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Protective Effect of Qnr on Agents Other than Quinolones That Target DNA Gyrase

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Qnr is a plasmid-encoded and chromosomally determined protein that protects DNA gyrase and topoisomerase IV from inhibition by quinolones. Despite its prevalence worldwide and existence prior to the discovery of quinolones, its native function is not known. Other synthetic compounds and natural products also target bacterial topoisomerases. A number were studied as molecular probes to gain insight into how Qnr acts. Qnr blocked inhibition by synthetic compounds with somewhat quinolone-like structure that target the GyrA subunit, such as the 2-pyridone ABT-719, the quinazoline-2,4-dione PD 0305970, and the spiropyrimidinetrione pyrazinyl-alkynyl-tetrahydroquinoline (PAT), indicating that Qnr is not strictly quinolone specific, but Qnr did not protect against GyrA-targeting simocyclinone D8 despite evidence that both simocyclinone D8 and Qnr affect DNA binding to gyrase. Qnr did not affect the activity of tricyclic pyrimidoindole or pyrazolopyridones, synthetic inhibitors of the GyrB subunit, or nonsynthetic GyrB inhibitors, such as coumermycin A1, novobiocin, gyramide A, or microcin B17.Thus, in this set of compounds the protective activity of Qnr was confined to those that, like quinolones, trap gyrase on DNA in cleaved complexes.

Qnr was discovered as a plasmid-encoded protein that reduces susceptibility to quinolones (1). Quinolones are synthetic compounds that target the essential bacterial enzymes DNA gyrase and topoisomerase IV, homologous tetramers composed of GyrA and GyrB or ParC and ParE subunits, respectively, that introduce negative supercoils or unknot and decatenate the DNA helix with energy from ATP hydrolysis (2).Qnr is a pentapeptide repeat protein that blocks quinolone inhibition of both topoisomerases and binds to each of their subunits as well as to the holoenzymes (3–5). Many bacteria have *qnr*-like genes on the chromosome, some, especially in aquatic bacteria, closely related to plasmid-determined *qnr* varieties (6–8). The native function of these proteins, which clearly antedate the clinical use of quinolones, is not known.

A number of other agents target topoisomerases. Well-studied natural products include coumermycin A1 (9, 10), gyramide A (11), microcin B17 (12), novobiocin (9, 10), and simocyclinone D8 (13, 14). From mutational and other studies the sites of action of many of these agents are known. For example, the primary site of resistance mutations for quinolones in Gram-negative bacteria is a region on the GyrA subunit known as the quinolone resistance-determining region (QRDR) (15), while novobiocin targets ATPase activity of the GyrB subunit (9). Qnr does not protect against novobiocin inhibition of gyrase (16), but the protective effect of Qnr on other natural products is not yet known. Medicinal chemists have synthesized synthetic compounds of various structures intended to act on gyrase at sites different from those directly affected by quinolones, especially the GyrB subunit. Whether Qnr protects gyrase from such compounds has not been investigated. The aim of this study was to use such natural and synthetic inhibitors as molecular probes to gain insight into how Qnr protects DNA gyrase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used are shown in Table 1. Strains were routinely grown in Luria-Bertani broth at 37°C. Culture plates contained Mueller-Hinton agar (Becton, Dickinson and Co., Sparks, MD). Plasmids were transferred to *Escherichia coli* J53 Azi^r (azide resistant) by transformation or conjugation using 100 μg/ml of ampicillin, 25 μg/ml of ceftazidime, or 25 μg/ml of chloramphenicol for selection and, where necessary, 200 μg/ml of sodium azide for counterselection. Isopropyl-β-D-thiogalactopyranoside (IPTG) at 100 μM was used to maximize QnrB production with M15 pREP3 pQE-60-QnrB1.

Susceptibility testing. Disk and agar dilution susceptibility testing was performed as described by the CLSI, using Mueller-Hinton agar, an inoculum of 10^4 CFU, and 16 to 20 h of incubation at 37°C (17). *E. coli* J53 Azi^r and ATCC 25922 were used for quality control. Blank disks were obtained from Becton, Dickinson, and Co.

Chemicals. Ciprofloxacin, coumermycin A1, and novobiocin came from Sigma-Aldrich Co., St. Louis, MO. ABT-719 was provided by Abbott Laboratories, Abbott Park, IL. PD 0305970 came from Pfizer Global Research and Development, Ann Arbor, MI. C3 and C4 came from Trius (subsequently acquired by Cubist). CB-220,404-AB-4 and CB-241,957-AD-2 came from Cubist Pharmaceuticals Inc., Lexington, MA. Simocyclinone D8 came from AdipoGen, San Diego, CA, and (*R*)-gyramide A came from Glixx Laboratories, Southborough, MA. Pyrazinyl-alkynyl-tetrahydroquinoline (PAT) was provided by AstraZeneca, Waltham, MA.

DNA gyrase supercoiling assay. DNA supercoiling assays were performed with *E. coli* DNA gyrase and relaxed plasmid pUC19 in gyrase reaction buffer at pH 7.5 containing 35 mM Tris-HCl, 24 mM KCl, 4 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1.75 mM ATP, 5 mM spermidine, 0.1 mg/ml of bovine serum albumin (BSA), and 6.5% glycerol, all from New England BioLabs Inc., Ipswich, MA. The extent of supercoiling was gauged by the intensity of the lowest DNA band as measured with a Gel Doc EZ Imager (Bio-Rad, Hercules, CA). QnrB1 was purified from *E. coli* BL21(DE3) pET28a:QnrB1 as previously described (16).

Received 2 June 2015 Returned for modification 18 July 2015
Accepted 30 July 2015
Accepted manuscript posted online 3 August 2015
Citation Jacoby GA, Corcoran MA, Hooper DC. 2015. Protective effect of Qnr on agents other than quinolones that target DNA gyrase. Antimicrob Agents Chemother 59:6689–6695. doi:10.1128/AAC.01292-15.

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Escherichia coli strains		
J53 Azi^r	Plasmid recipient	46
BL21(DE3)	Expression host	Agilent Technologies
M15 pREP4	Expression host	Qiagen
EW1b	Δ*tolC*	47
RYC1000 pMM39	Microcin B17 producer	48
Plasmids		
pMG252	*qnrA1*	1
pMG253	*qnrA1* cloned	3
pMG298	*qnrB1*	27
pET28a:QnrB1	*qnrB1* cloned, His_6 tagged, IPTG inducible	16
pQE-60-qnrB1	*qnrB1* cloned, His_6 tagged, IPTG inducible	27
pMG306	*qnrS1*	49

Cross-streak assay. The assay was adapted from a pyocin typing technique (18). The producer strain *E. coli* RYC1000 pMM39 was streaked diametrically across a glass petri dish containing Mueller-Hinton agar as a band about 1 cm wide. After overnight incubation, bacterial growth was removed with a glass slide, and 3 to 5 ml of $CHCl_3$ was placed in the inverted lid and covered with the bottom of the dish for 15 min to kill residual organisms. $CHCl_3$ in the lid was discarded, and the plate was opened for a few minutes to allow residual vapor to escape. Cultures of test organisms were then streaked at right angles to the original inoculum, and the plate was reincubated overnight.

RESULTS

Synthetic GyrA subunit inhibitors. ABT-719 (Fig. 1) is a 2-pyridone inhibitor of bacterial DNA gyrase differing from a fluoroquinolone by placement of the nitrogen atom in the ring juncture (19, 20). Ten micrograms of ABT-719 added to a 6-mm blank disk produced an inhibitory zone of 13 mm on a lawn of *E. coli* J53 Azi^r but a 6-mm (no inhibition) zone with J53 Azi^r pMG252, indicating that *qnrA1* protects against ABT-719.

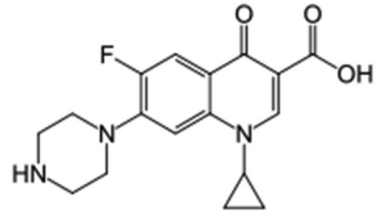
PD 0305970 is a quinazoline-2,4-dione (Fig. 1) that inhibits bacterial DNA gyrase and topoisomerase IV. It shares structural similarity with quinolones but remains effective against quinolone-resistant mutants of *Streptococcus pneumoniae* (21, 22). A 10-μg PD 0305970 disk produced an inhibitory zone of 29 mm on J53 Azi^r but only 18 mm with J53 Azi^r pMG252, indicating that *qnrA1* also protects against PD0305970.

Pyrazinyl-alkynyl-tetrahydroquinoline (PAT) is a spiropyrimidinetrione (Fig. 1) that inhibits DNA gyrase and topoisomerase IV and retains activity against quinolone-resistant mutants of *Staphylococcus aureus* and *S. pneumoniae* (23). A 10-μg disk of PAT produced a 28-mm zone of inhibition with EW1b Δ*tolC* and a 24-mm zone with EW1b pMG253 containing the cloned *qnrA1* gene. To confirm Qnr protection, the ability of purified QnrB1 to reverse PAT inhibition of gyrase supercoiling was studied *in vitro*. An 8 μM concentration of PAT inhibited gyrase activity by 78%, and QnrB reversed this inhibition with a 50% inhibitory concentration (IC_{50}) of 14 nM (Fig. 2). Hence, Qnr also protects against compounds with a PAT-like structure.

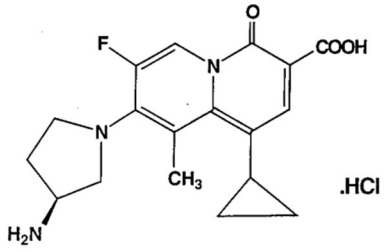
Synthetic GyrB subunit inhibitors. C3 and C4 are tricyclic pyrimidoindole inhibitors of the GyrB and ParE subunits (24). In *E. coli* J53 Azi^r or EW1b Δ*tolC*, QnrA, QnrB, or QnrS lacked a protective effect (Table 2).

CB-220 and CB-241 are pyrazolopyridones that are also dual inhibitors of GyrB and ParE (25). QnrA, QnrB, or QnrS did not block their activity against *E. coli* with or without a *tolC* deletion (Table 2).

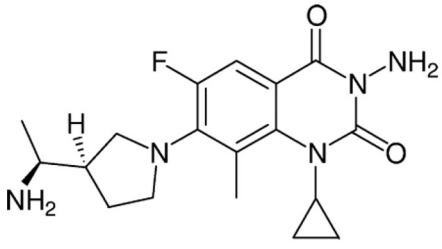
Natural product GyrA inhibitors. Simocyclinone D8 is a chlorinated aminocoumarin linked to an angucyclic polyketide via a



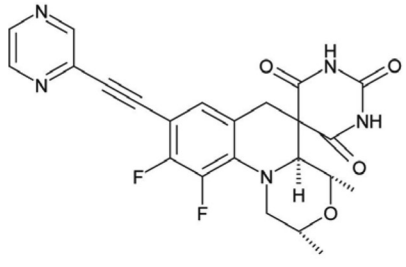
Ciprofloxacin



ABT-719



PD 0305970



Pyrazinyl-alkynyl-tetrahydroquinoline (PAT)

FIG 1 Chemical structures.

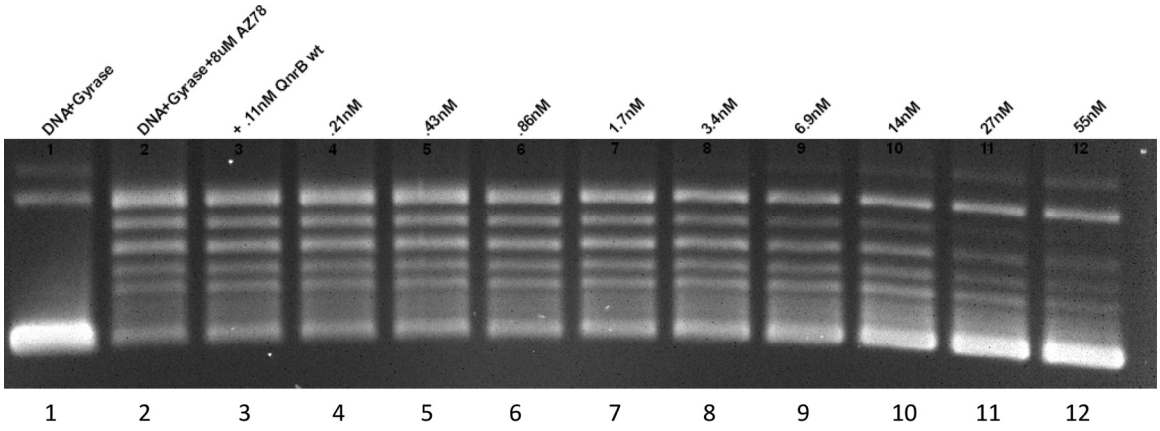


FIG 2 Protective effect of QnrB1 against gyrase inhibition by PAT. Lane 1, control reaction of relaxed pUC19 substrate and DNA gyrase. Lanes 2 to 12, DNA substrate, gyrase, and 8 μM PAT with no QnrB1 (lane 2) or with QnrB1 at concentrations of 0.11 nM (lane 3), 0.21 nM (lane 4), 0.43 nM (lane 5), 0.86 nM (lane 6),1.7 nM (lane 7), 3.4 nM (lane 8), 6.9 nM (lane 9), 14 nM (lane 10), 27 nM (lane 11), and 55 nM (lane 12).

tetraene linker and a D-olivose sugar produced by *Streptomyces antibioticus*. Despite its aminocoumarin moiety, it does not inhibit GyrB ATPase activity but rather binds to the GyrA subunit near the QRDR, preventing DNA binding (14, 26). No inhibitory activity was evident with a 25-μmol simocyclinone D8 disk on a lawn of *E. coli* J53 Azi^r or EW1b, but *in vitro*, simocyclinone D8 was at least as potent as ciprofloxacin in inhibiting DNA gyrase supercoiling. QnrB1 did not block simocyclinone D8 inhibition of DNA gyrase, and QnrB1 at a high concentration (27) still inhibited the enzyme in the presence of simocyclinone (Fig. 3).

Natural product GyrB inhibitors. Coumermycin A1 is an aminocoumarin produced by a *Streptomyces* sp. Qnr had no protective effect (Table 2).

Novobiocin is another aminocoumarin produced by *Streptomyces niveus*. Qnr was not protective in whole cells (Table 2), just as QnrB1 failed to block novobiocin inhibition of DNA gyrase *in vitro* (16).

Gyramide A is an *N*-benzyl-3-sulfonamidopyrrolidine produced by an *Streptomyces* sp. It inhibits GyrB ATPase activity and produces chromosome condensation halting DNA replication and segregation (11). Qnr did not protect against gyramide A inhibition (Table 2).

Microcin B17 is a plasmid encoded, peptide-derived antibiotic containing oxazoles and thiazoles. It was tested by cross-streaking a microcin B17 producer with test strains. Figure 4 shows that *E. coli* producing microcin B17 is protected from its action, but *E. coli* J53 Azi^r with *qnrA*, *qnrB*, or *qnrS* was just as susceptible as the plasmid-free strain, and addition of IPTG to stimulate QnrB production when the gene was cloned in an IPTG-inducible expression vector failed to disclose a protective effect.

DISCUSSION

Qnr protects against all fluoroquinolones tested (28, 29) and at a lower level against nalidixic acid, technically a naphthyridone. Reduced susceptibility to these compounds maps to the QRDR of GyrA, where in *E. coli* amino acid substitutions at specific residues between amino acids 51 and 106 affect susceptibility. This is the cleavage-ligation region of the enzyme where gyrase forms a covalent bond with DNA and quinolone intercalation into DNA increases the concentration of cleavage complexes and facilitates formation of lethal double-strand DNA breaks (30, 31). In Gram-negative bacteria, the GyrB subunit and topoisomerase IV are more resistant to quinolones than GyrA, but once GyrA becomes less susceptible by mutation, mutations in GyrB at residues 426 and 447, in ParC at positions 78, 80, and 84, and in ParE at residue 445 can further reduce susceptibility (32). Qnr is a pentapeptide repeat protein that dimerizes and folds into a rod-like molecule with a size and surface charge similar to those of B-form DNA (16). It competes with quinolone for gyrase *in vitro* (3) and in cells lowers susceptibility to the level of a GyrA mutation, suggesting that it may interact with GyrA as a DNA mimic in the QRDR region or DNA gate. However, in a gel displacement assay (4, 5) or bacterial two-hybrid system (33), Qnr binds to GyrB as well as to GyrA and to both subunits of topoisomerase IV as well as to the

TABLE 2 MICs of test compounds with Qnr-containing strains

E. coli strain	PMQR[a]	MIC (μg/ml)							
		Ciprofloxacin	Coumermycin A1	Novobiocin	Gyramide A	Cubist CB-220	Cubist CB-241	Trius C3	Trius C4
J53		0.016	8	2,048	>8	>10	>10	1.28	0.64
J53 pMG252	*qnrA1*	0.512	16	512	>8	>10	>10	1.28	0.64
J53 pMG298	*qnrB1 aac(6′)Ib-cr*	0.512	16	2,048	>8	>10	>10	0.64	0.64
J53 pMG306	*qnrS1*	2.048	16	512	>8	>10	>10	1.28	0.64
EW1b Δ*tolC*		0.004	4	1	1.024	0.32	0.16	0.008	0.04
EW1b pMG253	*qnrA1*	0.128	4	1	1.024	0.16	0.08	0.004	0.04

[a] PMQR, plasmid-mediated quinolone resistance.

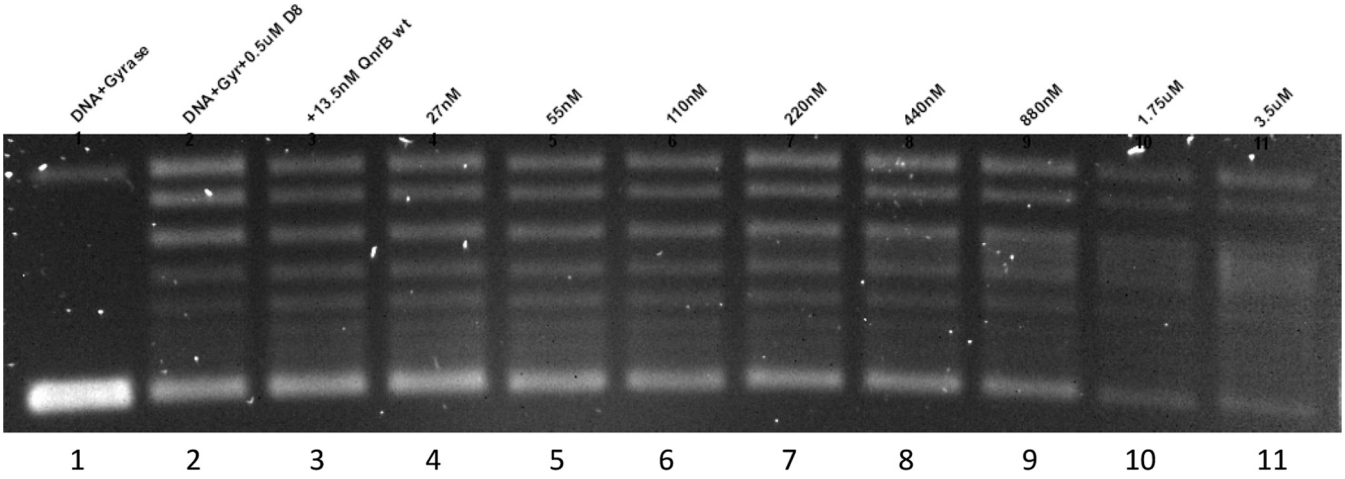


FIG 3 Lack of protection by QnrB1 against gyrase inhibition by simocyclinone D8. Lane 1, control reaction of pUC19 substrate and DNA gyrase. Lanes 2 to 11, DNA substrate, gyrase, and 0.5 μM simocyclinone D8 with no QnrB1 (lane 2) or with QnrB1 at concentrations of 13.5 nM (lane 3), 27 nM (lane 4), 55 nM (lane 5), 110 nM (lane 6), 220 nM (lane 7), 440 nM (lane 8), 880 nM (lane 9), 1.75 μM (lane 10), and 3.5 μM (lane 11).

holoenzymes, suggesting that a broader range of potential interactions is possible.

Table 3 summarizes information about the compounds tested to date for interaction with Qnr. Those that Qnr fails to protect against include coumermycin A1, novobiocin, and gyramide A, which act at the ATPase site of GyrB or ParE. Resistance to the aminocoumarin compounds in both *E. coli* (34) and *S. aureus* (35) map in GyrB at sites distinct from those contributing to quinolone resistance. Similarly, resistance to gyramide A, another competitive inhibitor of GyrB ATPase activity, occurs at sites separate from those for both quinolones and aminocoumarins. Qnr also failed to protect against synthetic GyrB ATPase inhibitors, such as pyrazolopyridone or tricyclic pyrimidoindoles compounds. Lack of protection against the GyrB targeting microcin B17 is interesting because organisms that produce this toxin protect themselves in part by coproduction of McbG, a pentapeptide repeat protein with antiquinolone activity (D. Hooper and J.-L. Yu, personal communication) that blocks gyrase inhibition by the microcin. Evidently the pentapeptide repeat Qnr proteins lack this microcin protective effect.

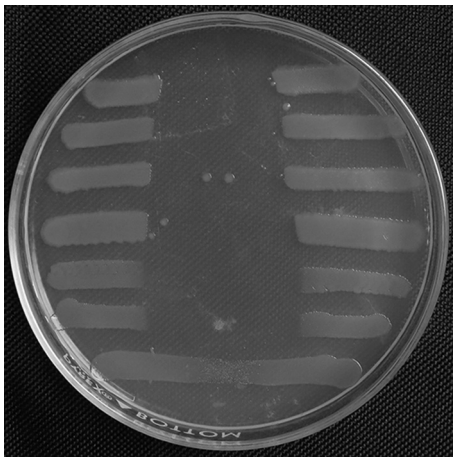


FIG 4 Cross-streak test for microcin B17 inhibition. The producer strain RYC1000 pMM39 was grown as a vertical band and removed, leaving microcin B17 in the agar, which was cross-streaked at right angles with (from the top) *E. coli* J53 Azi^r, J53 pMG298 (*qnrB1*), J53 pMG252 (*qnrA1*), J53 pMG306 (*qnrS1*), M15 pREP4 pQE-60, M15 pREP4 pQE-60-qnrB1, and RYC1000 pMM39 and reincubated. The Mueller-Hinton agar medium contained 100 μM IPTG to maximize QnrB1 production from expression plasmid pQE-60-qnrB1.

Qnr does protect against the synthetic compounds shown in Fig. 1, which have some structural similarity to a quinolone. Detailed structural analysis is available for the interaction of the dione PD 0305970 with a partially reconstructed topoisomerase IV of *Streptococcus pneumoniae* (30) and a similar quinazoline dione with topoisomerase IV of *Bacillus anthracis* (36). Quinolone and dione form similar cleavage complexes at the DNA gate but differ in their binding to neighboring amino acid residues, thus explaining the lack of cross-resistance between the two inhibitors. Evidently Qnr does not recognize such differences and blocks gyrase inhibition by both agents. ABT-719 and PAT probably have binding sites sufficiently near the quinolone binding site so that Qnr can block their action as well. The CcdB and ParE toxins of plasmid addiction systems have recently been shown to act without inhibition by Qnr (37). The mechanism of CcdB toxicity has been studied in greater detail than that of ParE (38, 39). CcdB binding requires gyrase to be in an open confirmation. The toxin also inhibits gyrase by stabilizing the cleavage complex but differs from quinolones in the site of resistance mutations. Lack of protection against simocyclinone D8 is intriguing because this agent binds to the N-terminal domain of GyrA, like quinolones, with mutations at such GyrA residues as 81, 83, 84, and 87 providing resistance to both agents (14). Furthermore, simocyclinone blocks DNA binding such that if Qnr functions as a DNA mimic, competition between Qnr and simocylinone would be expected. Nevertheless, none was seen. Not only did QnrB not prevent simocyclinone inhibition of gyrase supercoiling, but also it failed to prevent gyrase inhibition seen with QnrB at high concentrations (27, 40). Thus, no nonsynthetic agent has been found for which Qnr provides protection, although several potential candidates have not been available for testing, including albicidin (41, 42), clerocidin (43, 44), and cystobactamid (45).

Qnr reduces quinolone susceptibility but not to the CLSI-de-

TABLE 3 Summary of gyrase inhibitors tested for Qnr protection

Compound(s)	Target	Stabilization of covalent complex	ATPase inhibition	Inhibition of topoisomerase IV	Resistance mutations (in *E. coli* unless otherwise specified)	Qnr Protection	Reference(s)
CcdB	Binds as a dimer to the dimerization domain of GyrA while strand passage takes place	Yes		No	GyrA mutation at amino acid 462 with no cross-resistance with quinolone	No	37–39, 50
Coumermycin A1	Competitve inhibitor of GyrB and ParE ATPase activity	No	Yes	Yes	GyrB mutations at sites other than those associated with quinolone resistance: amino acids 73, 77, 136, 164, and 165	No	34, 35, 51, 52
Gyramide A	Competitive inhibitor of GyrB ATPase activity	No	Yes	No	At sites in GyrA (amino acids 34, 35, 45, 96, 97, 98, 169, 170, 172, 173, 267, and 335) and GyrB (amino acid 508) without effect on quinolone or novobiocin susceptibility	No	11, 53
Microcin B17	Binds to C-terminal domain of GyrB, slowing DNA strand passage	Yes		No	GyrB mutation at amino acid 751 confers resistance, but some quinolone resistance mutations at amino acid 83 in GyrA or amino acid 447 in GyrB provide partial resistance	No	12, 54, 55
Novobiocin	Competitve inhibitor of GyrB and ParE ATPase activity	No	Yes	Yes	GyrB mutations at sites other than those associated with quinolone resistance: amino acids 73, 77, 136, 164, and 165	No	16, 34, 35, 51, 52
ParE toxin	DNA gyrase	Yes				No	37, 56
Simocyclinone D8	N-terminal domain of GyrA, preventing DNA binding. Second weak binding site on C terminus of GyrB.	No	No	No	GyrA mutations (amino acids 42, 44, 45, 80, 81, 83, 84, 87, 91, and 120), some of which confer quinolone resistance	No	13, 14, 26, 57–59
ABT-719	DNA gyrase	Yes				Yes	19
PD 0305970	DNA gyrase	Yes		Yes	In *S. pneumoniae* mutations in toprim domain of GyrB at amino acids 456 and 474 or in ParE at 435 and 475. No cross-resistance with several quinolone resistance mutations.	Yes	21, 22, 30
PAT	DNA gyrase	Yes			No cross-resistance with quinolones in *S. aureus* or *S. pneumoniae*	Yes	23, 60, 61
Pyrazolopyridones	GyrB					No	25
Quinolones	GyrA, ParC, GyrB, ParE with gyrase more susceptible than topoisomerase IV for *E. coli*	Yes	No	Yes	QRDR GyrA: amino acids 51, 67, 81, 82, 83, 84, 87, and 106. QRDR GyrB: amino acids 426 and 447. QRDR ParC: amino acids 78, 80, and 84. QRDR ParE: amino acid 445.	Yes	3–5, 32
Tricyclic pyrimidoindoles	GyrB and ParE at the ATP binding site		Yes	Yes		No	24

fined breakpoint. It does, however, facilitate the selection of more quinolone-resistant mutants (1). Lack of protection by Qnr is thus a desirable property for a new therapeutic agent, and the results of this study suggest which compounds that target DNA gyrase are likely to escape this effect.

ACKNOWLEDGMENTS

We thank the suppliers of the proprietary compounds used in this study.

This work was supported by grant R01 AI057576 from the U.S. Public Health Service, National Institutes of Health, to D.C.H. and G.A.J.

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