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Comparative in-vitro activity of fleroxacin and other 6-fluoroquinolones against mycobacteria

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The susceptibility of 11 clinical isolates of *Mycobacterium tuberculosis,* 3 *M. kansasii,* 3 *M. xenopi,* 2 *M. scrofulaceum,* 2 *M. marinum,* 2 *M. malmoense* to fleroxacin, ciprofloxacin, norfloxacin, rifampicin, isoniazid, ethambutol, and streptomycin was determined by the standard proportion method (Middlebrook 7H10 agar). All *M. tuberculosis, M. kansasii, M. xenopi, M. scrofulaceum, M. marinum,* and *M, malmoense* isolates including those resistant to conventional antimycobacterials were inhibited by 0·5 mg/l of fleroxacin and ciprofloxacin, the lowest tested concentration. Fleroxacin and ciprofloxacin along with ofloxacin, pefloxacin, ansamycin, clofazimine and cycloserine were also tested against 14 isolates of the *M. avium* complex. Nine of 14 strains (64%) of the *M. avium* complex were found susceptible to 4 mg/l of fleroxacin and a similar percentage to the other quinolones. On the basis of its in-vitro potency and its favourable pharmacokinetic properties fleroxacin appears to be sufficiently active to warrant further experimental trials against difficult to treat mycobacteria.

Introduction

Fleroxacin (Ro 23-6240, AM-833), the 6,8 difluoro-1-(2-fluoroethyl)-1,4-dihydro-7-(4-methyl-1-piperazinyl)-4-oxo-quinoline-3-carboxylic acid has been shown to be highly active against a broad spectrum of Gram-positive and Gram-negative aerobic micro-organisms with the exception of many streptococci and anaerobes (Chin, Brittain & Neu, 1986). However, the antimycobacterial activity of this newly discovered 6-fluoroquinolone has not been as well characterized as other sectors of its antibacterial spectrum.

The purpose of the present study was to determine the in-vitro activity of fleroxacin in comparison with ciprofloxacin and norfloxacin against selected strains of susceptible and multiply resistant *Mycobacterium tuberculosis*, and against unselected isolates of the *M. avium* complex and of a further six clinically important species of mycobacteria other than tubercle bacilli (MOTT). Concurrently, the susceptibility of the strains belonging to the *M. avium* complex was also determined to ofloxacin, pefloxacin, ansamycin, clofazimine and D-cycloserine, and that of all the other mycobacteria mentioned to the first-line antituberculous compounds.

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Materials and methods

Antimicrobial drugs

The following compounds were provided as standard laboratory powders by the listed suppliers: fleroxacin [Ro 23-6240; AM-833 (F. Hoffmann-La Roche & Co. Ltd., Basle, Switzerland)]; ansamycin [(LM-427, a spiropiperidyl rifamycin); Farmitalia Carlo Erba, Milan, Italy]; ciprofloxacin (Bayer AG, Wuppertal West Germany); clofazimine (Ciba-Geigy, Basle, Switzerland); and D-cycloserine (F. Hoffmann-La Roche & Co. Ltd., Basle, Switzerland). Pefloxacin (Laboratoires Roger Bellon, Neuilly-s.-Seine, France), ofloxacin (Hoechst AG, Frankfurt a. Main, West Germany), norfloxacin (Merck, Sharp & Dohme, West Point, PA, USA) as well as rifampicin, streptomycin, ethambutol, and D-cycloserine were obtained from commercial sources.

Isolates

Thirty seven isolates (11 *M. tuberculosis*, 14 *M. avium* complex, 3 *M. kansasii*, 3 *M. xenopi*, 2 *M. scrofulaceum*, 2 *M. malmoense*, 2 *M. marinum*) were studied. All organisms were drawn from the stock culture collection of recent clinical isolates of the Department of Medical Microbiology of the University of Zürich except for those provided by Dr P. A. Jenkins, PHLS Mycobacterium Reference Unit, University Hospital of Wales, Cardiff, UK (3 strains of *M. avium* complex) and Dr K. H. Schroeder, Forschungsinstitut Borstel, West Germany (2 *M. avium* complex). The strains were identified by standard methods (Sommers & Good, 1985) and kept on Lowenstein-Jensen slants up to the time of use. Of the 11 strains of *M. tuberculosis*, 6 were susceptible and 5 resistant to both isoniazid and rifampicin. Of the 5 resistant isolates, 3 were also resistant to ethambutol and 2 to streptomycin. *M. tuberculosis* H37Rv was used as the control strain throughout the trial.

Susceptibility testing

Susceptibility testing was performed by the proportion method (Vestal, 1981; Sommers & Good, 1985; McClatchy, 1986). In brief, fleroxacin, ciprofloxacin, ofloxacin and norfloxacin were each solubilized in 0·06 ml of 1 *N* NaOH and 1 ml of methanol and, along with pefloxacin and D-cycloserine, diluted in sterile water to the desired final concentration. In contrast, ansamycin was dissolved in dimethylformamide and diluted in Sorensen buffer (pH 6·8), and clofazimine in absolute ethanol, respectively. Compounds were adjusted for solvent, water and impurity content.

Fresh solutions of the antibacterial agents were prepared on the day of the experiment, filter sterilized (0·22 μm Millipore) and, in the case of fleroxacin, stored in the dark up to the time of use. Appropriately concentrated aliquots (0·25 ml) of each drug were placed in three of four quadrants of each plate (the remaining quadrant being the antimicrobial-free control sector) and mixed with cooled molten (52°C) agar [Middlebrook 7H10 agar (Difco Laboratories, Detroit, MI, USA) containing 10% (v/v) Middlebrook OADC supplement (Difco)]. The final volume was 5 ml per quadrant (85 mm Petri dish), and final concentrations of antimicrobials tested were 0·5, 2 and 8 mg/l for the 6-fluoroquinolones against *M. tuberculosis* and most MOTT, and 2, 4, and 8 mg/l against the *M. avium* complex. Other final concentrations

employed were 2 mg/l for ansamycin and streptomycin, 1 mg/l for clofazimine, rifampicin and isoniazid, 10 mg/l for ethambutol, and 30 and 60 mg/l for D-cycloserine.

In preparation for inoculation mycobacteria were grown in Middlebrook 7H9 broth supplemented with Middlebrook OADC enrichment (Difco) at 37°C for five days, days, then diluted with saline and matched to a McFarland No. 1 standard. The resulting suspension was carefully vortexed for optimal homogenization prior to two consecutive 1 : 20 dilutions. Aliquots (150 μl per quadrant) from each of these three successive dilutions were used to seed the entire series of test plates set up for a particular strain including growth controls. Likewise, aliquots from the respective series of bacterial suspensions were used to demonstrate on other control plates that the solvents at the concentrations used in the trial were not inhibitory to the mycobacteria tested.

Cultures were incubated at 37°C in air with 5 to 10% CO_2 (except for *M. marinum* kept at 30°C in room air). Plates were read after three weeks' incubation with a stereomicroscope. Drug activity was assessed by dividing the number of colony forming units on quadrants with antimycobacterial by that of the respective drug-free control quadrant on the same plate growing preferentially 200–700 colonies.

Inhibitory activity of the test compounds was defined as the lowest concentration of antimycobacterial that reduced the colony count to less than 1% of that observed on the antimicrobial-free quadrant. Conversely, mycobacterial growth of 1% or greater on the plate with the highest listed or with the only stated concentration of antimycobacterial compound was interpreted as full resistance, and growth at the lower of two stated clinically achievable concentrations as partial resistance to a given compound.

Stability of fleroxacin in Middlebrook 7H9 broth (Difco, Lot. No. 673876) and 0·2% glycerol (Merck B 42522) was determined by high-performance liquid chromatography with a fluorimetric detector (SMF, Kontron) using 0·15% sodium dodecyl sulphate in acetonitrile–water–buffer pH 2 (Titrisol, Merck) as mobile phase for both the precolumn (Pellicular ODS®, 40 μm, Whatman) and the column (Novapack®, 4 μm; 150 × 3·9 mm ID, Waters). Aliquots of broth were removed from incubation at 37°C in the dark (fleroxacin being susceptible to degradation by light after exposure over several days) and sampled on days 0, 7, and 28. The coefficient of variation of our assay system was less than 5%.

Results

Stability

Experiments revealed that the potency of fleroxacin (10 mg/l) was fully maintained in the dark at 4°C in 0·9% sodium chloride and in 4% reconstituted human albumin over a four week storage period (data not shown). In experiments with Middlebrook 7H9 broth storage at 37°C with or without 0·025 mg % malachite green, the potency of fleroxacin degraded by 23% without and by 25% with malachite green over a 28 day period (data not shown).

Antimycobacterial activity

Results on the activity of fleroxacin and six comparative compounds against *M. tuberculosis* (with some strains being resistant to both rifampicin and isoniazid)

Table I Activity of fleroxacin and comparative compounds against *M. tuberculosis* and 5 MOTT species

Species (no. of isolates)	No. of susceptible isolates at an MIC (mg/l)[a] of: Fleroxacin 0·5	Fleroxacin 2·0	Fleroxacin 8·0	Ciprofloxacin 0·5	Ciprofloxacin 2·0	Ciprofloxacin 8·0	Norfloxacin 0·5	Norfloxacin 2·0	Norfloxacin 8·0	RMP[b] 1·0	INH[b] 0·2	INH[b] 1·0	EMB[b] 10·0	SM[b] 2·0
M. tuberculosis (11)	11	11	11	11	11	11	9	11	11	6	6	6	8	9
M. kansasii (3)	3	3	3	3	3	3	1	3	3	3	0	1	3	1
M. xenopi (3)	3	3	3	3	3	3	3	3	3	2	0	3	0	3
M. scrofulaceum (2)	2	2	2	2	2	2	1	2	2	2	0	0	1	1
M. malmoense (2)	2	2	2	2	2	2	0	2	2	2	0	0	1	1
M. marinum (2)	2	2	2	2	2	2	2	2	2	2	0	0	2	0

[a]Determined by the proportion method (7H10 Middlebrook agar).
[b]RMP, Rifampicin; INH, isoniazid; EMB, ethambutol; SM, streptomycin.

and five MOTT species other than *M. avium* complex are summarized (Table I). All 23 strains, including the control strain H37Rv, were found to be susceptible to fleroxacin and ciprofloxacin at 0·5 mg/l, the lowest concentration tested. In contrast, only 16 (70%) of 23 tested isolates were susceptible to norfloxacin at 0·5 mg/l even though 100% of strains were inhibited by a concentration of 2 mg/l. Thus, norfloxacin was less potent (W/W) than the other quinolones against *M. tuberculosis, M. kansasii, M. scrofulaceum* and *M. malmoense*. As expected, rifampicin and isoniazid were inactive at the tested concentrations against multi-resistant *M. tuberculosis* even though the latter were quinolone-susceptible. Isoniazid, but not rifampicin, was inactive also against most MOTT strains examined.

Some of the 14 strains of the *M. avium* complex were partially resistant to the 6-fluoroquinolones. The activity of fleroxacin as measured was similar to that of ciprofloxacin, norfloxacin and ofloxacin but slightly weaker than that of pefloxacin (Table II). At concentrations of 4 mg/l and 8 mg/l 64% and 79% of tested isolates were susceptible to fleroxacin and ciprofloxacin as against 71% and 93% to pefloxacin. All isolates resistant to 8 mg/l of fleroxacin were also resistant to 8 mg/l of ciprofloxacin, norfloxacin, and ofloxacin. One strain was even uniformly resistant to all five quinolones at the highest tested concentration of 8 mg/l.

Table II. Activity of fleroxacin and seven comparative compounds against *M. avium* complex (14 strains)

Drug	Concentration (mg/l)	Number susceptible	Strains[a] %
Fleroxacin	2	6	43
	4	9	64
	8	11	79
Ciprofloxacin	2	7	50
	4	9	64
	8	11	79
Norfloxacin	2	4	29
	4	7	50
	8	11	79
Ofloxacin	2	4	29
	4	8	57
	8	11	79
Pefloxacin	2	6	43
	4	10	71
	8	13	93
Ansamycin	2	14	100
Clofazimine	1	12	86
Cycloserine	30	10	71
	60	14	100

[a]Susceptible, determined by the proportion method (Canetti) on Middlebrook 7H10 agar.

Interestingly, the quinolone resistant strains of the *M. avium* complex were mainly susceptible in-vitro to the three non-quinolone drugs, and conversely, the two clofazimine resistant (MIC > 1 mg/l) isolates as well as the four D-cycloserine resistant (MIC >30 mg/l) strains of the *M. avium* complex were inhibited by all quinolones tested and by ansamycin. D-cycloserine was inhibitory to ten strains of the *M. avium* complex at a concentration of 30 mg/l and to all 14 strains at a concentration of 60 mg/l (peak serum level of D-cycloserine 20–50 mg/l). Similarly, 2 mg/l of ansamycin, a congener of rifamycin-S, were inhibitory *in vitro* to all *M. avium* complex isolates included in the trial.

Discussion

There still is an urgent need for new drugs to improve the prognosis of patients suffering from mycobacterial diseases due to drug-resistant *M. tuberculosis* or non-tuberculous mycobacteria. Cure rates of infections due to multiresistant *M. tuberculosis* (Costello, Caras & Snider, 1980; Bass, 1986; Jones *et al.*, 1987) and drug resistant MOTT have remained unsatisfactory despite combination therapy (Greene *et al.*, 1982; Masur *et al.*, 1987). Because of the recently discovered high incidence of mycobacterial infections in patients with AIDS (Goldman, 1987), there is an increasing demand for new first-line antimycobacterial agents that cure even the most severe forms of opportunistic *M. avium* complex infections in patients afflicted with the HIV-induced immunodeficiency (Horsburgh *et al.*, 1986), and of other mycobacterial diseases that are refractory to currently used antimycobacterial drugs as well.

The present study describes the in-vitro activity of the new 6-fluoroquinolone fleroxacin in comparison to that of ciprofloxacin, norfloxacin and other drugs against some very difficult to treat mycobacteria. In these susceptibility determinations, which were limited to antibacterial concentrations near or at the (proposed) susceptibility breakpoints of the various quinolones under study (Wise *et al.*, 1986, 1987), fleroxacin showed virtually the same degree of in-vitro activity (w/w) as ciprofloxacin against multiply resistant *M. tuberculosis* and six species of MOTT other than *M. avium* complex.

Fenlon & Cynamon (1986) found that the MIC_{90} of ciprofloxacin and norfloxacin were 0·5 and 4 mg/l for *M. tuberculosis* isolates, respectively. Thus, there was good agreement between their study and our results. Using 7H11 Middlebrook agar, Gay, DeYoung & Roberts (1984) and Berlin, Young & Bruckner (1987) reported somewhat higher MICs for ciprofloxacin (MIC_{90} of 1 mg/l) and norfloxacin and ciprofloxacin (MIC_{90} of 8 and 2·0 mg/l, respectively) against *M. tuberculosis*. Collins & Uttley (1985) reporting on the activities of ciprofloxacin against *M. tuberculosis* and its geographical variants, *M. xenopi, M. marinum, M. kansasii*, and *M. avium-intracellulare-scrofulaceum*, published that 164 out of 207 isolates (all except *M. avium-intracellulare-scrofulaceum*) had ciprofloxacin MICs of 1·56 mg/l or less in Lowenstein-Jensen medium. These values were somewhat higher than those observed by us. Methodological differences between the laboratories and varying susceptibility in different strains may account for the three-fold disparity in MICs. In the present study, two strains of *M. malmoense* were found susceptible to 0·5 mg/l of fleroxacin and ciprofloxacin, whereas others (Davies, Sparham & Spencer, 1987) have reported lower activity (MIC of 5 mg/l or greater) for ciprofloxacin against these organisms.

The results of fleroxacin and ciprofloxacin against 14 isolates of *M. avium* complex agreed well with those of Gay *et al.* (1984) who reported on the activity of ciprofloxacin (MIC_{90} at 16 mg/l) and of norfloxacin (MIC_{90} >16 mg/l) against these organisms. Fenlon and Cynamon (1986) found lower MICs to ciprofloxacin (MIC_{90} 2 mg/l) and to ofloxacin (MIC_{90} 8 mg/l) for *M. intracellulare* than our study. The observation of Tsukamura (1983) might explain the discrepancy. This author found that *M. intracellulare* was inhibited by lower concentrations of ofloxacin than *M. avium*. The reason for the divergence in activity of ofloxacin against the two species, *M. avium* or *M. intracellulare*, could account for correspondingly diverging activity of other quinolones. Yet irrespective of whether the choice of strains could have influenced the results in favour of the compounds, ofloxacin did not quite match the activity (w/w) of ciprofloxacin in Fenlon & Cynamon's study (1986) nor that of ciprofloxacin and fleroxacin in the present study. Pefloxacin was also active in the present trial against *M. avium* complex. We are unaware that this finding has been reported to date in the literature. Whether the pefloxacin activity would be confirmed in the clinical situation where the drug undergoes metabolism (Wise *et al.*, 1987) remains to be proven.

Heifets & Iseman (1985) tested 523 isolates of *M. avium* complex and found that 95% were susceptible to 2 mg/l of ansamycin. The MIC_{90} for 20 *M. intracellulare* isolates was 1 mg/l of ansamycin in the series reported by Cynamon (1985) and 2 mg/l in that published by Kiehn *et al.* (1985). In addition, the 35 isolates of Kiehn *et al.* (1985) were susceptible to cycloserine (30 and 60 mg/l) and clofazimine (1 mg/l). Our data agreed generally with these results but unlike these authors we encountered two strains resistant to clofazimine (1 mg/l) and four strains resistant to D-cycloserine (30 mg/l). The proven clinical results with ansamycin and clofazimine singly or in combination against *M. avium* infections (Whimbey, Kiehn & Armstrong, 1986) and the promising in-vitro activity of fleroxacin and the other new 6-fluoroquinolones raise the hope that with the advent of these new compounds the future of patients afflicted with *M. avium* complex infection could be less troublesome than at present.

Fleroxacin with its bioavailability of 100% after oral absorption (tablet), a low degree of metabolism and a long plasma elimination half-life of 9–10 h (Weidekamm, *et al.*, 1987) is known to combine not only a wide spectrum of activity similar to that of its congeners but also promising pharmacokinetics that distinguish it from the earlier quinolones such as the ones used comparatively in this trial (Wise *et al.*, 1986, 1987). Its rapid and uniform penetration into blister fluid in volunteers with peak levels in excess of 3 mg/l following a standard 400 mg oral dose suggests that some of the pathogenic mycobacteria tested might respond to the compound and that the compound is worth further experimental investigations against these organisms including killing tests (Yajko, Nassos & Hadley, 1987).

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