Pergamon

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Bioorganic & Medicinal Chemistry Letters 13 (2003) 1745–1749

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Piperazinyl-Linked Fluoroquinolone Dimers Possessing Potent Antibacterial Activity Against Drug-Resistant Strains of *Staphylococcus aureus*

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Received 11 December 2002; revised 17 February 2003; accepted 27 February 2003

Abstract—The synthesis of symmetric and asymmetric piperazinyl-linked dimers of the fluoroquinolone class of antibiotics is described. Specific dimers are shown to possess potent antibacterial activity against drug-resistant strains of *Staphylococcus aureus*, including strains possessing resistance due to the NorA multidrug efflux pump and a mutation in the quinolone resistance-determining region of topoisomerase IV.

The fluoroquinolone (FQ) class of antibiotics is widely used in the treatment of gram-positive and gram-negative bacterial infections.[1] The FQs exert their antibacterial action by interfering with the function of two bacterial type-II topoisomerase enzymes, DNA gyrase and topoisomerase IV.[1] In most gram-positive bacteria, including *Staphylococcus aureus* (*S. aureus*), topoisomerase IV is the primary target of the FQs. Resistance to FQs has occurred in virtually all bacteria that the FQs are used against, seriously challenging the clinical effectiveness of this class of antibiotics.[2]

The primary mechanism of resistance to FQs is the occurrence of point mutations resulting in amino acid substitutions within the topoisomerase enzymes DNA gyrase and/or topoisomerase IV.[2,3] Such mutations within the respective genes occur almost exclusively in a region encoding the interface region of the topoisomerase homodimer that has been termed the quinolone resistance-determining region (QRDR). The topoisomerase homodimer interface region, containing the QRDR, encompasses the enzyme active site and is where the putative FQ-topoisomerase–DNA complex forms.[4] The ineffectiveness of the FQs due to amino acid substitution is likely due to direct loss of binding contact, although other effects such as subtle changes to the topoisomerase–DNA complex that affect FQ binding to the DNA, the enzyme or both are also possible.

Bacterial resistance to FQs also occurs by way of membrane-based efflux pumps.[5] The development of efflux-mediated resistance to the FQ antibiotics, as well as other antibiotics, generally occurs through up-regulating the genes encoding transporters that efficiently expel the drug from the bacterial cell. Efflux-mediated resistance to the FQs in *S. aureus*, including methicillin-resistant strains, is mediated by the *norA*-encoded efflux pump.[5,6] As a direct resistance mechanism, the NorA efflux pump affords sub-therapeutic intracellular antibiotic concentrations by expelling substrates upon their entering the membrane or cytoplasm.[5,6] NorA also contributes indirectly to other types of resistance because exposure of bacteria to sub-therapeutic drug concentrations promotes the selection and expression of higher-level adaptive resistance mechanisms, such at target mutations.[7]

In the course of investigating novel strategies to prepare antibiotics that are refractory to efflux-mediated resistance

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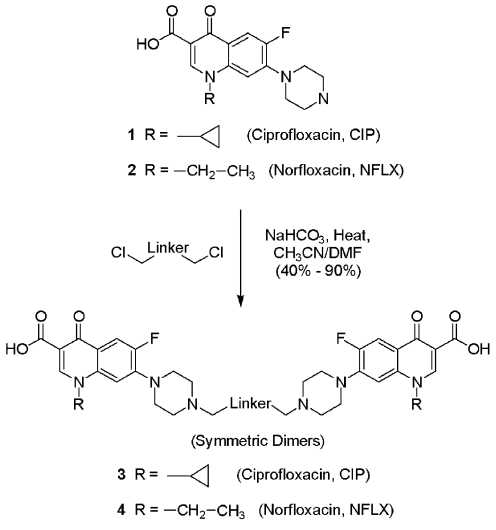
doi:10.1016/S0960-894X(03)00208-7

in gram-positive bacteria, a novel piperazinyl-linked FQ dimer was discovered to exhibit potent antibacterial activity against drug resistant strains of *S. aureus*, including strains overexpressing NorA and having a mutation in the QRDR of topoisomerase IV.[8] To begin investigating the structural features of the FQ dimer responsible for its potent antibacterial activity, a series of symmetric and asymmetric piperazinyl-linked FQ dimers was prepared. Synthesis of symmetric dimers of ciprofloxacin and norfloxacin employed a one-step strategy providing for the facile coupling of different monomers through different linkers (Scheme 1). Using this strategy, the piperazine-containing FQs and reactive bis-functional linkers were readily coupled to provide the desired dimers.[9]

Initially, symmetric *p*-xylenyl-linked and 1,4-*trans*-2-butenyl-linked dimers of ciprofloxacin and norfloxacin were prepared by employing α,α′-dichloro-*p*-xylene and *trans*-1,4-dichloro-2-butene respectively. Susceptibility testing (determination of MICs) with these dimers against a panel of drug resistant strains of *S. aureus* and comparison of activity to that of the parent monomers demonstrated that both the linker and the monomer used to make the dimer modulated antibacterial potency of the dimers, affording individual dimers with potent activity against each drug-resistant strain of *S. aureus* tested (Table 1). Most notable was the antibacterial activity profile of these symmetric ciprofloxacin and norfloxacin dimers against three defined strains of *S. aureus* (SA 1199, SA 1199-3 and SA 1199B).[6b–d] These strains possess varied levels of FQ resistance where SA 1199 is a wild-type isolate, SA 1199-3 is a laboratory-derived mutant of SA 1199 that inducibly overexpresses *norA* and has no DNA gyrase or topoisomerase IV mutations, and SA 1199B is a derivate of SA 1199 that constitutively overexpresses *norA* and harbors a topoisomerase IV A subunit substitution (A116E) known to correlate with raised FQ MICs.[10]

Interestingly, regardless of their inherent potency against wild-type SA 1199, the symmetric dimers of



Scheme 1. Synthetic strategy for the preparation of symmetric piperazinyl-linked fluoroquinolone dimers.[9]

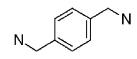
ciprofloxacin and norfloxacin typically maintained their antibacterial activity against both SA 1199-3 and SA 1199B, indicating that the dimers are not substrates for NorA and are not affected by an A116E substitution in topoisomerase IV.[12]

Direct comparison of the activity of ciprofloxacin and norfloxacin to their symmetric dimers against wild-type SA 1199 shows that antibacterial potency is greater for the ciprofloxacin dimer containing the *trans*-butenyl linker while the *p*-xylenyl linker afforded the more potent norfloxacin dimer. These results demonstrated that both the linker and the monomer used to construct the dimer play a role in dictating activity against a given strain of *S. aureus*. To further explore the effect of the monomer components on dimer activity a *p*-xylenyl linked ciprofloxacin-norfloxacin asymmetric dimer (CIP-XL-NFLX) and a *p*-xylenyl linked ciprofloxacin-pipemidic acid asymmetric dimer (CIP-XL-PIP) were prepared (Scheme 2). Since pipemidic acid alone is inactive at relevant concentrations against *S. aureus*, the *p*-xylenyl linked symmetric dimer of pipemidic acid was also prepared (Scheme 3), affording comparison of activity of the ciprofloxacin-pipemidic acid asymmetric dimer with the corresponding symmetric dimers of both ciprofloxacin and pipemidic acid.

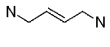
Susceptibility testing of the asymmetric dimers clearly demonstrates that antibacterial potency of a dimer does not correlate with the potency of the composing monomers (Table 1). The ciprofloxacin-norfloxacin asymmetric dimer (CIP-XL-NFLX) has greater antibacterial activity than the corresponding symmetric dimers of both ciprofloxacin (CIP-XL-CIP) and norfloxacin (NFLX-XL-NFLX) against most strains tested (Table 1). Furthermore, the virtually inactive pipemidic acid also afforded an extremely potent asymmetric dimer when linked to ciprofloxacin (CIP-XL-PIP) (Table 1). Surprisingly, even the symmetric dimer of pipemidic acid (PIP-XL-PIP) displayed relatively potent antibacterial activity, equaling or surpassing the activity of norfloxacin against these organisms (Table 1). These results indicate that each half of the piperazinyl-linked FQ dimers might participate in unique, non-equivalent, interactions with the topoisomerase, the DNA, or the putative topoisomerase–DNA complex.

Due to the differential effect of the linker on antibacterial activity observed with the *p*-xylenyl-linked and 1,4-*trans*-2-butenyl-linked ciprofloxacin and norfloxacin dimers, a series of symmetric ciprofloxacin dimers having different linkers were prepared as described in Scheme 1. The antibacterial activity of these dimers demonstrates that subtle structural differences in the linker impact dimer activity (Table 2). For example, the *p*-xylenyl linked ciprofloxacin dimer is equipotent with ciprofloxacin against wild-type SA 1199 while the 2,6-bis(chloromethy)pyridine and *m*-xylene linked ciprofloxacin dimers are more potent against SA-1199. As previously observed, these dimers maintain their increased potency against the ciprofloxacin and norfloxacin resistant strains SA 1199-3 and SA 1199B. Moreover, each of the symmetric ciprofloxacin dimers

Table 1. Antibacterial activity of symmetric and asymmetric dimers against drug-resistant strains of *S. aureus*.[11]



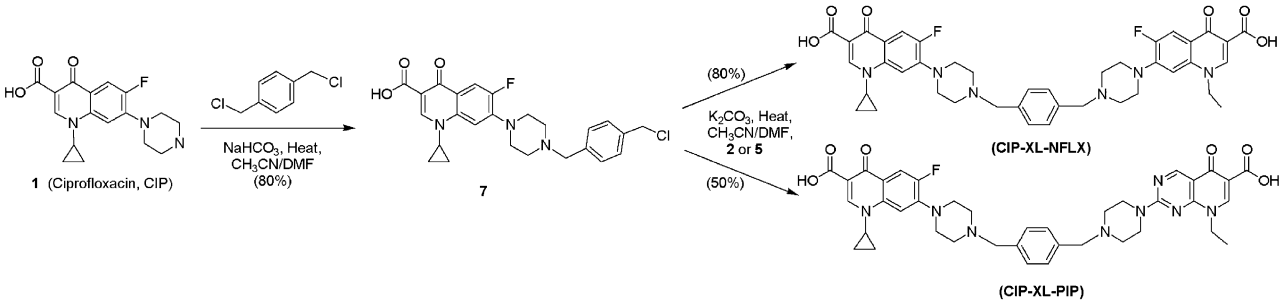
XL = *p*-Xylenyl Linked



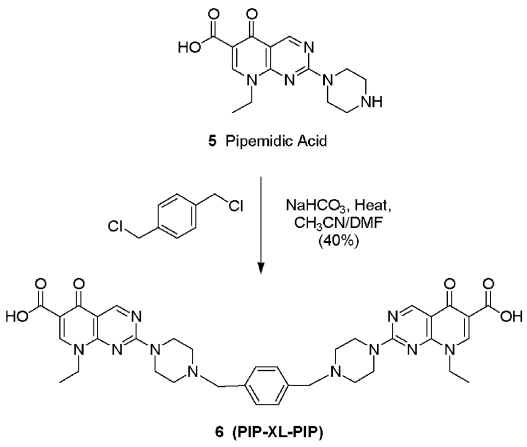
TB = *trans*-Butenyl Linked

	Minimum Inhibitory Concentrations (MICs, μg/mL)				
	Strains of *S. aureus*[a]				
Compd	SA 1199	SA 1199-3	SA 1199B	MRSA 494	GISA 992
1 (CIP)	0.125	1	8	0.5	32
2 (NFLX)	0.5	4	>16	2	>16
CIP-XL-CIP	0.125	0.125	0.125	0.125	4
CIP-TB-CIP	0.03	0.06	0.03	0.125	>16
NFLX-XL-NFLX	0.06	0.06	1	0.06	4
NFLX-TB-NFLX	0.25	0.125	0.125	0.125	>16
CIP-XL-NFLX	<0.03	<0.03	0.06	<0.03	4
5 (PIP)	>16	>16	>16	>16	>16
CIP-XL-PIP	<0.03	<0.03	<0.03	0.125	2
PIP-XL-PIP	0.5	0.5	4	2	8

[a]SA-1199, wild-type isolate; SA-1199-3, laboratory-derived mutant of SA-1199 that inducibly overexpresses *norA*, no gyrase or topoisomerase IV mutations; SA-1199B, constitutively overexpresses *norA* and harbors a topoisomerase IV A subunit substitution (A116E); MRSA 494, methicillin-resistant SA isolate; GISA 992, vancomycin-insensitive SA.



Scheme 2. Synthetic route employed to prepare asymmetric piperazinyl-linked dimers of ciprofloxacin with norfloxacin and pipemidic acid.[9]



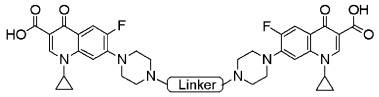
Scheme 3. Synthesis of the 1,4-xylenyl-linked symmetric dimer of pipemidic acid.[9]

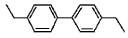
possessing the most potent activity against SA 1199, MRSA 494 and GISA 992 are composed of a different linker.

This effect of the linker group on antibacterial activity of the dimers is particularly notable since four piperazinyl-linked norfloxacin dimers composed of '*aliphatic*' linkers (e.g., hexyl) were reported to have very poor antibacterial activity, including against *S. aureus*, and were deemed not to be useful antibiotics.[13] The synthesis of a series of moderately active hybrid piperazine-linked bivalent quinolone derivatives was also reported.[14] In each of these cases, activity of the antibacterial quinolone dimers was worse than parent drug. Indeed, the unique and potent antibacterial activity of the dimers reported here would not have been initially observed if we had not been employing the unique series of drug-resistant strains of *S. aureus* (1199, 1199-3 and 1199-B) and studying resistance mechanisms.

FQ dimers and multimers are also reported in a series of patents based on 'multi-binding' compounds covering virtually all possible combinations of topoisomerase inhibitors having 2 to 10 ligands covalently connected, including FQs, but no antibacterial activities are reported.[15] In 1989 Shen et. al. reported the coupling of hydrophobic regions of norfloxacin together to test a model for FQ self-association and interaction with the DNA–DNA gyrase complex.[16] These dimers show linker dependant inhibition of DNA gyrase in vitro. This is interesting since the piperazinyl-linked dimers reported here are linked together in an entirely different manner and cannot directly adapt to this model, although the model was proposed well before the X-ray structure of DNA gyrase was reported.

Table 2. Comparison of the antibacterial activity of symmetric piperazinyl-linked ciprofloxacin dimers composed of different linking groups.[11]



	Minimum Inhibitory Concentrations (MICs, μg/mL)				
	Strains of *S. aureus*[a]				
Linker	SA 1199	SA 1199-3	SA 1199B	MRSA 494	GISA 992
	0.03	0.06	0.03	0.125	>16
	0.125	0.125	0.125	0.125	4
	0.06	0.06	0.125	<0.03	>16
	<0.03	<0.03	<0.03	0.06	8
	4	8	8	>16	>16
Ciprofloxacin monomer	0.125	1	8	0.5	>16

[a]See Table 1 for description of specific strains of *S. aureus*.

In summary, unique piperazinyl-linked dimers of the FQ class of antibiotics display increased antibacterial potency against drug-resistant strains of *S. aureus*, including FQ resistant strains possessing NorA efflux-mediated and topoisomerase IV substitution-mediated resistance mechanisms. Both the structure of the monomeric units and the linker comprising the dimer dramatically impact antibacterial activity. However, the effects on antibacterial activity do not correlate with the inherent activity of the parent monomer units used to form the dimer and the effect of a given linker on activity appears dependant on the monomers comprising the dimer. Therefore, the linker affording optimal activity for dimers of one FQ will not necessarily be optimal for dimers of a different FQ. Continued structure–activity-relationship studies, mechanistic and molecular interaction studies, and evaluation of antibacterial activity of the dimers against a broad spectrum of organisms are underway toward ascertaining their therapeutic potential, toward understanding their increased antibacterial potency and toward determining how these novel agents evade FQ resistance mechanisms in *S. aureus*.

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9. The coupling reactions outlined in Schemes 1 and 3 were performed by heating the piperazine-containing FQ with the bis-alkyl chloride in acetonitrile or DMF/acetonitrile mixture at 60–90 °C for 12 to 96 h. Similarly, stoichiometric excess of linker provided the partially coupled product **7** in Scheme 2, which was used to make the asymmetric dimers. Dimer-coupling reactions were followed using a combichem analytical reversed phase C-18 HPLC column since differentiation of products, intermediates and starting materials on TLC was difficult. For all final dimer products the reaction mixture was cooled, filtered, precipitated with excess diethyl ether, centrifuged and the recovered white solid dried. Purification of dimers was achieved using semi-preparative reversed-phase (C-18) HPLC employing a linear gradient of acetonitrile in water (0.1% TFA), affording separation of the desired products as a single peak. Acetonitrile was evaporated and the remaining aqueous sample lyophilized providing dimers in good to excellent isolated yield (40–90%). The lower yields were observed for dimers where attaching the second FQ to the linker is difficult, requiring higher reaction temperatures and affording recovery of partially linked products (e.g., **7**). Products were characterized by ^{1}H NMR, mass spectroscopic analysis and analytical HPLC showing a single peak at 220 nm and 254 nm.
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11. MICs were determined by broth microdilution following NCCLS guidelines using 2-fold dilutions of test compound. Error for MICs is therefore ± one dilution.
12. The symmetric norfloxacin dimers do not interfere with NorA-mediated efflux of ethidium bromide in SA 1199-B at concentrations up to five times the MIC. (Data not shown, see ref 6d for a description of this assay.) Therefore it is unlikely that the dimers are substrates for the NorA pump, accounting for their ability to evade this efflux mechanism.
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