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Synthesis and evaluation of dual-action kanglemycin-fluoroquinolone hybrid antibiotics

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ABSTRACT

Bacterial resistance threatens the utility of currently available antibiotics. Rifampicin, a cornerstone in the treatment of persistent Gram-positive infections, is prone to the development of resistance resulting from single point mutations in the antibiotic's target, RNA polymerase. One strategy to circumvent resistance is the use of 'hybrid' antibiotics consisting of two covalently linked antibiotic entities. These compounds generally have two distinct cellular targets, reducing the probability of resistance development and potentially providing simplified pharmacological properties compared to combination therapies using the individual antibiotics. Here we evaluate a series of semi-synthetic hybrid antibiotics formed by linking kanglemycin A (Kang A), a rifampicin analog, and a collection of fluoroquinolones. Kang A is a natural product antibiotic which contains a novel dimethyl succinic acid moiety that offers a new attachment point for the synthesis of hybrid antibiotics. We compare the activity of the Kang A hybrids generated via the acid attachment point to a series of hybrids linked at the compound's naphthoquinone ring system. Several hybrids exhibit activity against bacteria resistant to Kang A via the action of the partnered antibiotic, suggesting that the Kang scaffold may provide new avenues for generating antibiotics effective against drug-resistant infections.

Rifampicin, a member of the rifamycin class of antibiotics, is commonly used in the treatment of persistent Gram-positive bacterial infections, including those caused by *Mycobacterium tuberculosis*.[1] Rifampicin functions by inhibiting the bacterial RNA polymerase (RNAP).[2] Resistance to rifampicin most frequently occurs as the result of point mutations in the polymerase, with mutations in residues H451 and S456 of the RNAP RpoB subunit representing the most common resistance mutations in clinical isolates of *M. tuberculosis*.[3] Due to the risk of developing resistance, rifampicin is generally used in combination with additional antibiotics, which may create complicated dosing and pharmacological issues.[4] One strategy for circumventing resistance and potentially simplifying the pharmacological considerations involves the use of 'hybrid' antibiotics composed of two covalently linked antibiotic entities.[5,6] Hybrid antibiotics should retain efficacy in the event that resistance develops to either one of the two component antibiotics. Several rifamycin hybrids containing quinolones, macrolides, oxazolidinones, and nitroimidazoles are described in the patent literature.[7–11] One rifamycin-quinolone hybrid called TNP-2092 entered clinical trials for the treatment of GI tract infections by *Helicobacter pylori,* as well as prosthetic joint infections and other infections associated with biofilm formation.[12–14]

The kanglemycins (Kangs) represent a unique class of natural product rifamycin analogs that contain a dimethylsuccinic acid (K-acid) and a deoxysugar (K-sugar) appended at C-20 and C-27, respectively (Fig. 1).[15] While the K-acid and K-sugar confer activity against a common $RpoB^{S456L}$ mutation, the Kangs have poor activity against some other rifampicin resistance mutations including a frequently encountered $RpoB^{H451Y}$ mutation.[16,17] In the present study, we sought to circumvent these refractory mutations through the generation of Kang hybrid antibiotics. The Kangs had not been previously assessed as hybridization partners in this type of molecule.

There are several regions of the Kang structure that could be used as attachment points for a second antibiotic (Fig. 1). We first evaluated whether the K-acid could function in this capacity, as this structural feature is unique to the Kangs. As hybridization partners, we used fluoroquinolones, which were effective in a variety of previously generated hybrid antibiotics.[6] Fluoroquinolones interfere with DNA replication by inhibiting the bacterial DNA gyrase and topoisomerase IV.[18] We took advantage of the primary or secondary amine found in a number of fluoroquinolones to couple these antibiotics to the K-acid of Kang A (**1**),

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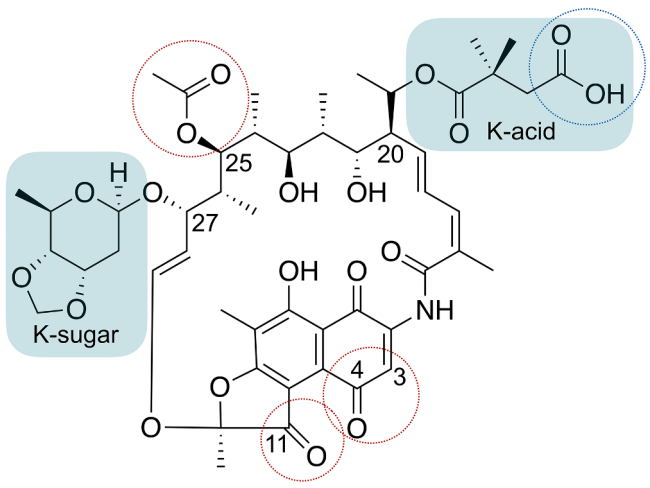

Figure 1. Potential attachment points for generating Kang A hybrids. The K-acid and K-sugar specific to the Kangs are highlighted in green. Potential entry points for hybridization are circled. The Kang-specific K-acid entry point is circled in blue.

the parent compound in the Kang family (Fig. 2). We expected the K-acid to function as a flexible linker between the core structures of the two antibiotics. We tested a total of nine fluoroquinolones as partners in our Kang hybrids.

The K-acid-based hybrids were screened against wild-type *Staphylococcus aureus* as well as rifampicin and ciprofloxacin resistant strains. The compounds were also screened against *Escherichia coli* as a representative Gram-negative bacterium. The rifampicin resistant *S. aureus* strain contained an RpoB^{H481Y} mutation (corresponding to *M. tuberculosis* RpoB^{H451Y}), which confers a very high level of resistance to rifampicin. The ciprofloxacin resistant strain contained common S80F

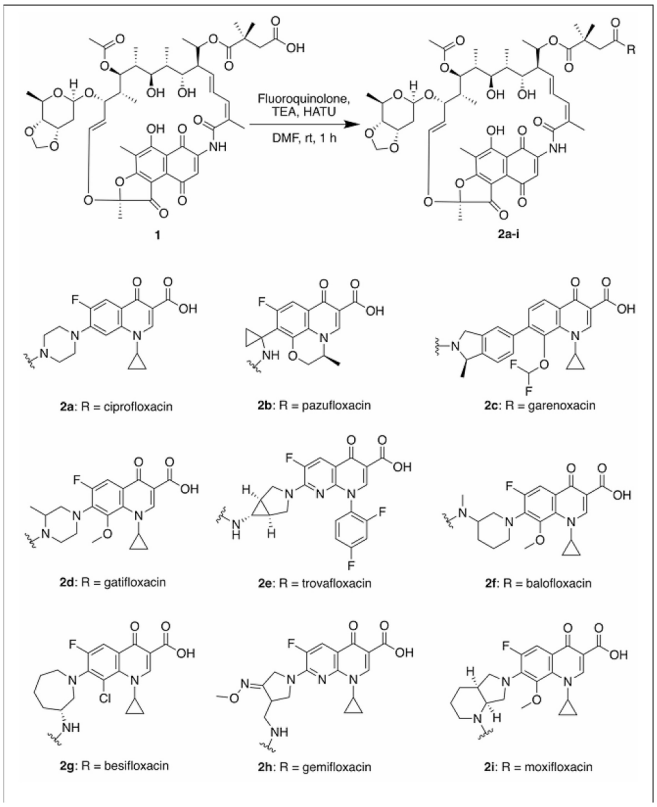

Figure 2. Synthesis of K-acid hybrids **2a-i**.

and S84L mutations in ParC and GyrA, respectively. The compounds were also tested against an additional *S. aureus* strain with a RpoB^{H481Y}/ParC^{S80F}/GyrA^{S84L} triple mutation. While Kang A was completely inactive against the RpoB^{H481Y} mutant (MIC > 64 μg/mL; Table 1), its activity compared to the wild-type *S. aureus* strain was unaffected by the ParC^{S80F}/GyrA^{S84L} double mutation. Kang A did not show Gram-negative activity against *E. coli*. The different fluoroquinolones showed distinct activities against the wild-type and ciprofloxacin resistant strains. Against wild-type *S. aureus* the MICs of the fluoroquinolones ranged from 0.25 μg/mL (ciprofloxacin) to 0.0078 μg/mL (trovafloxacin). The presence of the RpoB^{H481Y} mutation did not affect their activity. Against the strains containing the ParC^{S80F}/GyrA^{S84L} double mutation, the fluoroquinolones exhibited reductions in activity between 16- and 128-fold. Against *E. coli,* all of the fluoroquinolones exhibited MICs less than 1 μg/mL.

Of the K-acid hybrids, compounds **2a-i**, the most potent compounds against wild-type *S. aureus* were fusions of Kang A with pazufloxacin (**2b**), garenoxacin (**2c**), or trovafloxacin (**2e**), with MICs of 8 μg/mL (Table 1; since the hybrids differ considerably in size from the parent antibiotics, MIC values are also presented in Supplementary Table S1 in units of μM for ease of comparison). Several of the hybrids (**2c**, **2e**, and **2g**) acquired low-level activity (MIC = 16 μg/mL) against the strain containing the RpoB^{H481Y} mutation. This activity presumably results from the fluoroquinolone component of the hybrids, as the RpoB^{H481Y} strain shows a high level of resistance to Kang A (MIC >64 μg/mL). The K-acid hybrids also exhibited some activity against the ParC^{S80F}/GyrA^{S84L} fluoroquinolone resistant strain. Since this activity was lost against the RpoB^{H481Y}/ParC^{S80F}/GyrA^{S84L} triple mutant, it likely stems from the Kang A portion of the compounds. These results show that while some of the K-acid hybrids achieved a low level of activity against strains carrying mutations conferring either Kang A or ciprofloxacin resistance, utilization of the K-acid as a point of hybridization was accompanied by a significant decrease in antibiotic potency. It is possible that this finding reflects a limit to the size of the modifications to the K-acid that are tolerated without impairing activity. Consistent with this idea, in a previous study we found that small hydrocarbon substituents appended to the acid were well-tolerated while bulkier modifications led to reduced activity.[19]

We next compared the activity of the K-acid hybrids to a series of hybrids generated at the C-3/C-4 position of the Kang A structure (Fig. 1). This site has historically been the most common location of modifications in previously generated semi-synthetic rifamycins. We selected a subset of the fluoroquinolones used in the synthesis of the K-acid hybrids and fused them to Kang A via a benzoxazino modification introduced at C-3/C-4,[20] generating hybrids **5a-e** (Fig. 3). Since we were uncertain whether the rigidity of the benzoxazino moiety would limit the activity of the hybrids, in addition to directly fusing the fluoroquinolones to the ring system, we also generated a series of hybrids with a short flexible linker between the two antibiotic components (Fig. 3; compounds **6a-e**). Details of the synthesis of these compounds is described in the Supplementary Information associated with this manuscript.

The C-3/C-4 hybrids were screened against the same collection of strains as the K-acid hybrids. Against the wild-type *S. aureus* strain, the most potent C-3/C-4 fusions contained ciprofloxacin (**5a**) or moxifloxacin (**5e**), with MICs of 0.5 μg/mL, followed by garenoxacin (**5c**) or moxifloxacin with a flexible linker (**6e**), with MICs of 1 μg/mL (Table 2). Most of the hybrids had identical activities against the wild-type and ciprofloxacin resistant ParC^{S80F}/GyrA^{S84L} strains, suggesting that the observed activity against these strains was due to the Kang A portion of the molecules. The activity of the ciprofloxacin-containing hybrid **5a** against the ParC^{S80F}/GyrA^{S84L} strain provided further evidence that the Kang A component was active. This hybrid showed a 64-fold improvement in its activity compared to the 32 μg/mL MIC of ciprofloxacin against the same strain. This represents a ~277-fold improvement in its MIC in units of μM (Supplementary Table S1). As expected, this activity

Table 1
Activity of K-acid hybrids against wild-type and drug-resistant *S. aureus* and *E. coli*.

Compound	Hybridization partner	MIC (μg/mL)				
		S. aureus				*E. coli*
		WT	RpoB^{H481Y}	ParC^{S80F} GyrA^{S84L}	RpoB^{H481Y} ParC^{S80F} GyrA^{S84L}	
1	–	0.25	>64	0.25	>64	>64
Ciprofloxacin	–	0.25	0.25	32	16	0.016
Pazufloxacin	–	0.13	0.13	4	4	0.016
Garenoxacin	–	0.016	0.016	0.5	0.5	0.016
Gatifloxacin	–	0.063	0.063	2	2	0.016
Trovafloxacin	–	0.0078	0.016	1	1	0.0078
Balofloxacin	–	0.13	0.13	4	4	0.5
Besifloxacin	–	0.031	0.031	0.5	0.5	0.25
Gemifloxacin	–	0.016	0.016	1	1	0.0078
Moxifloxacin	–	0.016	0.016	1	1	0.031
2a	Ciprofloxacin	16	>64	32	>64	>64
2b	Pazufloxacin	8	>64	8	>64	>64
2c	Garenoxacin	8	16	8	>64	>64
2d	Gatifloxacin	64	>64	32	>64	>64
2e	Trovafloxacin	8	16	8	>64	>64
2f	Balofloxacin	32	>64	16	>64	>64
2g	Besifloxacin	16	16	16	>64	>64
2h	Gemifloxacin	16	>64	8	>64	>64
2i	Moxifloxacin	32	>64	32	>64	>64

[a]MIC assays were performed in duplicate with the same results obtained for each trial.

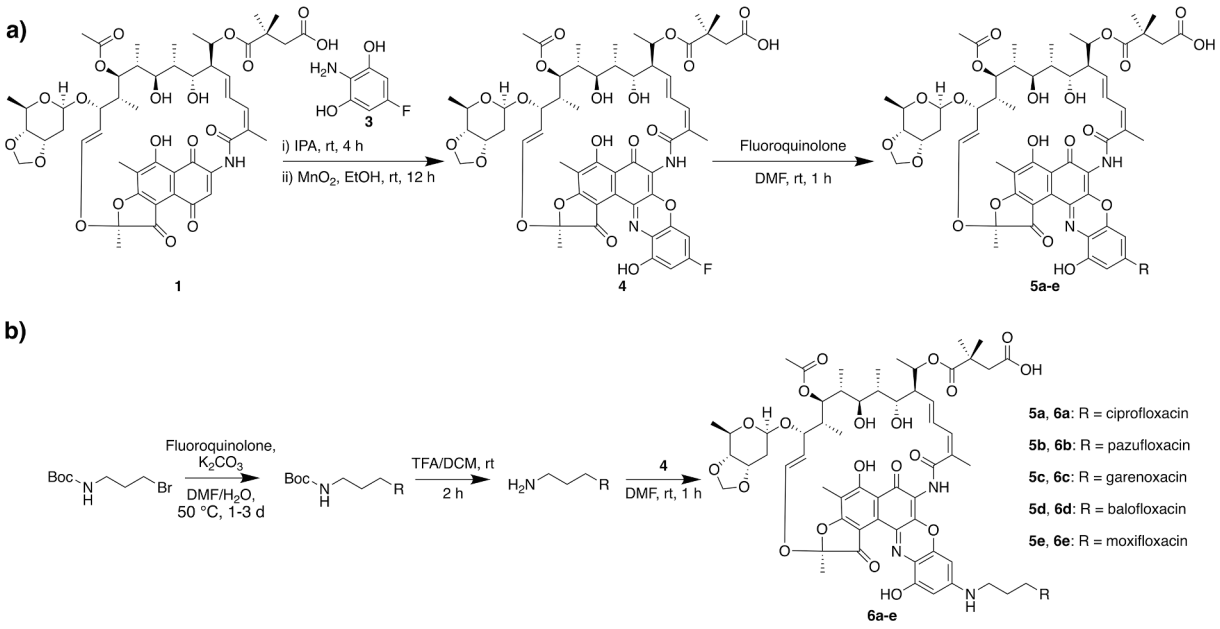

Figure 3. Synthesis of a) C-3/C-4 hybrids **5a-e** and b) linker-containing hybrids **6a-e**.

was lost when the RpoB^{H481Y} mutation was added in the RpoB^{H481Y}/ParC^{S80F}/GyrA^{S84L} background. Several of the other hybrids (**5b**, **5c**, **5e**, and **6e**) also surpassed the potency of their fluoroquinolone parents against the ParC^{S80F}/GyrA^{S84L} strain when the MIC data were analyzed in units of μM, again highlighting the activity of the Kang A component of these molecules (Supplementary Table S1).

Importantly, several of the hybrids acquired activity against the Kang A resistant RpoB^{H481Y} strain, implying that the fluoroquinolone components function in cells resistant to Kang A (Table 2). Consistent with this hypothesis, these hybrids lost activity when the ParC^{S80F}/GyrA^{S84L} mutations were added to the RpoB^{H481Y} mutation in the RpoB^{H481Y}/ParC^{S80F}/GyrA^{S84L} background. The most potent hybrid against the RpoB^{H481Y} strain was the garenoxacin fusion (**5c**), with an MIC of 2 μg/mL. Interestingly, some of the C-3/C-4 hybrids also acquired modest activity against *E. coli* (Table 2), with the most potent compounds containing pazufloxacin (**5b**; MIC = 4 μg/mL) or ciprofloxacin (**5a**; MIC = 8 μg/mL). This activity may be the result of improved Gram-negative cell penetration conferred by the fluoroquinolone component of the hybrids.

The addition of a short flexible linker led to notable variations in the activities of the C-3/C-4 hybrids (Table 2). To further explore the effects of a linker, we generated six different garenoxacin-linker conjugates with flexible or semi-rigid linkers of variable length (**7a-f**) and used these to produce an additional set of Kang A-garenoxacin hybrids (compounds **8a-f**; Fig. 4). We used garenoxacin in these hybrids as the garenoxacin fusion, **5c**, exhibited the best activity against the RpoB^{H481Y} strain in our previous round of synthesis. We found that the activity of the hybrids containing the different linkers varied significantly (Table 3). While some of the hybrids, in particular those with bulkier

Table 2
Activity of C-3/C-4 hybrids against wild-type and drug-resistant *S. aureus* and *E. coli*.

Compound	Hybridization partner	Flexible linker	MIC (μg/mL)[a]				
			S. aureus				*E. coli*
			WT	RpoB^{H481Y}	ParC^{S80F} GyrA^{S84L}	RpoB^{H481Y} ParC^{S80F} GyrA^{S84L}	
5a	Ciprofloxacin	no	0.5	32	0.5	>64	8
5b	Pazufloxacin	no	8	32	8	>64	4
5c	Garenoxacin	no	1	2	1	>64	16
5d	Balofloxacin	no	8	16	16	>64	64
5e	Moxifloxacin	no	0.5	8	0.5	>64	32
6a	Ciprofloxacin	yes	4	8	4	>64	16
6b	Pazufloxacin	yes	4	>64	4	>64	64
6c	Garenoxacin	yes	4	8	16	>64	>64
6d	Balofloxacin	yes	8	16	8	>64	>64
6e	Moxifloxacin	yes	1	16	1	>64	>64

[a] MIC assays were performed in duplicate with the same results obtained for each trial.

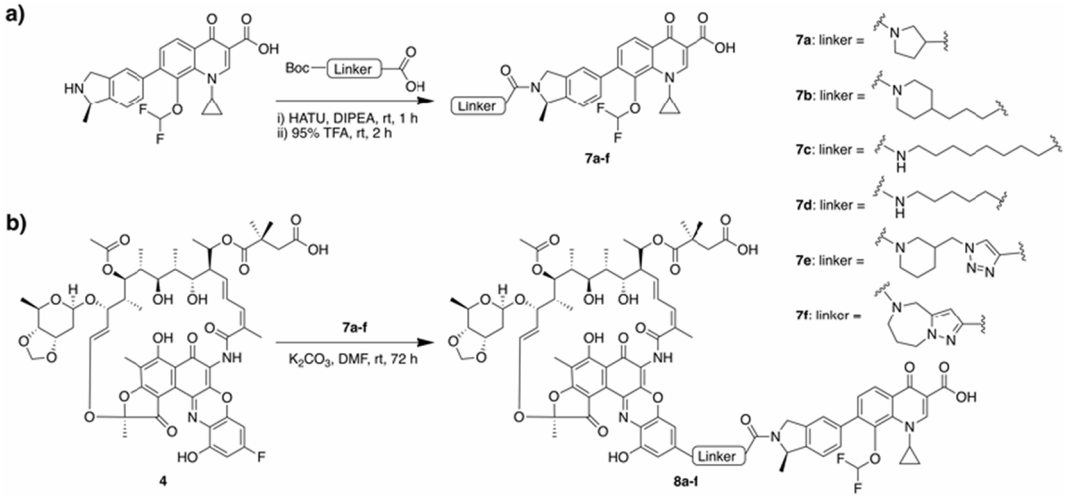

Figure 4. Synthesis of a) garenoxacin-linker conjugates **7a-f** and b) hybrid antibiotics **8a-f**.

Table 3
Activity of garenoxacin-containing hybrids with different linkers against wild-type and drug-resistant *S. aureus* and *E. coli*.

Compound	MIC (μg/mL)[a]				
	S. aureus				
	WT	RpoB^{H481Y}	ParC^{S80F} GyrA^{S84L}	RpoB^{H481Y} ParC^{S80F} GyrA^{S84L}	*E. coli*
8a	16	64	16	>64	>64
8b	64	>64	64	>64	>64
8c	4	8	4	>64	>64
8d	1	4	2	>64	>64
8e	8	16	16	>64	>64
8f	16	>64	16	>64	>64

[a] MIC assays were performed in duplicate with the same results obtained for each trial.

semi-rigid linkers had poor activity against the different strains tested, compound **8d**, which contained a flexible 6-carbon linker, was active against both the Kang A resistant strain (MIC = 4 μg/mL) and ciprofloxacin resistant strain (MIC = 2 μg/mL).

Finally, all hybrid antibiotics generated in this study were assessed for cytotoxicity against HEK293 cells (Table 4). None of the hybrids exhibited a high level of cytotoxicity. Only mild inhibition of cell growth was detected for K-acid hybrids **2a, 2c**, **2d**, **2f**, and **2i** (with IC_{50} values between 19 and 28 μg/mL) and C-3/C-4 hybrids **5d**, **6a**, and **6e** (with IC_{50} values between 36 and 44 μg/mL). The remaining compounds did not exhibit cytotoxicity at the highest concentration tested (64 μg/mL).

In summary, we generated a series of Kang A-fluoroquinolone hybrids with the principal aim of overcoming common resistance mutations. We used Kang A as a hybridization partner as the compound had never been tested in this capacity and because it possesses a carboxylic acid functionality that could be used as a new entry point for attaching a second antibiotic entity. While some of the K-acid hybrids exhibited a low level of activity against both Kang A and fluoroquinolone resistant bacteria, it appears that fusion of the two antibiotics at the K-acid may be suboptimal for retention of potency. A subset of the C-3/C-4 hybrids we generated showed better activity in both resistance backgrounds. While the TNP-2092 rifamycin C-3/C-4 hybrid that entered clinical trials shows even more potent activity against resistant bacteria, it has undergone an extensive optimization program involving the screening of a much larger collection of quinolones, linkers, and modes of attachment.[12,13] Indeed, we found that alteration of any one of these features had dramatic effects on the activity of our Kang A hybrids. Our second round of synthesis of a collection of additional garenoxacin-containing Kang A hybrids demonstrates the importance of the linker alone in determining activity and suggests that broader optimization of our compounds could lead to Kang hybrids with improved potency.

Table 4
Cytotoxicity of hybrid antibiotics against HEK293 cells.

Compound	IC_{50} (μg/mL)[a]
2a	23 ± 5
2b	>64
2c	19 ± 3
2d	21 ± 7
2e	>64
2f	23 ± 5
2g	>64
2h	>64
2i	28 ± 5
5a	>64
5b	>64
5c	>64
5d	36 ± 8
5e	>64
6a	44 ± 7
6b	>64
6c	>64
6d	>64
6e	38 ± 7
8a	>64
8b	>64
8c	>64
8d	>64
8e	>64
8f	>64

[a] Cytotoxicity assays were performed in triplicate. Results shown represent the mean ± standard deviation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.128484.

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