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Relationships among Antibacterial Activity, Inhibition of DNA Gyrase, and Intracellular Accumulation of 11 Fluoroquinolones

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A series of 11 fluoroquinolone antibacterial agents, including 8 newly synthesized molecules and 3 reference compounds (pefloxacin, ciprofloxacin, and sparfloxacin), were tested for their MICs against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The intracellular accumulation of fluoroquinolones by these microorganisms was measured by centrifugation through silicone oil and a fluorescence assay. The minimal effective dose (MED) was determined for all agents in a supercoiling assay with *E. coli* DNA gyrase. The hydrophobicities of the quinolones were determined and expressed as the logarithm of the coefficient of distribution (log *D*) between 1-octanol and phosphate buffer (pH 7.2). No correlation was found between MICs and cell accumulation for the quinolones studied. A correlation was found between log *D* and accumulation by *S. aureus* ($r = 0.71$, $n = 11$), and an inverse correlation was found between log *D* and accumulation by *E. coli* ($r = 0.73$, $n = 11$) and *P. aeruginosa* ($r = 0.64$, $n = 10$). The correlation coefficients between MICs and MED for *E. coli*, which were 0.60, 0.64, and 0.74 ($n = 11$) for *E. coli*, *P. aeruginosa*, and *S. aureus*, respectively, rose to 0.85, 0.74, and 0.74 ($n = 11$) for the same microorganisms, respectively, when the accumulation of the drug by the cell was taken into account. It was concluded that the inhibitory activity against DNA gyrase remains the most important parameter for quinolone potency, but that intracellular accumulation must be taken into account, since, for a given organism, both parameters are under the control of the physicochemical properties of the quinolones.

The MIC of a quinolone against a particular organism is determined by at least two factors: its rate of penetration across the bacterial cell envelope (31, 34) and its inhibitory activity against the supercoiling reaction catalyzed by its target, the enzyme DNA gyrase (15). Although both of these activities should be at their optimum to ensure the maximal antibacterial potency of a compound, it is evident that the second of these properties is the more important, since a quinolone devoid of activity against DNA gyrase is not active against a bacterium, despite sufficient drug accumulation. Moreover, it has been documented that most high-level quinolone resistance arises from gyrase modifications (13, 30, 35, 38, 44). Conversely, poor penetration into bacteria may impair the effectiveness of a quinolone that is highly active against gyrase. This could explain the lack of a clear correlation between antibacterial activity and inhibition of isolated DNA gyrase observed for some quinolones (2, 15). Several studies dealing with the structure-activity relationships of fluoroquinolones have been published (3, 10–12, 15, 16, 18, 24, 36), and the investigators sometimes attempted to identify separately those factors that are necessary for gyrase inhibition from those that are needed for penetration into the cell. With regard to penetration into the bacterial cell, physical properties such as relative hydrophobicity (23, 28) or charge or molecular weight (7, 8, 31) are important. As a general rule, bulky size, negative charge, and increasing hydrophobicity retard the penetration of antibacterial agents into gram-negative organisms through porin channels (9, 20, 21), although hydrophobic molecules appear to use an alternative route, through lipopolysaccharide (8). For gram-positive organisms, the molecular weight of the antimicrobial agent has little influence on the agent's potency, and positively charged hydrophobic molecules have been shown to have a better rate of penetration (17, 19). The structural features necessary for enzyme inhibition appear to be different from those required for penetration, and analysis of these features is more complex (10, 24).

We have at our disposal a series of fluoroquinolones with a wide range of hydrophobicities (logarithm of the coefficient of distribution [log *D*], between −1.18 and 1.05). We therefore examined whether a more direct relationship could be established between hydrophobicity, intracellular penetration, gyrase inhibition, and the antibacterial potencies of these fluoroquinolones against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

(Part of this research was presented at the 4th International Symposium on New Quinolones, Munich, Germany, 27 to 29 August, 1992.)

MATERIALS AND METHODS

Antibiotics. Most of the quinolones used in this study were synthesized at Bristol-Myers Squibb Research Laboratories. Ciprofloxacin was from Bayer; pefloxacin, sparfloxacin, and [^{14}C]sparfloxacin were provided by Rhône-Poulenc Rorer. Their UV and fluorescence spectra were recorded on Kontron Uvikon 810 and F-2000 Hitachi spectrometers, respectively. Standard dosage curves were established at the maximum wavelength of each compound to allow further determination of concentration.

Quinolone hydrophobicity. The hydrophobicity of each compound was measured as described by Bouzard et al. (4). Briefly, a solution of 10 μg of the test compound per ml in 0.1

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M phosphate buffer (pH 7.2) saturated with 1-octanol was mixed with an equal volume of 1-octanol saturated with buffer. The mixture was stirred for 12 h at 25°C, and the two phases were separated. The concentration in each phase was determined by UV absorbance. Log *D* was measured as the logarithm of the ratio of the concentration of the organic phase to the concentration of the aqueous phase.

Bacterial strains. We used laboratory reference strain *E. coli* K-12 J53 (F^- *pro met aziR*; from the Institut Pasteur collection) and clinical isolates of *P. aeruginosa* S 678 and *S. aureus* GAB S that were susceptible to quinolones. The susceptibilities of the two clinical isolates were compared with those of strains PAO1 and 209P, respectively.

MICs. Bacteria were routinely grown in Trypticase soy broth from Biomérieux, Craponne, France. MICs were determined by using twofold dilutions of antibiotic in Trypticase soy broth, to which 10^6 CFU of bacteria per ml was added. Cultures were incubated for 18 h at 37°C. Three experiments were carried out by using various initial fluoroquinolone concentrations to increase accuracy. As a consequence, MICs were not multiples of one another.

Chemicals. Silicone oils DC 550 and DC 556 were from Touzart et Matignon, Vitry sur Seine, France. Oil with a density of 1.043 was made by mixing 3 parts of DC 550 and 7 parts of DC 556. Topoisomerase I was purchased from Bethesda Research Laboratories, Cergy Pontoise, France. Plasmid pBR322 was obtained from Boehringer, Meylan, France.

Buffers. Buffer A consisted of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1 mM EDTA, 6 mM β-mercaptoethanol, 200 mM KCl, and 10% ethylene glycol (pH 7.6); buffer B consisted of 50 mM KH_2PO_4, 0.2 mM EDTA, and 6 mM β-mercaptoethanol (pH 7.6); buffer C consisted of 75 mM KCl, 60 mM HEPES, 12 mM dithiothreitol, 5 mM spermidine, 18 mM magnesium acetate, 1.5 mM EDTA, 9% ethylene glycol, and 100 μg of tRNA per ml (pH 7.6); phosphate-buffered saline (PBS) consisted of 80 mM KH_2PO_4 and 77 mM NaCl (pH 7.2).

Intracellular accumulation of quinolones. The method described by Celesk and Robillard (7), with minor modifications, was used to measure the intracellular accumulation of the quinolones. Cells were grown in Trypticase soy broth to 180 Klett units (blue filter), washed with PBS, and resuspended in the same medium to an A_{530} of 25. Accumulation was assayed at 37°C in PBS upon the addition of 10 μg of the drug per ml. At 0, 5, 10, and 20 min, a 500-μl sample was placed onto the surface of 500 μl of ice-cold silicone oil (density, 1.043) in a 1.5-ml conical plastic tube, and the mixture was centrifuged for 2 min at 10,000 × *g* by using a 5415C Eppendorf centrifuge. The tubes were frozen and then cut in the middle of the oil layer and were inverted to eliminate excess oil from the pellet. The pellet was suspended in 1 ml of PBS, boiled for 7 min, and centrifuged at 10,000 × *g* for 5 min. The fluoroquinolone level in the supernatant was estimated by fluorimetry. For sparfloxacin, a ^{14}C-labeled product (5 μCi/mg) was used, and the quinolone concentration was estimated by liquid scintillation counting. The results given in Table 1 are the means of at least three determinations at 20 min and varied by no more than 10%.

Gyrase purification. DNA gyrase was purified by affinity chromatography on novobiocin by the technique of Staudenbauer and Orr (39), with some modifications. *E. coli* K-12 cells were used; they were suspended in Tris hydrochloride buffer containing 2 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (pH 7.5) and were stored at −80°C until required. Cell lysis and subsequent experiments were carried out at 4°C. The cell suspension was thawed and lysis was effected with glass beads (diameter, 0.25 to 0.30 mm) in a cell homogenizer (Braun, les Ulis, France). The suspension was adjusted to 5 mM magnesium acetate and ultracentrifuged for 1.5 h at 150,000 × *g* in a Beckman 45 Ti rotor. Nucleic acids were precipitated from the supernatant with 4% (wt/vol) streptomycin sulfate. Ammonium sulfate fractionation and affinity chromatography were carried out as described previously (39), but elution of the enzyme was carried out in one step with buffer A containing 5 M urea (40). The urea fractions were dialyzed overnight against buffer B and concentrated with solid polyethylene glycol 20,000 for 3 h. The specific activity was 10^4 supercoiling units per mg of protein. Aliquots were kept in liquid nitrogen and were stable for at least 1 year.

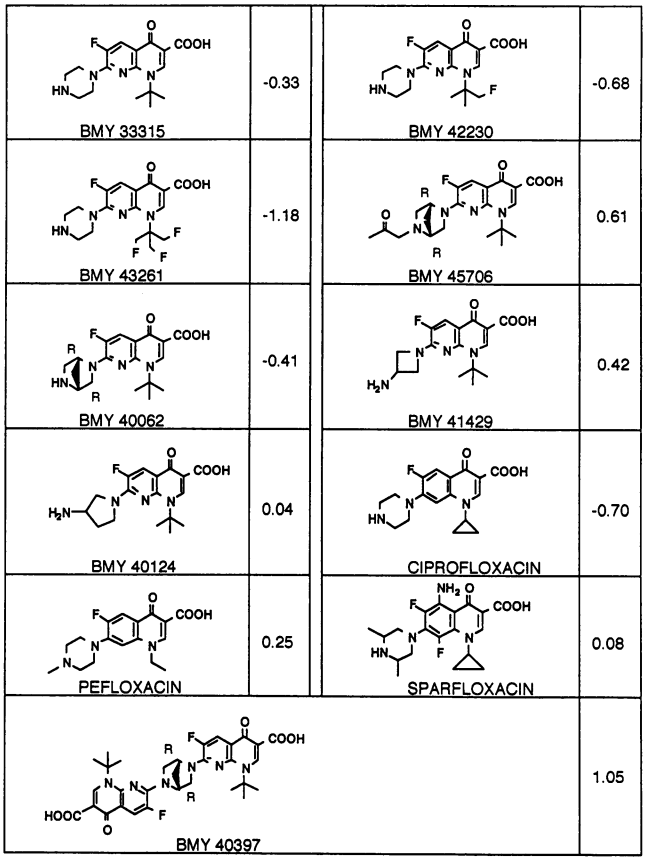

FIG. 1. Structure and log *D* of the assayed quinolones.

DNA supercoiling assay and MED determination. DNA supercoiling activity was assayed in buffer C as described previously (29), using 150 ng of relaxed pBR322 plasmid and 1 U of DNA gyrase. One unit of activity is the amount of gyrase that can supercoil 150 ng of relaxed pBR322 plasmid in 30 min at 37°C. Results were expressed as minimal effective dose (MED; the minimum amount of drug required to cause any inhibition of activity). We have found this parameter easier to evaluate on electrophoresis gels than the classical 50% inhibitory dose; the MED gives more accurate values that can differ by a factor of 2.

TABLE 1. Antibacterial activities and intracellular accumulation for *S. aureus*, *E. coli*, and *P. aeruginosa* and MED against DNA gyrase[a]

Compound[b]	*S. aureus* GAB S		*P. aeruginosa* S 678		*E. coli* K-12 J53		
	MIC (μg/ml)	Accumulation (ng/10^9 cells)	MIC (μg/ml)	Accumulation (ng/10^9 cells)	MIC (μg/ml)	Accumulation (ng/10^9 cells)	MED (μg/ml)
BMY 33315	0.15	262	1.6	48	0.11	78	1
BMY 42230	0.60	158	3.2	41	0.22	104	10
BMY 43261	24	108	30	36	3.5	90	40
BMY 45706	0.15	290	1.6	19	0.45	44	1.2
BMY 40062	0.25	220	0.25	111	0.03	120	0.6
BMY 40397	0.06	340	60	19	1.75	40	1.2
BMY 41429	0.25	134	1	11	0.11	42	0.4
BMY 40124	0.25	224	2	31	0.30	108	0.4
CIPRO	0.25	54	0.5	36	0.05	100	0.1
PEFLO	1	224	4	15	0.20	89	1
SPFX	0.06	170	1	48	0.05	80	0.2

[a] Assay conditions are described in the text.
[b] CIPRO, ciprofloxacin; PEFLO, pefloxacin; SPFX, sparfloxacin.

RESULTS

Hydrophobicities of the fluoroquinolones. The structures of the assayed quinolones (4) together with their log *D* values are given in Fig. 1. Log *D* varied from −1.18 for BMY 43261 up to 1.05 for BMY 40397. The variation was well distributed among the various compounds studied.

Antibacterial activity. The MICs for *S. aureus*, *E. coli*, and *P. aeruginosa* are given in Table 1. The assayed compounds had marked activities against *S. aureus*, with only three compounds being less active than ciprofloxacin (MIC, 0.25 μg/ml): BMY 42230, pefloxacin, and BMY 43261, with MICs of 0.60, 1, and 24 μg/ml, respectively. The presence of a *tert*-butyl group at the N-1 position has been shown to be very important for activity against gram-positive organisms (4). Two compounds, BMY 40062 and sparfloxacin, were more active or as active as ciprofloxacin against *E. coli*. One compound (BMY 40062) was more active than ciprofloxacin against *P. aeruginosa*, and two compounds, BMY 43261 and BMY 40397, were relatively inactive (MICs, 30 and 60 μg/ml, respectively). BMY 43261 was the least active quinolone against the three strains that were assayed. BMY 40397 was specific for *S. aureus* and was essentially inactive against both the *E. coli* and *P. aeruginosa* strains used. *P. aeruginosa* was generally the least susceptible organism, particularly to BMY 33315 and BMY 40397. The latter compound, (1*R*,4*R*)-*N*,*N*′-bis [3-carboxy-1,4-dihydro-1-(1,1-dimethylethyl)-6-fluoro-1,8-naphthyridin-7-yl]-2,5-diazobicyclo [2.2.1] heptane, is called the "bis compound" throughout this report, since it is composed of two BMY 40062 quinolone moieties symmetrically disposed relative to the piperazine ring. The bis compound was inactive against both gram-negative organisms, whereas it was most active against *S. aureus*. It is noteworthy that BMY 40062 retained activity against *P. aeruginosa* (MIC of BMY 40062, 0.25 μg/ml).

Accumulation experiments. Kinetic studies of accumulation were performed at 0, 5, 10, and 20 min. Data from the 20-min time were chosen to express the results, which are given in Table 1. Results were expressed as nanograms of drug per 10^9 bacteria, but the internal concentration, in micrograms per milliliter, was also calculated by assuming an internal volume of 2.3×10^{-9} μl for *E. coli* (37) and 1.14×10^{-9} μl for *S. aureus* (26). *S. aureus* was able to accumulate at least 100 ng of drug per 10^9 bacteria for all assayed compounds except ciprofloxacin. Among the 11 assayed molecules, 6 were taken up at concentrations of >200 ng/10^9 bacteria, with BMY 40397, the more bulky and hydrophobic drug, being accumulated the most (340 ng/10^9 bacteria) in *S. aureus*. Because the external concentration was 10 μg/ml, this led to a concentration factor of between 5 for ciprofloxacin and 34 for BMY 40397. The accumulation by *E. coli* was less for all compounds tested except ciprofloxacin, with a maximum of 120 ng/10^9 bacteria for BMY 40062 and a minimum of 40 ng/10^9 bacteria for BMY 40397, the bis compound. This corresponded to concentration factors of 5 and 2, respectively. Only four compounds, BMY 42230, BMY 40062, BMY 40124, and ciprofloxacin, were accumulated at a level of more than 100 ng/10^9 *E. coli* cells. In the case of *P. aeruginosa*, the accumulation was always less than 50 ng/10^9 bacteria, except for BMY 40062, which was taken up to an extent of 111 ng/10^9 bacteria. This compound was accumulated well by all the strains (220 ng/10^9 bacteria for *S. aureus*, 120 ng/10^9 bacteria for *E. coli*, and 111 ng/10^9 bacteria for *P. aeruginosa*).

Inhibitory activity on *E. coli* DNA gyrase. The results of testing of drug inhibitory activity on *E. coli* DNA gyrase are given in Table 1. They are expressed as MED, the minimum amount of drug required to cause any inhibition. Only two molecules produced an MED of ≥10 μg/ml; BMY 42230 had an MED of 10 μg/ml, and BMY 43261 had an MED of 40 μg/ml. BMY 43261 was the least active against the three assayed strains.

DISCUSSION

Figure 2 shows the relationship between intracellular accumulation in *S. aureus*, *E. coli*, and *P. aeruginosa* and log *D* for the quinolones tested. For *S. aureus*, there was a direct correlation between accumulation and hydrophobicity ($r = 0.71$). Such a relationship has previously been observed with quinolones by other investigators, but never with more than a few compounds (45). For *S. aureus*, the size of the molecule is not a limiting factor, since in the case of the quinolones BMY 40062 and BMY 40397, the more bulky and hydrophobic analog BMY 40397 entered the cell more easily.

In the case of *E. coli*, there was an inverse correlation between accumulation and hydrophobicity ($r = 0.73$), which was in good agreement with results of previous studies on quinolones (28). It is now generally recognized that the less hydrophobic a compound, the better its diffusion (33). Chapman and Georgopapadakou (8) found a positive correlation

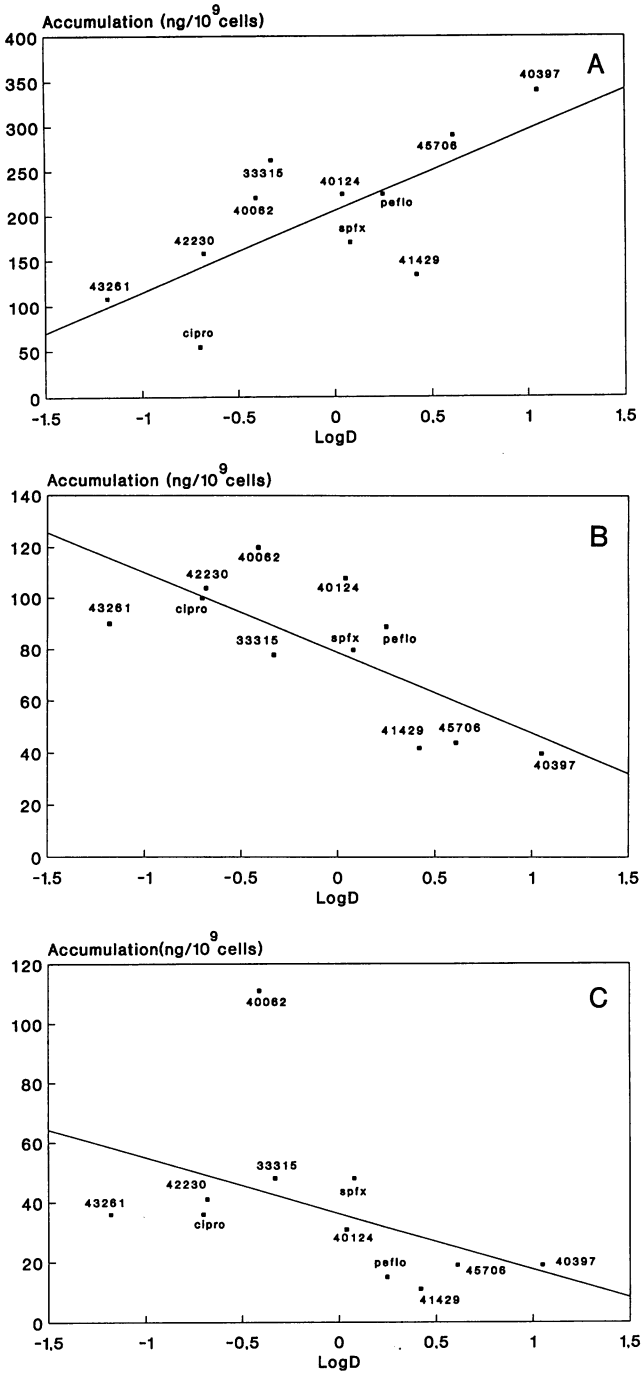

FIG. 2. Relationship between accumulation and log D for *S. aureus* (A), *E. coli* (B), and *P. aeruginosa* (C). The values are from Table 1 and Fig. 1. The curves were traced by least-squares analysis using the Harvard Graphic computer program. peflo, pefloxacin; spfx, sparfloxacin; cipro, ciprofloxacin. Numbers indicate BMY compounds.

of 0.73 between quinolone hydrophobicity and antagonism of antibacterial activity in the presence of 5 mM $MgCl_2$ for a series of 12 compounds when *E. coli* CS1293 ($OmpF^-$ $OmpC^-$ $OmpA^-$) was used. They suggested that the excess magnesium present prevented the quinolones from chelating lipopolysaccharide-associated magnesium, thus limiting their entry into the porin pathway. Our results with *E. coli* K-12 J53 are in agreement with those conclusions.

For *P. aeruginosa*, there was an inverse relationship between accumulation and log D, not taking into account compound BMY 40062 (r = 0.64). Compound BMY 40062 was accumulated to the highest degree by the two gram-negative organisms, with surprisingly high levels found in *P. aeruginosa*. It is possible that the bridged piperazine ring in compound BMY 40062 modifies its basic properties and increases the role of the charge factor, which is not taken into account. The existence of a correlation coefficient of only 0.44 between accumulation and log D for *P. aeruginosa* when BMY 40062 is included is in agreement with the more complex mechanism of quinolone accumulation by this strain, with important roles for both outer membrane proteins and lipopolysaccharide (5, 7, 25, 27). It is probable that hydrophobicity, size, and charge contribute to the accumulation of fluoroquinolones; the charge factor was not taken into account, since it is not easy to determine the percentage of the possible different ionic states in equilibrium at physiological pH (41). Comparison of Fig. 2B and C indicates that the accumulation of fluoroquinolones was less in *P. aeruginosa* than in *E. coli*. It is now well established that antibiotic permeation is low through the porin pathway of *P. aeruginosa* (7, 32). It has been suggested that porin F, the principal porin of this organism, may be substantially smaller than those of other gram-negative bacteria (22, 43), with a cutoff of 340 Da compared with a cutoff 650 Da for *E. coli* (6). Ashby et al. (1) did not find a relationship between molecular size and potency against *P. aeruginosa* for a series of quinolones with molecular weights of between 233 and 361. They concluded that the appropriate factor to be taken into account is not the molecular weight but rather the three-dimensional shape of the molecule. Other investigators (32) suggested that the lack of permeability of *P. aeruginosa* might be due to the low number of open porins.

There was no direct relationship between antibacterial activity and accumulation with the three microorganisms tested (r = 0.38, 0.37, and 0.24 for *S. aureus*, *E. coli*, and *P. aeruginosa*, respectively; data not shown). This suggests that other factors must be taken into account. For *E. coli*, the comparison of MICs and MED for gyrase showed a positive correlation (n = 11, r = 0.6), but with certain exceptions. BMY 40397, for example, has an MED of 1.2 μg/ml and a MIC of 1.75 μg/ml, while pefloxacin and BMY 33315, with MEDs of 1 μg/ml, have MICs of 0.2 and 0.11 μg/ml, respectively, which is in line with general results. If we consider the accumulation of BMY 40397, it is half those of pefloxacin and BMY 33315. This could explain how such a low MED can be associated with an unexpectedly high MIC. In contrast, BMY 42230 and BMY 40062 have high MEDs (10 and 0.6 μg/ml, respectively), whereas they have low MICs (0.22 and 0.03 μg/ml, respectively), which can be explained by their good accumulation by *E. coli* (104 and 120 ng/10^9 bacteria, respectively). These results are in good agreement with the conclusions of others (5, 11, 14) and indicate that the major factor in determining the potency of a quinolone is its activity against DNA gyrase, although other targets, such as interference with membrane or cell division, could be considered (42). If we correct the MED by taking into account the extent of drug accumulation and look at the correlation between MICs and MED and accumulation, the correlation coefficient rises to 0.85 (Fig. 3B).

We attempted to correlate the MICs of *S. aureus* and *P. aeruginosa* using the MED for *E. coli*. We found correlation coefficients of 0.74 and 0.64, respectively. Using the cor-

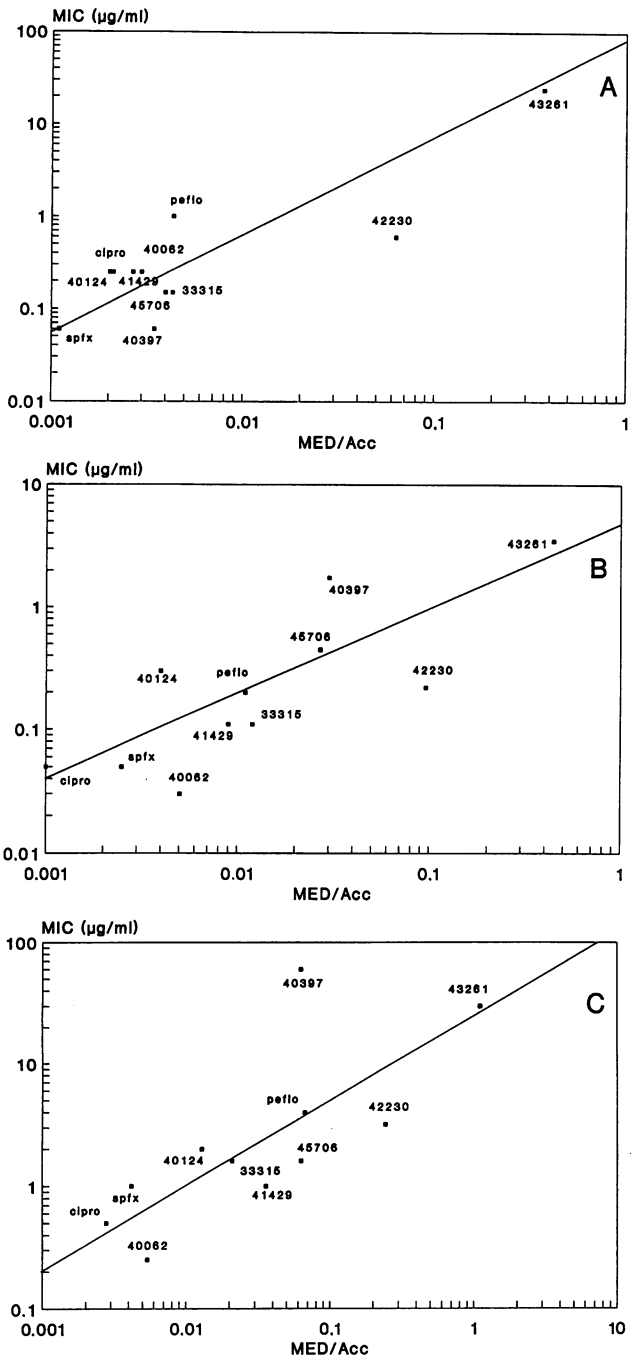

FIG. 3. Relationship between MIC and MED/accumulation (MED/Acc) for *S. aureus* (A), *E. coli* (B), and *P. aeruginosa* (C). MIC and accumulation were for all strains; MED was for *E. coli* only. The values are from Table 1, and least-squares linear regression analysis was used as described in the legend to Fig. 2. peflo, pefloxacin; spfx, sparfloxacin; cipro, ciprofloxacin. Numbers indicate BMY compounds.

rected MEDs, these coefficients were unchanged for *S. aureus* and rose to 0.74 for *P. aeruginosa* (Fig. 3). MEDs were not determined with *P. aeruginosa* or *S. aureus* DNA gyrase, but for the MED determined with the *E. coli* enzyme, the good correlations observed may indicate that the gyrases of the three organisms have comparable ranges of susceptibility to these fluoroquinolones. For *P. aeruginosa*, the correlation was better (0.85) without the bis compound BMY 40397, although BMY 40397 had a high MIC (60 μg/ml) and poor accumulation (19 ng/10^9 bacteria); the latter parameter is possibly overestimated, since such low values are difficult to measure with accuracy.

Compounds BMY 40062 and BMY 40397 differ with respect to molecular weight (336 and 574, respectively) and hydrophobicity; the bis analog does not share a basic nitrogen atom and, consequently, is considerably less charged at physiological pH. The bis compound was only twofold less active against DNA gyrase than the parent BMY 40062 was, but it was poorly active against *E. coli* because of a further threefold loss in accumulation (40 compared with 120 ng/10^9 bacteria); increases in both hydrophobicity and size perhaps explain this phenomenon. In the case of *S. aureus*, there was a 1.5-fold increase in accumulation between the two compounds, with a corresponding improvement in activity (Table 1).

The combined data for the series of quinolones examined in the present study strongly suggest that both activity against DNA gyrase and degree of accumulation are important factors governing the antibacterial activities of fluoroquinolones, with the activity against gyrase being the major contributing factor. With regard to intracellular accumulation, hydrophobicity appears to be a major feature for both gram-negative and gram-positive organisms. High molecular weight is a limiting factor only in gram-negative organisms, and it is dramatically so in *P. aeruginosa*. Consideration of both factors leads to useful correlations between the MIC and the MED for DNA gyrase when corrected for accumulation in *E. coli*, *S. aureus*, and *P. aeruginosa*. This could indicate that the DNA gyrases from the three assayed organisms have parallel affinities for these fluoroquinolones. Additional experiments are in progress to further explain the conclusions of the present study.

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