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Effect of the efflux pump inhibitor Phe-Arg-β-naphthylamide on the MIC values of the quinolones, tetracycline and chloramphenicol, in *Escherichia coli* isolates of different origin

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Sir,

The main mechanisms of quinolone resistance in *Escherichia coli* include mutations in the *gyrA* and *parC* target genes and decreased intracellular accumulation.[1] Active efflux pumps are important for intrinsic and acquired antibiotic resistance, and overexpression of efflux pumps affecting quinolones is becoming increasingly common in *E. coli.* Recently, efflux pump inhibitors have been investigated with a view to improving and potentiating the activity of exported antibiotics.[2] Phe-Arg-β-naphthylamide has been described as a broad-spectrum efflux pump inhibitor in *Pseudomonas aeruginosa*.[2] The effect of this inhibitor on fluoroquinolone susceptibilities in *Enterobacter aerogenes*,[3] *E. coli*,[4] *P. aeruginosa*,[2] *Acinetobacter baumannii*[5] and *Stenotrophomonas maltophilia*[5] has also been studied, but not for nalidixic acid and other antimicrobial agents in *E. coli*. Therefore in this study, we analysed the influence of the Phe-Arg-β-naphthylamide (Sigma) inhibitor on the activity of quinolones, chloramphenicol and tetracycline against a collection of 60 *E. coli* isolates previously characterized with respect to amino acid changes in both GyrA and ParC proteins.[6,7] The collection includes the strain Nor5, an *in vitro* selected quinolone-resistant mutant, in which the overexpression of MarA, SoxS and AcrA has been shown (J. Vila, B. Peter, J. Sánchez-Cespedes & M. Tario, unpublished data). The remaining 59 isolates were recovered from foods ($n = 11$), humans ($n = 25$) and animals ($n = 23$).

Susceptibilities to nalidixic acid, norfloxacin, chloramphenicol, tetracycline and ciprofloxacin were studied by agar dilution in the presence or absence of 20 mg/L of Phe-Arg-β-naphthylamide, in accordance with NCCLS guidelines. All of the 60 *E. coli* isolates showed an MIC value of >256 mg/L to Phe-Arg-β-naphthylamide. Table 1 shows the effect of 20 mg/L of the inhibitor Phe-Arg-β-naphthylamide on the MIC values of the five tested antibiotics in 60 *E. coli* isolates with different numbers of amino acid changes in GyrA and

Table 1. MIC values (in mg/L) of the five tested antibiotics in the presence or absence of 20 mg/L of Phe-Arg-β-naphthylamide in a series of 60 *E. coli* isolates organized by number of amino acid changes in GyrA and ParC proteins

No. of amino acid changes in			MIC_{50} (MIC ranges)								
GyrA	ParC	No. isolates	NAL	NAL+I	CIP	CIP+I	NOR	NOR+I	CHL	CHL+I	TET	TET+I
0	0	11	4 (2–4)	0.5 (<0.25–0.5)	0.06 (0.06)	0.06 (0.03–0.06)	0.125 (0.06–0.125)	0.125 (0.06–0.125)	16 (16)	4 (4)	2 (2–4)	2 (2–4)
1	0	23	128 (64–1024)	8 (4–16)	0.25 (0.125–1)	0.25 (0.125–0.25)	1 (0.5–4)	1 (0.5–2)	32 (8–512)	4 (1–256)	128 (2–256)	128 (1–256)
1	1	10	4096 (2048–>4096)	128 (64–512)	1 (1–4)	1 (0.5–2)	4 (4–32)	4 (4–32)	16 (4–512)	4 (1–128)	4 (2–256)	4 (2–256)
1	2	1	>4096	256	8	8	32	32	16	4	256	256
2	1	13	>4096 (≥4096)	256 (128–512)	8 (4–32)	4 (2–16)	16 (8–128)	16 (8–32)	16 (4–256)	4 (2–32)	128 (1–256)	128 (1–256)
2	2	2	≥4096	256	32, >64	32, 64	128	128	128, 512	64, 256	256	128, 256

NAL, nalidixic acid; I, Phe-Arg-β-naphthylamide; CIP, ciprofloxacin; NOR, norfloxacin; CHL, chloramphenicol; TET, tetracycline.

ParC proteins. The results show that the use of 20 mg/L of Phe-Arg-β-naphthylamide decreased the MIC of nalidixic acid (from two to five serial two-fold dilution steps) in all isolates tested, even in those with the nalidixic acid-susceptible phenotype. This high reduction in the MIC values was observed independent of the number of amino acid changes in GyrA and ParC. This effect was especially noticeable among isolates carrying one single GyrA substitution, in which the MIC_{50} of nalidixic acid decreased from resistant to susceptible levels (from 128 to 8 mg/L). This result indicates that one amino acid change in GyrA by itself (without an additional mechanism of resistance) is unable to confer a resistant phenotype to nalidixic acid in *E. coli*. On the other hand, almost no decreases in the MIC_{50} were detected in the fluoroquinolones tested. Nevertheless, Phe-Arg-β-naphthylamide affected the MIC of ciprofloxacin in 10 of the 60 *E. coli* isolates whose MIC values were lowered by at least two serial dilution steps. Eight of these 10 isolates also decreased the MIC of norfloxacin (in both cases including mutant Nor5). Interestingly, fluoroquinolone MIC values for these 10 isolates were higher than the MIC_{50} that corresponds with the number of substitutions present in the targets. The addition of the inhibitor decreased these high MIC values to expected MIC_{50} values (for example, the MIC of ciprofloxacin was decreased from 1 to 0.25 mg/L in one isolate with only a mutation in GyrA). The presence of 20 mg/L of Phe-Arg-β-naphthylamide also affected the MIC of chloramphenicol in 51 isolates whose MICs decreased by two or three serial dilution steps. Finally, no effect was observed when the MIC of tetracycline was analysed.

The effect of different concentrations of Phe-Arg-β-naphthylamide (40, 80 or 160 mg/L) on nalidixic acid, ciprofloxacin and norfloxacin MICs was also studied in 25 of the *E. coli* isolates. The influence of the Phe-Arg-β-naphthylamide was homogeneous in the range 20–160 mg/L of the inhibitor in all the isolates tested.

These results might be explained by the activity of the inhibitor against two or more efflux systems. The effect on the MIC of nalidixic acid in all the strains (both susceptible and resistant) suggests the presence of a constitutive efflux pump able to discharge the antibacterial agent and confer a basal level of resistance. Moreover, chloramphenicol might be another substrate of this hypothetical efflux pump. The Phe-Arg-β-naphthylamide only affects the MIC of fluoroquinolones in isolates with levels of resistance higher than those expected according to the presence of alterations in their targets. These high MIC values might be explained by the overexpression of the AcrAB efflux pump, as previously described,[2,3] although the overexpression of other efflux pumps cannot be ruled out. A proposal to be considered is the possible inhibitor effect over the outer membrane protein of the efflux system.

In summary, the inhibitor Phe-Arg-β-naphthylamide produces a decrease in the MIC of nalidixic acid in *E. coli* isolates with different susceptibilities to this quinolone. This result suggests the inability of a single amino acid substitution in the GyrA protein to confer resistance phenotypes to this antimicrobial agent. Moreover, the presence of a constitutive efflux pump able to discharge nalidixic acid easily is strongly suggested. These facts may pave the way to the design of novel combination therapies that could be used against strains with amino acid substitutions in GyrA.

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False-positive extended-spectrum β-lactamase tests for *Klebsiella oxytoca* strains hyperproducing K1 β-lactamase

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Sir,

The growing complexity of extended-spectrum β-lactamases (ESBLs) presents new detection challenges. Until recently, the predominant ESBLs in the UK were TEM- and SHV-derived ceftazidimases, occurring mostly in *Klebsiella* spp. On this basis it seemed adequate to screen with ceftazidime and to perform ceftazidime–