to albendazole (Didier 1997, Desportes-Livage 2000).

MATERIALS AND METHODS

Microsporidia. *E. intestinalis* and *V. corneae* were grown in RK-13 rabbit kidney cells (50506, 50505, and CCL-37, respectively, American Type Culture Collection, Manassas, VA) and supernatants containing the microsporidia were washed successively in sterile distilled water, Tris-buffered saline containing 0.3% Tween 20 (TBS-Tw), and TBS followed by density centrifugation in 50% Percoll (Pharmacia, Picatoway, NJ) as previously described (Didier et al. 1996, Didier 1997).

Drugs. All drugs were purchased from Sigma Chemical (St. Louis, MO) with the exception of ciprofloxacin, gatifloxacin, levofloxacin, and moxifloxacin, which were provided through a contract with National Institute of Allergy and Infectious Diseases (N.I.A.I.D.) from Southern Research Institute (Frederick, MD). Stock solutions of drugs were prepared at 10 mM in dimethylsulfoxide (DMSO) and further diluted in medium for testing in the *in vitro* screening assays or in saline for treating mice.

***In vitro* drug assay.** RK-13 cells were plated onto 96-well tissue culture plates at 5×10^4 cells per well in RPMI 1640 medium containing 2 mM L-glutamine and 5% foetal bovine serum overnight at 37°C with 5% CO_2 to allow host cells to reach confluency. The next day, medium was replaced with 100 µl fresh medium containing microsporidia (1.5×10^5 organisms resulting in a 3:1 ratio of parasites to cells) and 100 µl freshly-diluted drugs in medium to generate final concentrations of 100, 10, 1, and 0.1 µM. Control cultures with microsporidia were treated with DMSO at the same dilution used to generate the 100 µM drug dilution (i.e. 1:1,000). Fresh medium containing drugs or diluent was replaced in each well on days 3 and 7. On day ten, 20 µl of 10% (w/v) sodium dodecyl sulfate was added to each well with microsporidia to release organisms from the host cells and the number of microsporidia was counted on a haemacytometer. Each treatment was assayed in quadruplicate and the percent inhibition of microsporidia replication was calculated as equal to; 100–[(number of microsporidia in each treatment well / mean number of microsporidia in the non-treated wells) × 100].

Measurement of drug toxicity *in vitro.* Quadruplicate sets of RK-13 host cells not inoculated with microsporidia were treated with drug only as described above and host cell viability was measured using an MTT assay (3(4,5-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide; Sigma, St. Louis, MO) as a measure of drug toxicity as described previously (Didier 1997). On day 10 of the assay, 50 µl of MTT (5 mg/ml stock) was added to each well and the cultures were incubated an additional 4 h at 37°C. The supernatants were then removed and the formazan was dissolved by addition of 200 µl acidified isopropanol. Absorbance values were measured using an ELISA spectrophotometer at a test wavelength of 570 nm and a reference wavelength of 630 nm. The percent of host cell viability was calculated as equal to; [absorbance of drug-treated host cells / mean absorbance of non-treated host cells] × 100. Drugs were considered toxic at concentrations that caused host cell viability to fall below 75% of diluent-treated control cultures.

Mice. Seven-week-old athymic CRL:CD-1nuBR male mice were purchased from Charles Rivers Laboratories (Wilmington, MA) and housed in groups of four in sterile filter-topped cages on HEPA-filtered shelf racks with sterile water and food provided *ad libitum*. Mice were allowed to acclimate for one week prior to initiation of the experiments. All protocols and procedures were approved by the Tulane National Primate Research Center Institutional Animal Care and Use Committee.

Microsporidia infection and treatment of mice. Mice were inoculated intraperitoneally with 1 ml saline containing 1×10^7 *V. corneae*. Drug or diluent injections were initiated 24 h later using doses and routes as described for each experiment. Mice were monitored for changes in activity, general appearance, body weight, and survival. If animals became moribund due to infection, or at the completion of the experiment, mice were euthanized by carbon dioxide overdose and necropsied. Gross abnormalities were recorded and histopathology was performed. Major organs including the brain, heart, liver, spleen, kidney, pancreas, gall bladder, stomach, lymph nodes, and adrenals were fixed by immersion in 10% formalin for histochemisty. In addition, the lung and the entire intestinal tract were perfused with fixative via the trachea and lumen prior to immersion. Tissues were embedded in paraffin for routine histologic processing and multiple sections were stained with H&E and Gram stain. Without prior knowledge of treatment, one section of each tissue from each mouse received a lesion score (subjective score of 0–4) and a parasite density score based on counting aggregates of Gram-positive microsporidia.

Statistical analyses. Means were compared by Student's *t*-test when comparing two groups, or by Analysis of Variance when comparing more than two groups. Approximate 50%

minimum inhibitory concentration (MIC_{50}) values were determined either experimentally or by linear regression and interpolation. The software programs of Graphpad Instat and Graphpad Prism software (San Diego, CA; website: www.graphpad.com) were used for these statistical analyses.

RESULTS

In vitro screening

Fifteen (fluoro)quinolones were assayed for their ability to inhibit replication of *E. intestinalis* and *V. corneae* in culture (Table 1). Norfloxacin and ofloxacin inhibited *E. intestinalis* replication by more than 70% compared with non-treated control cultures, while gatifloxacin, lomefloxacin, moxifloxacin, and nalidixic acid (sodium salt) inhibited both *E. intestinalis* and *V. corneae* by at least 60% at concentrations not toxic to the host cells. The lowest MIC_{50} values were observed for lomefloxacin, norfloxacin, and ofloxacin. In some cases, the MIC_{50} values needed to be interpolated to concentrations that were higher than could be tested *in vitro* due to toxicity to host cells.

In vivo evaluation of test compounds

Athymic mice inoculated i.p. with 1×10^7 *V. corneae* succumbed to infection about two to three weeks later. Affected animals usually demonstrated weight loss, and ascites terminally. Livers were mottled with pinpoint grey foci and congested centrolobular zones. In infected animals that survived more than three weeks, hepatic lesions were inapparent, but the kidneys contained pinpoint grey-white foci in the polar cortex. Histologically during the acute disease typically 14–21 days after inoculation with *V. corneae*, lesions were primarily identified as peritonitis of the intestine and spleen, and necrosis and inflammation of the liver and pancreas (not shown). Acute lesions of the visceral peritoneum were characterized by deposition of fibrin, variable accumulations of polymorphonuclear leucocytes and mononuclear cells, and dense intracellular accumulations of Gram-positive microsporidial spores that could often be detected and enumerated at low microscopic power (× 4). In the liver, random foci of necrosis and/or neutrophilic infiltrations were accompanied by individual spores and clusters of microsporidia within hepatocytes. In the pancreas, individual acinar cells to entire acini were necrotic or distended by dense aggregates of microsporidia. In chronic infections where mice survived at least 21 days, scattered renal tubules were necrotic, infiltrated with neutrophils, and contained distended epithelial cells filled with microsporidia (not shown). The survival curves of athymic mice inoculated with *V. corneae* and treated with (fluoro)quinolones are shown in Fig. 1.

Gatifloxacin

Athymic mice infected with *V. corneae* survived an average of 15.8 (± 0.4) days in this experiment. Gatifloxacin administered at doses of 25 and 50 mg/kg i.p. daily statistically significantly prolonged survival of the infected athymic mice to a mean of 20.1 ± 1.8 days ($P < 0.05$) and a mean of 21.3 ± 1.2 days ($P < 0.01$), respectively, while 100 mg/kg i.p. daily resulted in a mean survival time of 16.6 ± 0.7 days which was not statistically significantly longer than the untreated infected mice. The mean numbers of parasite-associated lesions in one section of all tissues of all mice were compared between groups of treated and non-treated *V. corneae*-infected athymic mice. An average of 126.0 ± 35.9 lesions were observed in the untreated infected mice. Infected mice treated with gatifloxacin at 25 mg/kg i.p. daily expressed a lower mean number of lesions at 89.7 ± 34.4 per mouse, but this was not statistically significantly less than those observed in the untreated control mice. Statistically significantly fewer lesions were observed in the infected mice treated with gatifloxacin at 50 mg/kg i.p. daily (53.9 $\pm$ 40.9 lesions; $P < 0.01$) and 100 mg/kg i.p. daily (25.6 ± 16.5 lesions; $P < 0.001$) compared with those observed in the untreated control mice. The observation that mice treated with the higher dose of gatifloxacin at 100 mg/kg i.p. daily failed to survive longer, even though parasite-associated lesions were significantly reduced, suggested that this drug was toxic at this dose. This was further supported by findings that five of the eight uninfected athymic mice in the toxicity control group treated with gatifloxacin 100 mg/kg i.p. daily failed to survive the four-week duration of the experiment and had developed suppurative and chronic peritonitis as observed at necropsy and by histopathology.

Lomefloxacin

In this experiment, the athymic mice inoculated with *V. corneae* survived an average of 21.4 ± 1.6 days. The mean survival time of the infected athymic mice treated with lomefloxacin at 50 mg/kg i.p. daily was 22.8 ± 1.0 which was not significantly longer than the controls. The mean survival times were significantly longer for the infected athymic mice treated with lomefloxacin at 100 mg/kg i.p. daily (25.7 ± 5.1 days; $P < 0.05$) and 200 mg/kg i.p. daily (25.8 ± 3.6 days; $P < 0.05$). The mean numbers of parasite-associated lesions observed histologically were lower in the treated versus untreated infected athymic mice, but these values were not statistically significant. Toxicity control athymic mice treated with lomefloxacin at 200 mg/kg i.p. daily survived the length of the experiment, but mild focal peritonitis was noted in six of the eight mice.

Moxifloxacin

The mean survival time of the untreated *V. corneae*-infected athymic mice in this experiment was 22.8 ± 2.3 days. The infected athymic mice treated with moxifloxacin at 25, 50, and 100 mg/kg i.p. daily survived an average of 25.6 ± 2.1, 25.3 ± 2.6, and 20.9 ± 7.6 days, respectively, and none of these survival times were significantly longer than the average survival time of the

Table 1. Screening of (fluoro)quinolones for antimicrosporidial activity *in vitro*.

Compound	Highest non-toxic dose tested [a]	Percent inhibition (mean ± SD) [b]		Approximate MIC_{50} (μM) [c]	
		Encephalitozoon intestinalis	*Vittaforma corneae*	*Encephalitozoon intestinalis*	*Vittaforma corneae*
Cinoxacin	10 μM	1.7 ± 6.6	12.1 ± 9.2	55	49
Ciprofloxacin	10 μM	2.9 ± 3.6	4.2 ± 3.9	84	66
Enoxacin	10 μM	28.6 ± 3.6	10.4 ± 12.8	69	60
Flumequine	0.1 μM	0.5 ± 5.6	12.8 ± 2.82	359	132
Gatifloxacin	100 μM	60.4 ± 2.3	66.1 ± 2.6	85	73
8-Hydroxyquinolone	0.1 μM	–0.5 ± 4.9	7.6 ± 3.6	53	52
Levofloxacin	< 0.1 μM	toxic	toxic	n.d. [d]	n.d.
Lomefloxacin	100 μM	98.4 ± 0.1	98.3 ± 0.2	3	3
Moxifloxacin	100 μM	66.5 ± 3.7	64.3 ± 4.1	76	77
Nalidixic acid (free acid)	10 μM	20.6 ± 4.1	14.3 ± 9.2	50	66
Nalidixic acid (sodium salt)	100 μM	98.9 ± 0.1	73.6 ± 10.0	23	66
Norfloxacin	10 μM	85.7 ± 1.8	43.6 ± 2.8	1	24
Novobiocin	100 μM	–7.3 ± 3.8	8.7 ± 5.0	>100	>100
Ofloxacin	10 μM	78.2 ± 2.3	23.3 ± 5.3	2	54
Pipemidic acid	10 μM	0.9 ± 7.0	2.6 ± 6.8	>100	>100

[a]Test compounds were considered toxic if host cell viability fell below 75% of control host cells that were incubated in compound-free medium.
[b]The inhibition of microsporidian growth was reported for the highest concentration of drug treatment that was not toxic to the host cells. At higher drug concentrations, it was not possible to determine if microsporidia growth inhibition was due to direct effects of drug on the microsporidia or indirect effect from loss of host cells for supporting growth of microsporidia.
[c]MIC_{50} values were measured by linear regression of experimental values and interpolation using Graphpad Prism 4. In some cases, the estimated values exceeded the highest non-toxic concentrations tested in this screening assay.
[d] n.d.; not determined due to toxicity at all doses tested in this screening.

infected untreated control mice. In addition, no statistically significant reduction in the mean numbers of parasite-associated lesions were observed for any of the treatment groups. The uninfected toxicity control mice treated with moxifloxacin at 100 mg/kg i.p. daily survived the term of the experiment and chronic peritonitis was observed in three of the eight mice.

Nalidixic acid (sodium salt)

The untreated infected athymic control mice survived an average of 23.7 ± 0.8 days in this experiment. Infected athymic mice treated with nalidixic acid at 25, 50, and 100 mg/kg i.p. daily survived for 23.0 ± 0, 25.3 ± 4.1, and 25.9 ± 4.5 days, respectively, which were not statistically significantly longer than the mean survival time of the untreated infected athymic mice. The mean numbers of parasite-associated lesions, likewise were not significantly lower than that of the untreated infected control mice. Toxicity control athymic mice treated with nalidixic acid at 100 mg/kg i.p. daily survived the length of the experiment and no lesions associated with drug toxicity were observed.

Norfloxacin

In this experiment, the mean survival time of the untreated athymic mice inoculated with *V. corneae* was 19.0 ± 1.9 days. Norfloxacin statistically significantly prolonged survival times of mice treated at 50 mg/kg i.p. daily (25.9 ± 1.8 days; $P < 0.001$), 100 mg/kg i.p. daily (26.8 ± 0.7 days; $P < 0.001$), and 200 mg/kg i.p. daily (25.4 ± 2.6 days; $P < 0.01$). The mean numbers of parasite-associated lesions also were significantly reduced in mice treated with norfloxacin at 50 mg/kg i.p. daily (30.1 ± 26.6 lesions; $P < 0.05$), 100 mg/kg i.p. daily (17.9 ± 25.6 lesions; $P < 0.01$), and 200 mg/kg i.p. daily (16.1 ± 17.1; $P < 0.01$) compared with untreated infected mice that had a mean of 73.9 ± 43.2 lesions per mouse. Although the toxicity control athymic mice treated with norfloxacin at 200 mg/kg i.p. daily survived the length of this experiment, suppurative peritonitis was observed in all of these mice.

Ofloxacin

The mean survival time of the untreated *V. corneae*-infected athymic mice in this experiment was 23.7 ± 0.8 days and all treatment groups survived statistically significantly longer. Infected athymic mice treated with ofloxacin at 25, 50, and 100 mg/kg i.p. daily survived an average of 28.4 ± 2.2 days ($P < 0.001$), 28.3 ± 2.1 days ($P < 0.001$), and 28.3 ± 2.5 days ($P < 0.001$), respectively. The mean number of parasite-associated lesions, however, were not significantly reduced in tissues of the treated mice. The toxicity control athymic mice treated with ofloxacin at 100 mg/kg i.p. daily survived the length of the experiment and no lesions associated with drug toxicity were observed in any of these mice.

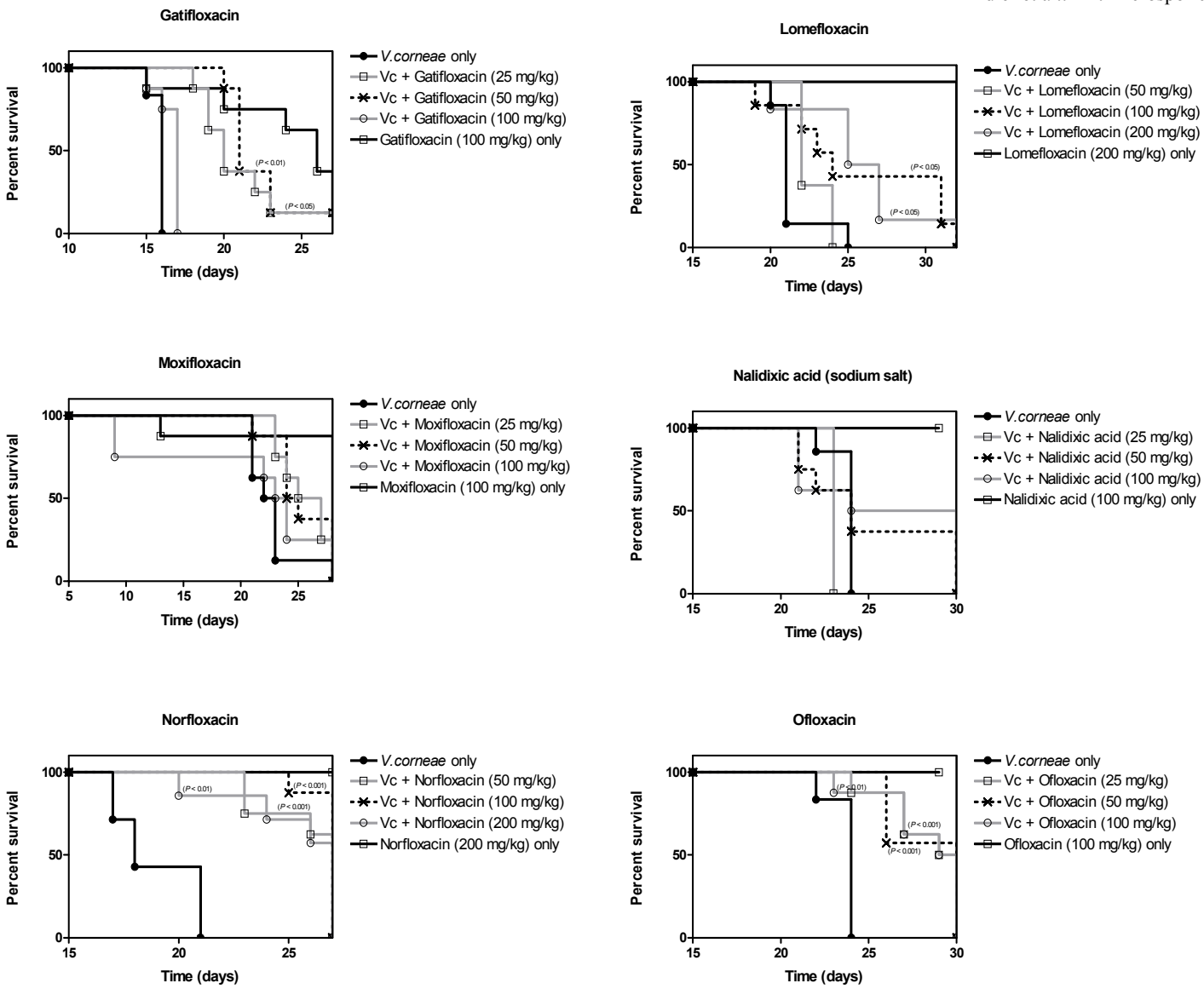

Fig. 1. Survival curves of *Vittaforma corneae*-infected mice treated with fluoroquinolones.

DISCUSSION

Fluoroquinolones are antimicrobial compounds that are structurally related to the antimalarial quinolone drugs (Andersson and MacGowan 2003, Emmerson and Jones 2003). Nalidixic acid was the first quinolone developed and applied for treating urinary tract infections due to Gram-negative bacteria. Modification of nalidixic acid led to the development of new quinolones, including the fluoroquinolones, which also are active against systemic infections due to specific Gram-positive, Gram-negative, and atypical bacteria. The fluoroquinolones are currently known to target two bacterial enzymes, DNA gyrase and topoisomerase IV. DNA gyrase is a tetramer composed to two A and two B subunits encoded by genes designated *gyrA* and *gyrB*, respectively, that introduces negative superhelical twists in DNA for initiation of DNA replication (Drlica and Zhao 1997, Ball 2000, Hooper 2000, Hawkey 2003). In addition, DNA gyrase removes superhelical twists that are generated ahead of the replication fork or during gene transcription. Topoisomerase IV is composed of two C and two E subunits encoded by genes designated *parC* and *parE*, respectively. This enzyme causes decatenation of intertwined daughter chromosomes to allow for segregation of daughter cells and also relaxes negative DNA supercoils (Ball 2000, Hooper 2000, Hawkey 2003). The fluoroquinolones inhibit DNA gyrase and topoisomerase IV by preventing religation of the broken DNA strands through formation of quinolone-enzyme-DNA complexes. Fluoroquinolones vary in their antibacterial activity by preferentially targeting DNA gyrase, DNA topoisomerase IV, or both. Bacterial resistance to fluoroquinolone activity may also occur through alteration in quinolone targets, decreased uptake of the drugs, expression of efflux pump systems, and mobile elements which may confer resistance (Ince and Hooper 2003, Ruiz 2003).

Topoisomerases that cleave one strand of DNA are classified as type I enzymes and those that cleave both strands of DNA are classified as type II topoisomerases and subtypes are defined by their linkage to the 5' or 3' phosphate or other structural considerations (Champoux 2001). Bacteria (e.g. *Escherichia coli*) typically contain two type IA topoisomerases (topoisomerase I and topoisomerase III) and two type IIA topoisomerases (DNA gyrase and topoisomerase IV). Some eubacteria may lack one of these enzymes due to redundant activities of their existing topoisomerases. Yeasts (e.g. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) contain topoisomerase I (belonging to topoisomerase type IB), topoisomerase II (of the topoisomerase type IIA), and topoisomerase III (of topoisomerase type IA). Higher eukaryotes typically contain topoisomerase I, two type IIA topoisomerases (topoisomerases IIα and IIß), and two topoisomerase III enzymes (topoisomerases IIIα and IIIß) which belong to the topoisomerase type I family.

Microsporidia are eukaryotic organisms that were recently reclassified with the fungi due to numerous related characteristics (Cavalier-Smith 1998, Weiss et al. 1999, Keeling and Fast 2002, Vossbrinck et al. 2004). The microsporidia, however, also share some characteristics with prokaryotes including the smaller size and organisation of their ribosomes and relatively few introns in the genome (Vossbrinck et al. 1987, Weiss et al. 1999). During a limited sequence survey of the *Vittaforma corneae* genome, a partial gene sequence encoding for 193 deduced amino acid residues was found to share a high level of identity to the DNA topoisomerase IV C subunit (type II; GenBank acc. nos. AZ694766, AZ694767, BH614712) of *Borrelia burgdorferi* (Mittleider et al. 2002). Furthermore, this *V. corneae* sequence was found to be more similar to the prokaryotic type II topoisomerase than to the *Encephalitozoon cuniculi* or eukaryotic type II topoisomerase. Since the entire *V. corneae* genome has yet to be determined, it is unknown if *V. corneae* contains other topoisomerases. The only microsporidian whose genome has been completely sequenced is *E. cuniculi*, and this species was found to encode genes for topoisomerase I (type I; GenBank acc. no. ECU06 1520), topoisomerase II (type II; GenBank acc. no. ECU04 0350), and topoisomerase III (type I; GenBank acc. no. ECU04 1070) (Katinka et al. 2001). A gene encoding for topoisomerase II (sequence 14VII-9F) was described for *Spraguea lophii*, but no gene encoding for topoisomerase IV was reported (Hinkle et al. 1997).

Since *V. corneae* was found to carry at least a partial gene encoding for the ParC subunit of topoisomerase IV, these studies were performed to determine if fluoroquinolones could exert antimicrosporidial activity. Of the 15 (fluoro)quinolones tested *in vitro*, two compounds, norfloxacin and ofloxacin, inhibited *Encephalitozoon intestinalis* by more than 70%. Four of these compounds, gatifloxacin, lomefloxacin, moxifloxacin, and nalidixic acid (sodium salt), inhibited both *E. intestinalis* and *V. corneae* by more than 60%. These drugs were then tested *in vivo*, and gatifloxacin, lomefloxacin, norfloxacin, and ofloxacin, were found to statistically significantly prolong survival of *V. corneae*-infected athymic mice. Moxifloxacin and nalidixic acid failed to prolong survival. Ciprofloxacin was tested *in vivo*, as well, because it is the most widely prescribed fluoroquinolone, but it did not significantly prolong survival of the *V. corneae*-infected athymic mice at 25, 50, or 100 mg/kg i.p. daily (data not shown). The wide variation in activity against *V. corneae* and *E. intestinalis* that was noted among the different fluoroquinolones thus may be due to the possibility that these two species of microsporidia may contain different types of topoisomerases and that the fluoroquinolones have different

mechanisms of action (Ball 2000, Hooper 2000, Hawkey 2003).

(Fluoro)quinolones are primarily used for treating specific bacterial infections and are only rarely applied to studies on parasitic protozoan infections (Nenortas et al. 1999, Ball 2000, Hooper 2000, 2001). These compounds are structurally related to antimalarial quinolones, and a few fluoroquinolones, including trovafloxacin, ciprofloxacin, enoxacin, ofloxacin, and norfloxacin were shown to inhibit *Plasmodium falciparum* and *Trypanosoma brucei brucei* growth *in vitro* (Divo et al. 1988, Tripathi et al. 1993, Nenortas et al. 1998, 1999, Hamzah et al. 2000). Norfloxacin also was reported to be effective for treating *P. falciparum* and *Plasmodium vivax* infections in India (Sarma 1989, Tripathi et al. 1993), but ciprofloxacin was ineffective against multidrug resistant *P. falciparum* infections in Thailand (Watt et al. 1991). Trovafloxacin, grepafloxacin, gatifloxacin, and moxifloxacin were among the most effective of 24 (fluoro)quinolones tested for their ability to inhibit *Toxoplasma gondii* replication *in vitro* (Gozalbes et al. 2000). Ciprofloxacin also was found to inhibit *in vitro* replication of *Giardia lamblia* (Sousa and Poiares-da-Silva 2001) and *T. gondii* (Fichera and Roos 1997), particularly during the second infectious cell cycle in the latter case which was associated with reduction in plastid replication. Ciprofloxacin was ineffective *in vivo*, however, for treating mice with experimental *T. gondii* infection (Khan et al. 1996). *P. falciparum* mitochondrial DNA topoisomerase II and DNA gyrase activities were not inhibited *in vitro* in the presence of ofloxacin, ciprofloxacin, and norfloxacin suggesting to the investigators that the *in vitro* activity of these fluoroquinolones for inhibiting *P. falciparum* was instead probably due to targeting of the plastid DNA topoisomerase II, as was reported for *T. gondii* (Chavalitshewinkoon-Petmir et al. 2001).

The only published application of fluoroquinolones for treating microsporidiosis was by Chan et al. (2003) in which norfloxacin was used topically for microsporidial keratoconjunctivitis in combination with systemic albendazole therapy. The results of the studies presented in this report suggest that although none of the fluoroquinolones cleared the *V. corneae* infection from the athymic mice, several fluoroquinolones did statistically significantly prolong survival and might be more efficacious when used in combination with other drugs. For example, fumagillin, though active against microsporidia, was found to be somewhat toxic in humans (Molina et al. 2002), but perhaps when administered at lower non-toxic doses in combination with fluoroquinolones, may show greater efficacy. In practice, the combination of enrofloxacin in combination with metronidazole was effective for treating canine leishmaniasis (Bianciardi et al. 2004) and trovafloxacin, in combination with clarithromycin, pyrimethamine, or sulfa-diazine, was more effective than each compound alone for treating acute murine toxoplasmosis (Khan et al. 1997). These results would therefore support continued studies to determine if fluoroquinolones can be applied for successfully treating microsporidiosis and to further characterize and identify the topoisomerase drug targets in the microsporidia.

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