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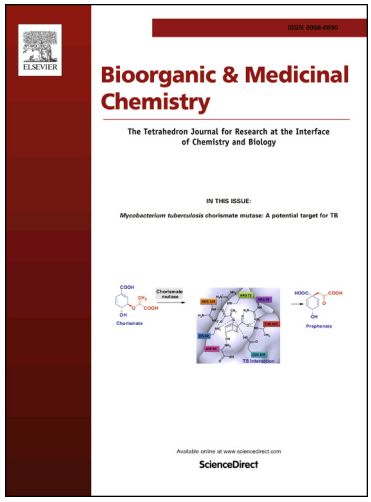

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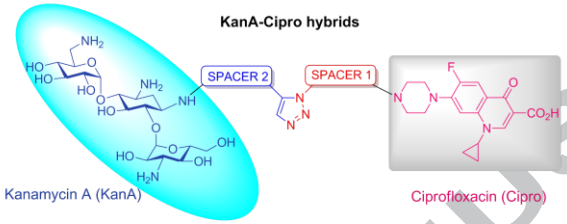
The Edith and Joseph Fischer enzyme inhibitors laboratory, Schulich Faculty of Chemistry, Technion – Israel Institute of Technology, Haifa 3200003, Israel



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ABSTRACT

To address the growing problem of antibiotic resistance, a set of 12 hybrid compounds that covalently link fluoroquinolone (ciprofloxacin) and aminoglycoside (kanamycin A) antibiotics were synthesized, and their activity was determined against both Gram-negative and Gram-positive bacteria, including resistant strains. The hybrids were antagonistic relative to the ciprofloxacin, but were substantially more potent than the parent kanamycin against Gram-negative bacteria, and overcame most dominant resistance mechanisms to aminoglycosides. Selected hybrids were 42-640 fold poorer inhibitors of bacterial protein synthesis than the parent kanamycin, while they displayed similar inhibitory activity to that of ciprofloxacin against DNA gyrase and topoisomerase IV enzymes. The hybrids showed significant delay of resistance development in both *E. coli* and *B. subtilis* in comparison to that of component drugs alone or their 1:1 mixture. More generally, the data suggest that an antagonistic combination of aminoglycoside-fluoroquinolone hybrids can lead to new compounds that slowdown/prevent the emergence of resistance.

1. Introduction

Since most of the currently known antibiotics target a single essential process in pathogenic bacteria, the development of resistance against these antibacterial substances by either spontaneous mutations or by horizontal transfer of resistance genes[1] is rather fast. In an attempt to slow down the evolution of resistance, one approach that has been used with some clinical success is combination therapy[2]; a combination (cocktail) of two or more different antibiotics employing distinct mechanisms of action. Since the drugs act with different mechanisms, there is a very low probability that any cell will simultaneously gain resistance to both drugs[3,4]. However, the combination therapy effects in vitro do not necessarily correlate to in vivo outcomes due to the varied pharmacokinetic properties of the different drugs in the combination[5,6]. Furthermore, this strategy cannot address the problem of multiple drug resistance (MDR), strains exhibiting resistance to both drugs in combination, and thus requires employment of other families of drugs.

To address some of the limitations of combination therapy, another intriguing approach named "hybrid antibiotics" has been developed[7,8,9,10]. The strategy is to chemically connect two drugs that target bacterial cells through different modes of action into a single hybrid molecule. The covalent connection of two drugs can make the pharmacokinetic properties of the hybrid molecule more predictable, can improve its toxicity profile and can lead to increased retention[11,12]. Furthermore, rationally designed linkers that connect two drugs may lead to better inhibition of both drug targets, overcoming or mitigating existing resistance mechanisms to individual drugs, and may even decrease the incidence of resistance mutations development[7].

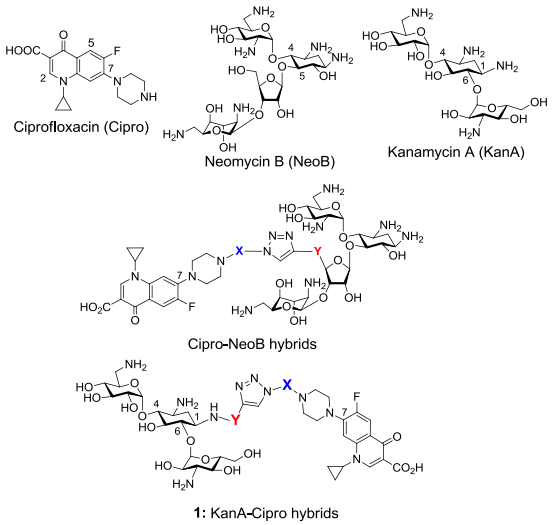
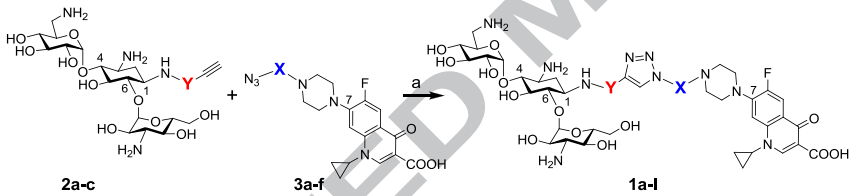


Figure 1. Structures of ciprofloxacin, neomycin B, kanamycin A, Cipro-Neo B hybrids and the designed KanA-Cipro hybrids.

With the motivation of these potential advantages, we recently reported on hybrids that had been synthesized by linking two commonly used antibiotics, ciprofloxacin (Cipro) that belongs to the fluoroquinolone class of antibiotics, and neomycin B (NeoB)

a representative of the aminoglycoside (AG) class of antibiotics[13] (Fig. 1). The obtained Cipro-NeoB hybrids were active against a wide range of wild-type Gram-negative and Gram-positive bacteria, and were also able to overcome common resistance mechanisms to AGs. Furthermore, tested Cipro-NeoB hybrids have demonstrated a significant delay of resistance formation in both Gram-negative (*Escherichia coli*) and Gram-positive (*Bacillus subtilis*) bacteria in comparison to that of each component drug separately or their 1:1 mixture.

To understand the mechanism by which the Cipro-NeoB hybrids modulate the evolution of resistance, one such hybrid, and the combination of its component drugs were further investigated in terms of phenotypic and genotypic evolution of resistance in *E. coli*, by using integrated high-throughput resistance measurements and genomic sequencing[14]. The observed data indicated that the Cipro-NeoB hybrids delay resistance development mainly because of its ability to evade resistance mediated by the multiple antibiotic resistance (*mar*) operon that regulates efflux systems. The data also demonstrated that the component drugs in the hybrid are responsible for two different but complementary functions: The Cipro moiety inhibits bacterial growth whereas the NeoB moiety, being highly hydrophilic, diminishes the effectiveness of *mar* activation. Since the antibacterial activity of these hybrids does not rely on the NeoB moiety binding to the ribosome, it was hypothesized that the NeoB component may be substituted with chemically similar structures, in order to try and find a compromise between permeability of the resulting hybrid and evasion of the *mar* pathway.



[a] (a) [(CH3CN)4Cu]PF6, 7% Et_3N in water, microwave 40 sec.

Scheme 1. Synthetic strategy for the assembly of KanA-Cipro Hybrids **1a-l**.

To test this hypothesis, we have designed, synthesized and biologically evaluated new variants of the previously studied Cipro-NeoB hybrid structures, in which the NeoB moiety is replaced by another common AG antibiotic, kanamycin A (KanA). We report that the resulted KanA-Cipro hybrids (**1**, Fig. 1) are active against both Gram-negative and Gram-positive bacteria, overcome the most prevalent types of resistance mechanisms associated with AGs, and significantly delay resistance acquisition in both Gram-negative and Gram-positive bacteria.

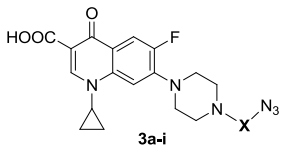
2. Results and Discussion

2.1. Design and synthesis of KanA-Cipro hybrids

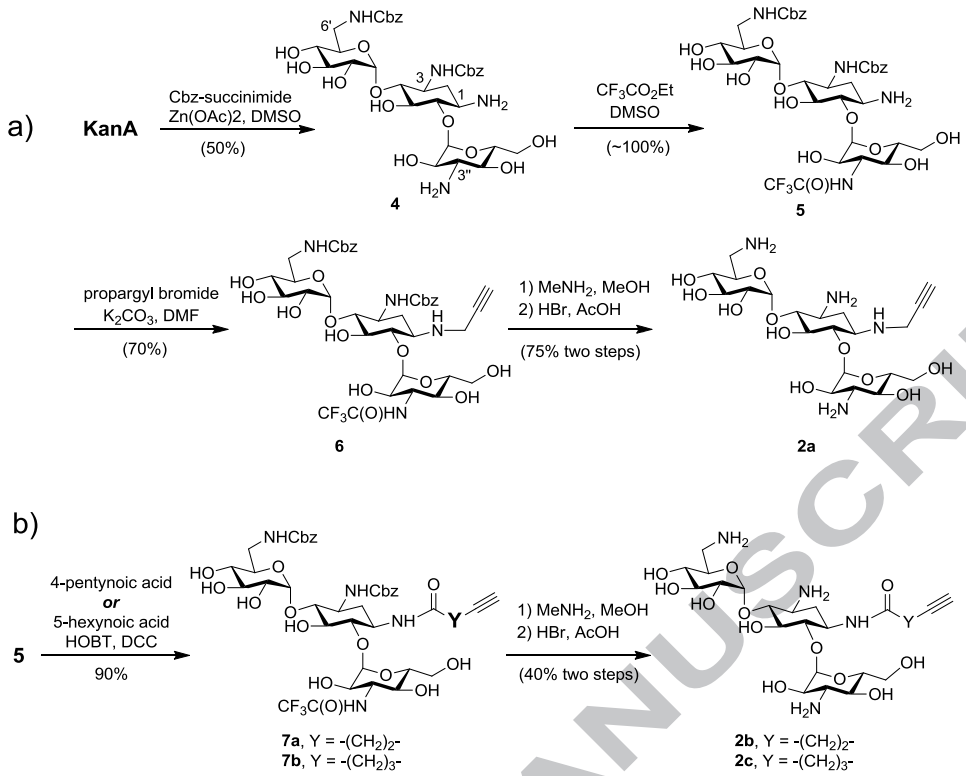
We chose KanA as a target AG molecule for the preparation of new hybrids for the following reasons. Firstly, while KanA is highly hydrophilic it significantly differs from NeoB (Fig. 1). KanA consists of 3 rings and four amino groups, while NeoB has 4 rings and six amino groups. KanA belongs to the 4,6-disubstituted 2-deoxystrepamine family of AGs while NeoB is the representative of the 4,5-disubstituted 2-deoxystrepamine family of AGs. Secondly, although KanA possesses similar antibacterial activity to that of the previously used NeoB, KanA (LD_{50}=280 mg/kg) is significantly less toxic than NeoB (LD_{50}=24 mg/kg). Therefore, the target KanA-Cipro hybrids are more likely to be less toxic than the parallel Cipro-NeoB hybrids. Thirdly, the clinical AG amikacin, which is derived from KanA by installation of (*S*)-4-amino-2-hydroxybutanoyl (AHB) moiety at N-1 position, is one of the currently used AGs that has low toxicity (LD_{50}=300 mg/kg) and good activity against bacterial strains resistant to KanA[15]. It is suggested that the attached flexible AHB moiety in amikacin interferes effectively with its binding to the AG inactivating enzymes and prevents its acetylation, phosphorylation and adenylation[16]. Furthermore, previous studies have shown that for the 4,6-disubstituted 2-deoxystrepamine family of AGs (like KanA) the best tolerance for structural variations was observed at position N-1[17–19]. Based on these collective data, we anticipated that attachment of KanA through the N-1 position to Cipro will result in a new series of KanA-Cipro hybrids with potentially low toxicity, good activity against AG resistant strains, and with good potential to delay new resistance development.

We used three different alkyne derivatives of KanA (compounds **2a-c**) and six azido derivatives of Cipro (compounds **3a-f**) that were straightforwardly coupled (via click reaction) using microwave-assisted heating to yield a library of 12 different KanA-Cipro hybrids **1a-l** (Scheme 1). Importantly, the spacers X (Table 1, the Cipro moiety) and Y (the KanA moiety) were similar to our previously reported Cipro-NeoB hybrids[13] and were selected to vary both the length and chemical nature of the linkages between the two drugs. Scheme 2 illustrates the synthesis of the alkyne derivatives of KanA, compounds **2a-c**. All these compounds were synthesized from the common intermediate derivative of KanA, compound **5**, which was obtained in two chemical steps from the commercial KanA according to the previously published procedure[20,21]. Briefly, commercial KanA was first selectively protected with the benzyloxycarbonyl (Cbz) protection at its two amino groups: N-6' and N-3 positions to afford compound **4**. Treatment of **4** with ethyl trifluoroacetate (in DMSO) afforded compound **5** in quantitative yield. Reaction with propargyl bromide in the presence of K_2CO_3 gave the protected alkyne derivative of KanA, compound **6**. Two deprotection steps,

Table 1. Ciprofloxacin-Azido Derivatives **3a-f**[13] that were used in this study.



Compound	X
3a	-$(CH_2)_2$-
3b	-$(CH_2)_6$-
3c	-$CH_2CH(OH)CH_2$-
3d	-$(CH_2)_2$-O-$(CH_2)_2$-
3e	-CH_2-*m*C_6H_4-CH_2-
3f	-CH_2-*p*C_6H_4-CH_2-



Scheme 2. Synthesis of KanA alkyne derivatives **2a-c**.

removal of the trifluoroacetate ester with methyl amine followed by Cbz deprotection in the presence of HBr in acetic acid, yielded the alkyne derivative of KanA, compound **2a**. In the synthesis of the other two alkyne derivatives of KanA, compounds **2b** and **2c**, the alkyne moiety is connected to the KanA moiety via an amide linkage. Therefore, for the assembly of these compounds, the intermediate compound **5** was directly coupled with either 4-pentynoic acid or 5-hexynoic acid in the presence of 1-hydroxybenzotriazole hydrate (HOBT) and dicyclohexyl carbodiimide (DCC) to give the corresponding protected alkyne derivatives of KanA, compounds **7a** and **7b**, respectively (Scheme 2). Finally, removal of the ester ($MeNH_2$, MeOH) and Cbz (HBr, HOAc) protections afforded the alkyne derivatives **2b** and **2c**, respectively.

The azide derivatives of Cipro, compounds **3a-f** (Table 1) were synthesized by direct coupling of the commercial Cipro with the corresponding bromoazides/chloroazides as described previously by us[13]. Finally, the KanA-alkyne derivatives **2a-c** were reacted with selected Cipro-azide derivatives **3a-f** under microwave conditions (~40 seconds) in the presence of organic base (7% Et_3N in water) and Cu(I) catalyst to afford 12 new KanA-Cipro hybrids **1a-l** in 25-95% isolated yields (Scheme 1, Table 2). The structures of **1a-l** were confirmed by a combination of different analytical tools, including 2D ^{1}H-^{13}C HMQC and HMBC, 2D COSY, and 1D selective TOCSY experiments, along with mass spectral analysis.

2.2. *Antibacterial activity tests of the KanA-Cipro hybrids*

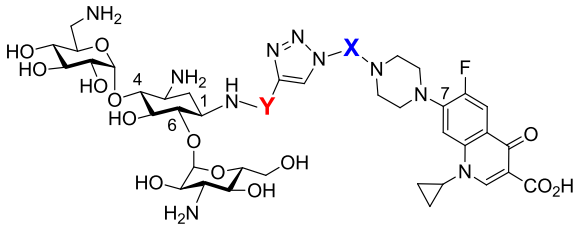
The hybrids **1a-l**, along with KanA and Cipro (that were used as controls), were initially tested against a panel of AG susceptible and AG resistant strains by measuring minimal inhibitory concentration (MIC) values (Table 2). Susceptible strains included *E. coli* R477-100 and 25922 (Gram-negative), and *B. subtilis* and *Staph. Epidermis* (Gram-positive). Resistant strains included *E. coli* AG100A and AG100B; these are kanamycin resistant laboratory strains that harbor Kan^{r} transposon Tn903 (and also upregulate the active efflux system)[22].

The MIC data in Table 2 shows that all the hybrids (**1a-l**) exhibit significant antibacterial activity. The activity of the hybrids in Gram-negative bacteria was improved in comparison to that of KanA, while all the hybrids exhibited significantly reduced activity compared to that of Cipro in both Gram-negative and Gram-positive bacteria. The hybrids displayed up to 4 fold and 32fold activity improvement in comparison to KanA, against *E. coli* R477-100 and *E. coli* 25922, respectively. The activity improvement was especially high against the resistant *E. coli* AG100A (32-260 fold) and *E. coli* AG100B (8-32 fold) strains. In contrast, most of the hybrids showed similar or lower activity than KanA against the Gram-positive *B. subtilis* and *Staph. Epidermis*, strains that are susceptible to AGs.

In general, we would like to note that the observed antibacterial data in Table 2 of the KanA-Cipro hybrids **1a-l** are very similar to that previously reported by us with the parallel Cipro-NeoB hybrids[13], in the following ways: (1) their better performance against Gram-negative versus Gram-positive bacterial strains tested; (2) SAR in terms of the nature of the X and Y spacers used to connect Cipro with the AG moiety; and (3) their especially high improvement in activity against the AGs-resistant strains *E. coli* AG100A and *E. coli* AG100B.

Since the physio-chemical properties of the hybrids are very different from those of their component drugs, one could argue that the observed improved activity of the hybrids against AGs-resistant strains *E. coli* AG100A and *E. coli* AG100B (Table 2) could be the result of the improved cell permeability of the hybrids versus that of the parent drugs.

Table 2. Chemical yields and MIC values of the hybrids **1a-l** against selected non-pathogenic bacterial strains[a]



1a-l

Compound	X	Y	Yield (%)	MIC (µg/mL) *E.coli R477-100*	*E.coli 25922*	*E.coli* AG100B[b]	*E.coli AG100A*[b]	*Bacillus subtilis*	*Staph. Epidermis*
Cipro	-	-	-	0.02	0.02	0.05	<0.005	0.02	0.094
KanA	-	-	-	12	48	384	96	1.5	1.5
1a	-$(CH_2)_2$ -	-CH_2-	55	6	3	-	-	1.5	6
1b	**-$CH_2CH(OH)CH_2$-**	**-CH_2-**	77	**3**	**1.5**	**12**	**0.37**	**1.5**	**6**
1c	**-CH_2-pC_6H_4-CH_2-**	**-CH_2-**	75	**6**	**6**	**12**	**0.28**	**3**	**3**
1d	-$(CH_2)_6$-	-CH_2-	35	12	12	24	1.5	6	24
1e	-CH_2-mC_6H_4-CH_2-	-CH_2-	45	36	24	36	1.5	12	18
1f	-$CH_2CH(OH)CH_2$-	-$CO(CH_2)_2$-	35	12	6	24	1.5	9	36
1g	**-$(CH_2)_2$ -**	**-$CO(CH_2)_2$-**	70	**6**	**3**	**24**	**0.75**	**6**	**18**
1h	-$(CH_2)_2$ -O-$(CH_2)_2$ -	-$CO(CH_2)_2$-	15	12	6	24	0.75	9	24
1i	-CH_2-mC_6H_4-CH_2-	-$CO(CH_2)_2$-	45	24	18	48	1.5	9	18
1j	-CH_2-pC_6H_4-CH_2-	-$CO(CH_2)_2$-	90	36	24	48	3	6	18
1k	**-CH_2-pC_6H_4-CH_2-**	**-$CO(CH_2)_3$-**	30	**6**	**3**	**24**	**0.75**	**3**	**6**
1l	-$CH_2CH(OH)CH_2$-	-$CO(CH_2)_3$-	25	12	6	-	-	1.5	24

[a]The MIC values represent the results obtained in parallel experiments with two different starting concentrations of the tested compound (384 µg/ml and 1.5 µg/mL). [b]Kanamycin resistant E. coli strains expressing increased active efflux system.

Table 3: Antibacterial activities of the hybrids **1b-k** against *E. coli* XL1 blue and BL21 background strains and their engineered variants expressing resistance enzymes

Compd	MIC (µg/mL) *BL21*	*BL21 psf815*	MIC ratio[a]	MIC (µg/mL) *BL21 pET11d*	MIC ratio[a]	MIC (µg/mL) *BL21 pETSACG1*	MIC ratio[a]	MIC (µg/mL) *XL1 blue*	*XL1 Blue pET9d*	MIC ratio[a]
Cipro	0.003	0.003	**1**	0.003	**1**	0.003	**1**	0.006	0.006	**1**
KanA	12	>384	**>32**	>384	**>32**	>384	**>32**	3	>384	**>128**
1b	0.2	0.3	**1.5**	0.3	**1.5**	0.3	**1.5**	3	3	**1**
1c	0.09	0.1	**1**	0.1	**1**	0.1	**1**	3	3	**1**
1d	0.8	0.8	**1**	0.8	**1**	0.8	**1**	12	12	**1**
1e	0.8	0.8	**1**	0.8	**1**	0.8	**1**	12	24	**2**
1f	1.5	1.5	**1**	1.5	**1**	1.5	**1**	24	24	**1**
1g	0.2	0.4	**2**	0.4	**2**	0.6	**3**	24	18	**1**
1h	0.8	0.8	**1**	0.8	**1**	0.8	**1**	12	12	**1**
1i	0.8	1.5	**2**	1.5	**2**	1.5	**2**	12	24	**2**
1j	0.8	1.5	**2**	1.5	**2**	0.8	**1**	24	24	**1**
1k	0.8	0.8	**1**	0.8	**1**	0.8	**1**	6	-	-

[a] The MIC ratios were calculated by dividing the MIC values against resistant strain by that against background strain

To test this issue, selected hybrids (compounds **1b-k**) were examined against six isogenic *E. coli* strains (Table 3): two background strains (*E. coli* BL21 and *E. coli* XL1 blue) and four strains with harbored resistance enzymes APH(3')-Ia, AAC(6')-APH(2"), APH(3')-IIIa, and APH(3')-IIb. Among the resistant strains used were the *E. coli* (pSF815), *E. coli* (pET11d) and *E. coli* (pETSACG1); these are laboratory resistant strains derived by transformation of *E. coli* BL21 (background strain) with the pSF815, pET11d and pETSACG1 plasmids, respectively. The pSF815 encodes for the bifunctional AAC(6′)/APH(2″) resistance enzyme, which catalyzes acetylation of the amino group at 6′-NH_2 and phosphorylation at the 2″-OH. The pET11d and pETSACG1 plasmids encode for the APH(3')-IIb and APH(3′)-IIIa resistance enzymes, respectively. The last resistant strain used was *E. coli* (pET9d), derived by transformation of *E. coli* XL1 blue with the pET9d plasmid that encodes for the APH(3′)-Ia resistance enzyme, which catalyzes phosphorylation at the 3′-OH of both neomycin and kanamycin families of aminoglycosides. These enzymes are among the most prevalent modes of resistance found in aminoglycosides resistance strains [23–25]. The data in Table 3 show, that while the MIC values of KanA dramatically rose in the case of resistant strains, the activities of the hybrids were almost identical against background and resistance-carrying strains. These observations indicate that the enzymes modifying the majority of AG antibiotics are ineffective in the case of the hybrid molecules. These data also suggest that the reason for the observed sensitivity of AG resistant strains, *E. coli* (pSF815), *E. coli* (pET11d), *E. coli* (pETSACG1) and *E. coli* (pET9d), is the

reduced activity of the resistance enzymes against the **1b-k** hybrids rather than the improved cell permeability of these hybrids.

Table 4: Activity of Selected Hybrids as Inhibitors of DNA Gyrase, TopoIV, and Bacterial Protein Synthesis**.**

Compound	IC_{50} (μM)		
	DNA gyrase[a]	TopoIV[b]	Protein synthesis[c]
Cipro	1.3 ± 0.1	8.6 ± 0.3	inactive
KanA	inactive	inactive	0.03 ± 0.01
1a	-	-	8.14 ± 1.04
1b	5.9 ± 0.4	19.8 ± 0.6	1.27 ± 0.25
1c	2.6 ± 0.3	7.9 ± 0.4	10.69 ± 1.55
1d	-	-	6.46 ± 0.50
1g	9.9 ± 0.4	>100	19.18 ± 1.64
1k	-	-	2.19 ± 0.33

[a]Supercoiling assay with *E. coli* DNA gyrase. The IC_{50} was defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%. See experimental section for assay details.
[b]Relaxation assay with *E. coli* TopoIV. The IC_{50} was defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%. See experimental section for assay details.
[c]In vitro transcription/translation assay with *E. coli* S30 extract system. See experimental section for assay details.

2.3. Biological evaluation of the KanA-Cipro hybrids.

The selected hybrids were further tested for their potential to function with a dual mode of action. For this purpose, the ability of the hybrids **1a-d**, **1g** and **1k** to inhibit protein synthesis was examined by using an in vitro transcription/translation assay[13]. In parallel, the hybrids **1b-c** and **1g** were also tested for the inhibition of the enzymes that are targeted by the quinolones, DNA gyrase and TopoIV [13,26,27]. The observed data in Table 4 show that the tested hybrids are 42-640 times poorer inhibitors of bacterial protein synthesis than the parent KanA, suggesting that their interaction with the bacterial ribosome is significantly reduced. However, the tested hybrids **1a-d**, **1g** and **1k** displayed better antibacterial activity compared to that of KanA (Table 2), likely due to the antibacterial activity that is mediated through the Cipro moiety rather than the KanA moiety. Furthermore, all three hybrids displayed similar activities to that of Cipro in both the DNA gyrase and TopoIV assays, except for **1g** that did not show activity on the TopoIV target, lending further support to this hypothesis. The IC_{50} values measured here for Cipro against both the DNA gyrase and TopoIV, are very similar to those previously reported[26,17,27]. Finally, the apparent contradiction between the observed inferior antibacterial activity of the tested hybrids **1b-c** and **1g** compared with Cipro (Table 2), and their similar activity against both the DNA gyrase and TopoIV targets (Table 4), can be explained by the reduced cell penetration of the hybrid structures in comparison to Cipro. Indeed, the bigger size and net charge of the hybrids compared to Cipro, could contribute to their reduced cellular uptake.

The ability to slow down the emergence of resistance is probably one of the most important advantages of the hybrid drugs[7,28,29]. To evaluate the potential of Cipro-KanA hybrids to delay resistance development, we used a well-established procedure of selective pressure[30,31], which was successfully implemented by us[13]. Briefly, two different bacterial strains, one Gram-negative (*E. coli* ATCC 35218) and one Gram-positive (*B. subtilis* ATCC 6633), were exposed to sub-inhibitory (1/2 MIC) amounts of the tested hybrids **1b** and **1c**, along with the Cipro, KanA and the cocktail of Cipro/KanA (1:1 molar ratio) during 15 successive subcultures. The observed data are shown in Figure 3 as a ratio of the measured MIC values after the 15th and first passages. The individual components of the hybrids show a high tendency towards resistance development: the relative MIC values are ~30-fold for Cipro and over 15-fold for KanA in both *E. coli* and in *B. subtilis*. A relatively high tendency towards resistance development was also seen for the cocktail Cipro + KanA (1:1 molar ratio): in the *E.coli* the level of resistance development was in between the levels of Cipro and KanA, while in *B. subtilis* it was somewhat lower than that of both. The hybrids **1b** and **1c**, however, showed very low propensity to resistance development in both bacterial strains with a ratio of 1.33 and 2.0 respectively for **1b**, and for **1c** the ratio was 4 in both strains under the same experimental conditions. We note that earlier studies from our[13] and other laboratories[31,30] have reported the similar, high level resistance development under same experimental conditions for Cipro and for AGs. To the best of our knowledge, however, the KanA-Cipro hybrids studied here, and the Cipro-NeoB hybrids reported recently by us[13,14] are the first AG-fluoroquinolone hybrids for which the delay of resistance development has been demonstrated[7,28,32].

2.4. Summary and conclusions

The present study was designed to further scrutinize the concept of hybrid drugs especially in regards to their ability to modulate the evolution of resistance. Indeed, previous reports by Kishony and co-workers have demonstrated that while the "synergistic" antibacterial drug combinations can actually enhance the development of resistance, "antagonistic" drug combinations have significantly slowed the evolution of resistance[33,34]. Such a benefit of antagonistic drug combination in regards to the hybrid antibiotic was first demonstrated by the synthesis and evaluation of Cipro-NeoB hybrids[13,7]; the hybrids were antagonistic relative to Cipro and showed significant delay of resistance evolution in both Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria. However, it remained unclear whether or not the ability of these hybrid drugs to delay the development of resistance was a general phenomenon for the aminoglycoside-fluoroquinolone hybrids.[35]

These accumulative data for the KanA-Cipro hybrids correlate very well with the mechanism by which the Cipro-NeoB hybrids limit the evolution of resistance[14], and support the notion that the antagonistic mechanism of overcoming resistance is a general phenomenon for aminoglycoside-fluoroquinoline hybrids. We anticipate that this approach will be beneficial for future drug design of hybrid molecules intended to slowdown/prevent the emergence of multidrug resistance not only in infectious diseases[36] but also in cancer[7,28].

3. Experimental section

3.1. General Methods.

^{1}H NMR spectra (including DEPT, 2D-COSY, 2D TOCSY, 1D TOCSY, HMQC, HMBC) were routinely recorded on a Bruker Avance^{TM} 500 spectrometer or 400 spectrometer, and chemical shifts reported (in ppm) are relative to internal Me_4Si (δ =0.0) with $CDCl_3$ as the solvent, and to HOD (δ =4.63) with D_2O as the solvent. ^{13}C NMR spectra were recorded on a Bruker Avance^{TM} 500 spectrometer or at 125.8 MHz, and the chemical shifts reported (in ppm) relative to the residual solvent signal for $CDCl_3$ (δ =77.00), or to external sodium 2,2-dimethyl-2-silapentane sulfonate (δ =0.0) for D_2O as the solvent. Mass spectra were obtained either on a Bruker Daltonix Apex 3 mass spectrometer under electron spray ionization (ESI), or by a TSQ-70B mass spectrometer (Finnigan Mat). Reactions were monitored by TLC on Silica Gel 60 F_{254} (0.25 mm, Merck), and spots

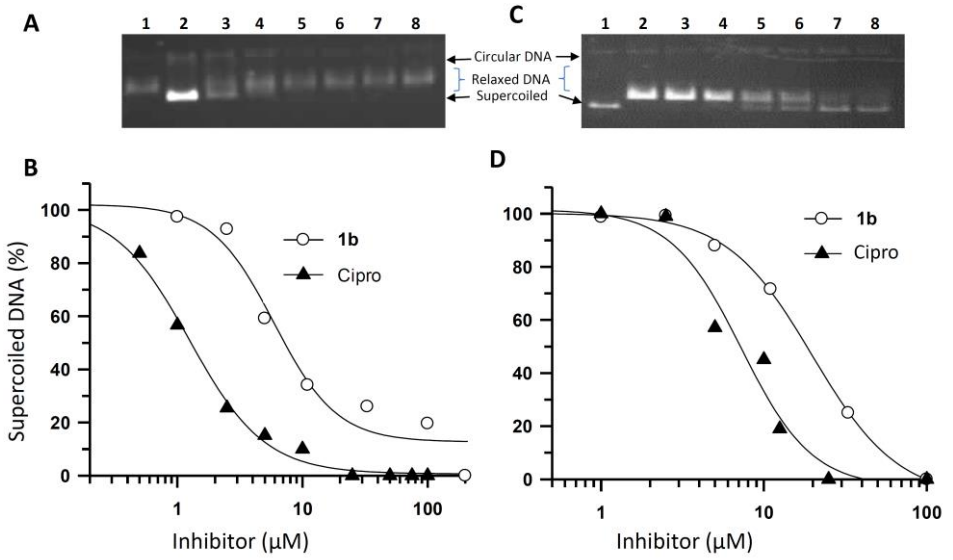


Figure 2. Representative comparative data for the inhibition of DNA gyrase (panels A and B) and TopoIV (panels C and D) with Cipro and compound **1b**. (A) A 1% agarose gel shows the inhibitory activity of **1b** against DNA gyrase. Lane 1, relaxed DNA; lane 2, supercoiling reaction by DNA gyrase without presence of inhibitor; lanes 3-8 are the same as lane 1 but in the presence of 1, 2.5, 5, 11, 33, and 100 μM of inhibitor **1b**. (B) Semilogarithmic plot of in vitro DNA gyrase supercoiling reaction inhibition, measured for Cipro and **1**b. (C) A 1% agarose gel shows the inhibitory activity of **1**b against TopoIV. Lane 1, supercoiled DNA; lane 2, relaxation reaction by TopoIV without the presence of inhibitor; lanes 3-8 are the same as lane 1 but in the presence of 1, 2.5, 5, 11, 33, and 100 μM of **1b**. (D) Semilogarithmic plot of TopoIV inhibition, measured for Cipro and **1b**. The percentages of the supercoiled DNA were calculated from the electrophoresis images by using GelAnelyzer program, and plotted as functions of drug concentration. Each data point represents the average of 2-3 independent experimental results

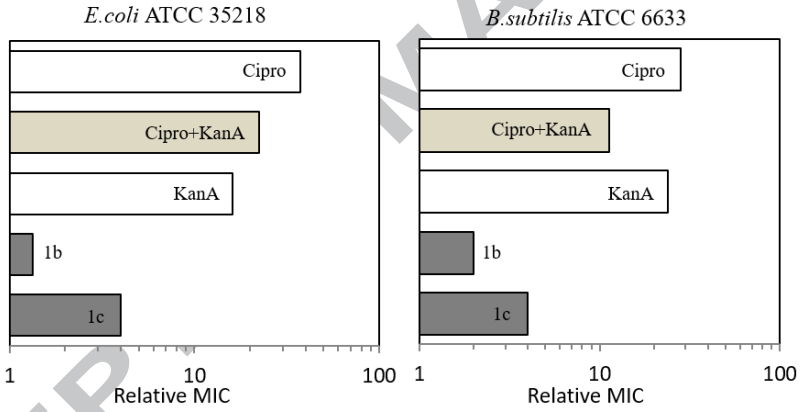


Figure 3*:* Comparative study on the emergence of resistance in *E. coli* and *B. subtilis* after 15 serial passages in the presence of Cipro, KanA, Cipro+KanA mixture (1:1 molar ratio), and hybrid structures **1b** and **1c**. Relative MIC in the normalized ratio of MIC obtained for a given subculture to MIC obtained upon first exposure.

were visualized by charring with a yellow solution containing $(NH_4)Mo_7O_{24}{\cdot}4H_2O$ (120 g) and $(NH_4)_2Ce(NO_3)_6$ (5 g) in 10% H_2SO_4 (800 mL). All reactions were carried out under an argon atmosphere with anhydrous solvents, unless otherwise noted. Microwave assisted reactions were carried out in domestic microwave oven *Sauter* SG251. Compound **5** (Scheme 2) was prepared from commercially available KanA (Shanghai FWD Chemicals Limited) according to previously published procedure[20,21]. All chemicals unless otherwise stated, were obtained from commercial sources.

*3.2. Synthesis of Kanamycin-alkyne derivatives **2a-c**.*

Compound **5** (100 mg, 0.115 mmol) was dissolved in dry DMF (5 mL) and to the resulting solution were added potassium carbonate (19 mg, 0.140 mmol) and propargyl bromide (11.3 μL, 0.126 mmol). Reaction progress was monitored by TLC (MeOH/DCM 1:5), which indicated completion after overnight stirring. The crude mixture was then purified by flash chromatography (silica, MeOH/DCM) to yield compound **6** (72 mg, 70%). ^{1}H NMR (500 MHz, $CD_3OD/CDCl_3$) δ_H 2.44-2.45 (t, *J*=2.5 Hz, 1H, CH of triple bond), 3.63-4.08 (m, 2H, -CH_2-triple bond), 4.96-5.10 (m, 4H, CH_2 of Cbz), 7.23-7.35 (m, 10H, aromatic); ring I δ_H 3.37-3.54 (m, 5H, H-2, H-3, H-4, H-6, H-6'), 3.63-4.08 (m, 1H, H-5), 4.96-5.10 (m, 1H, H-1); ring II δ_H 1.29-1.36 (ddd, J_1=J_2=J_3=12.5 Hz, 1H, H-2ax), 2.23-2.27 (dt, *J*=4.0, 12.5 Hz, 1H, H-2eq), 2.96-2.98 (m, 1H, H-1), 3.37-3.54 (m, 1H, H-5), 3.63-4.08 (m, 3H, H-3, H-4, H-6); ring III δ_H 3.22-3.26 (dd, *J*=4.0, 13.0 Hz, 1H, H-6), 3.31-3.34 (m, 1H, H-6'), 3.37-3.54 (m, 2H, H-2, H-4), 3.63-4.08 (m, 2H, H-3, H-5), 4.96-5.10 (m, 1H, H-1); ^{13}C NMR (125 MHz, $CD_3OD/CDCl_3$) δ_C 31.2 (C-2), 35.0 (C-6''), 40.9 (C-6'), 50.1, 55.7, 61.2 (triple bond), 66.7, 66.8, 67.7, 69.7, 70.4, 71.4, 72.5, 72.8, 73.4, 73.9, 75.2, 84.0, 85.5, 100.41 (C-1'), 101.3 (C-1''), 114.87, 117.2, 127.8, 127.9, 128.0, 128.1, 128.4, 136.1, 136.2, 156.5, 157.9, 158.9, 159.2. MALDI TOFMS calcd for $C_{39}H_{49}F_3N_4O_{19}Na$ ($[M+Na]^+$) *m/e* 909.3; measured *m/e* 909.3. Compound **2a.** Compound **6** (100 mg, 0.113 mmol) was dissolved in 33% solution of $MeNH_2$ in EtOH (10 mL) and the resulting solution was stirred at room temperature for 12 h. The reagent and the solvent were removed by evaporation and the residue was dissolved in AcOH (2 mL) and stirred at 15°C for 10 minutes after which 30% solution of HBr in AcOH (0.5 mL) was added.

Reaction progress was monitored by TLC (CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH) 10:15:6:15), which indicated completion after 1 hour. 1N NaOH solution was added to the reaction mixture until pH became neutral, and then the crude mixture was purified on a short column of Amberlite CG-50 (H^+-form). The column was sequentially washed by: MeOH, MeOH/$MeNH_2$ (33% solution in EtOH) 95:5, MeOH/$MeNH_2$ (33% solution in EtOH) 9:1 and MeOH/$MeNH_2$ (33% solution in EtOH) 4:1. Fractions containing the product were combined, evaporated, re-dissolved in water and evaporated again to afford the free amine form of the product (50 mg, 75%). This product was then dissolved in water; the pH was adjusted to 7.5 with 0.01 M H_2SO_4 and lyophilized to give the sulfate salt of **2a** as a white foamy solid. ^{1}H NMR (500 MHz, D_2O, pH=3.17) δ_H 3.00 (t, 1H, CH of triple bond), 3.94-3.98 (dd, *J*=2.5, 15.5 Hz, 1H, -*CH*$_2$-triple bond), 4.07-4.11 (dd, *J*=2.5, 15.5 Hz, 1H, -*CH*$_2$-triple bond); ring I δ_H 3.03-3.08 (dd, *J*=9.0, 13.5 Hz, 1H, H-6), 3.24-3.28 (t, *J*=9.5 Hz, 1H, H-4), 3.35-3.38 (dd, *J*=3.5, 9.0 Hz, 1H, H-6'), 3.60-3.76 (m, 1H, H-2), 3.81-3.93 (m, 2H, H-3, H-5), 5.55-5.56 (d, *J*=4.0 Hz, 1H, H-1); ring II δ_H 1.94-2.01 (ddd, J_1=J_2=J_3=12.5 Hz, 1H, H-2ax), 2.56-2.58 (dt, *J*=4.0, 12.5 Hz, 1H, H-2eq), 3.40-3.45 (m, 1H, H-3), 3.60-3.76 (m, 1H, H-5), 3.81-3.93 (m, 3H, H-1, H-4, H-6); ring III δ_H 3.40-3.45 (m, 1H, H-3), 3.55-3.58 (dd, *J*=4.0, 10.5 Hz, 1H, H-4), 3.60-3.76 (m, 2H, H-6, H-6'), 3.81-3.93 (m, 2H, H-2, H-5), 5.10-5.11 (d, *J*=3.0 Hz, 1H, H-1). ^{13}C NMR (125 MHz, D_2O) δ_C 25.4 (C-2), 35.9 (-*CH*$_2$-triple bond), 40.4 (C-6'), 47.7, 50.0, 54.9, 55.6, 59.8 (C-6''), 65.2, 68.1, 68.6, 70.7, 71.8, 72.4, 73.0, 73.7, 76.9, 78.8, 83.0, 96.0 (C-1'), 100.4 (C-1''). MALDI TOFMS calcd for $C_{21}H_{39}N_4O_{11}$ ([M+H]$^+$) *m*/*e* 523.2; measured *m*/*e* 523.3.

Compound 2b. Compound **5** (200 mg, 0.23 mmol) was dissolved in dry Et_3N (2 mL) and cooled to -10ºC. The HOBT (217.42 mg, 1.61 mmol), 4-pentynoic acid (67.6 μL, 0.69 mmol) and DDC (332.22 mg 1.61 mmol) were dissolved in DMF (5 mL), and stirred for 1hr at 0ºC. The two solutions were combined at -10ºC and the reaction progress was monitored by TLC (MeOH/DCM 1:4) which indicated completion after 2 hours. A solution of $MeNH_2$ (20 ml, 33% methylamine in MeOH) was then added, and the resulting solution was stirred over night at room temperature. The crude mixture was evaporated to dryness and then purified by flash chromatography (silica, MeOH/DCM) to yield compound **7a** (170 mg, 90%). ^{1}H NMR (500 MHz, CD_3OD/$CDCl_3$) δ_H 2.48-2.55 (t, *J*=3.5 Hz, 1H, CH of triple bond), 3.34-3.35 (m, 4H, -*CH*$_2$-triple bond), 4.96-5.10 (m, 4H, CH_2 of Cbz), 7.26-7.35 (m, 10H, aromatic); ring I δ_H 3.45-3.49 (m, 5H, H-2, H-3, H-4, H-6, H-6'), 3.77-3.85 (m, 1H, H-5), 5.00-5.08 (m, 1H, H-1); ring II δ_H 0.88-0.92(ddd, J_1=J_2=J_3=12.5 Hz, 1H, H-2ax), 2.27-2.29 (dt, *J*=4.0, 12.5 Hz, 1H, H-2eq), 2.95-2.03 (m, 1H, H-1), 3.37-3.54 (m, 1H, H-5), 3.63-4.08 (m, 3H, H-3, H-4, H-6); ring III δ_H 3.22-3.26 (dd, *J*=4.0, 13.0 Hz, 1H, H-6), 3.31-3.34 (m, 1H, H-6'), 3.37-3.54 (m, 2H, H-2, H-4), 3.63-4.08 (m, 2H, H-3, H-5), 4.96-5.10 (m, 1H, H-1); ^{13}C NMR (125 MHz, CD_3OD/$CDCl_3$) δ_C 29.3, 29.5, 31.5 (C-2), 34.9 (C-6''), 41.1 (C-6'), 50.1, 53.6, 61.4 (triple bond), 66.5, 66.6, 67.7, 69.6, 70.5, 71.3, 72.5, 72.9, 73.4, 73.6, 75.4, 81.2, 84.0, 85.6, 100.6 (C-1'), 101.1 (C-1''), 114.9, 117.3, 127.8, 127.9, 128.0, 128.1, 128.4, 136.2, 136.4, 156.9, 157.5, 158.7, 159.0. MALDI TOFMS calcd for $C_{41}H_{51}F_3N_4O_{17}Na$ ([M+Na]$^+$) *m*/*e* 951.3; measured *m*/*e* 951.2.

Compound **7a** from the previous step (100 mg, 0.113 mmol) was dissolved in 33% solution of $MeNH_2$ in EtOH (10 mL) and the mixture was stirred at room temperature for 12 h. The reagent and the solvent were removed by evaporation and the residue was dissolved in acetic acid (10 mL), cooled to 16ºC and the 30% solution of HBr in AcOH (1 ml) was added. Reaction progress was monitored by TLC [CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH) 10:15:6:15], which indicated completion after 2 hours. The pH of the reaction was then adjusted to neutral with 1N NaOH. The crude mixture was evaporated to dryness and then purified by flash chromatography (silica, MeOH/$MeNH_2$) to yield compound **2b** (40 mg, 40%). ^{1}H NMR (500 MHz, D_2O, pH=3.00) δ_H 3.00 (t, 1H, CH of triple bond), 3.94-3.98 (dd, *J*=2.5, 15.5 Hz, 1H, -*CH*$_2$-triple bond), 4.07-4.11 (dd, *J*=2.5, 15.5 Hz, 1H, -*CH*$_2$-triple bond); ring I δ_H 3.03-3.08 (dd, *J*=9.0, 13.5 Hz, 1H, H-6), 3.24-3.27 (t, *J*=9.5 Hz, 1H, H-4), 3.35-3.39 (dd, *J*=3.5, 9.0 Hz, 1H, H-6'), 3.59-3.60 (m, 1H, H-2), 3.80-3.85 (m, 2H, H-3, H-5), 5.55-5.56 (d, *J*=4.0 Hz, 1H, H-1); ring II δ_H 1.94-2.01 (ddd, J_1=J_2=J_3=12.5 Hz, 1H, H-2ax), 2.55-2.59 (dt, *J*=3.9, 12.5 Hz, 1H, H-2eq), 3.40-3.45 (m, 1H, H-3), 3.60-3.76 (m, 1H, H-5), 3.81-3.93 (m, 3H, H-1, H-4, H-6); ring III δ_H 3.40-3.45 (m, 1H, H-3), 3.55-3.58 (dd, *J*=4.0, 10.5 Hz, 1H, H-4), 3.60-3.76 (m, 2H, H-6, H-6'), 3.81-3.93 (m, 2H, H-2, H-5), 5.10-5.11 (d, *J*=3.0 Hz, 1H, H-1). ^{13}C NMR (125 MHz, D_2O) δ_C 25.4 (C-2), 35.9 (-*CH*$_2$-triple bond), 40.4 (C-6'), 47.7, 49.8, 50.0, 54.9, 55.6, 59.8 (C-6''), 65.2, 68.1, 68.6, 70.7, 71.8, 72.4, 73.0, 73.7, 76.9, 78.8, 83.0, 96.0 (C-1'), 100.4 (C-1''). MALDI TOFMS calcd for $C_{21}H_{39}N_4O_{11}$ ([M+H]$^+$) *m*/*e* 587.3; measured *m*/*e* 587.3.

Compound 2c. Compound **5** (200 mg, 0.23 mmol) was dissolved in dry Et_3N (2 mL) and cooled to -10ºC. The HOBT (217.42 mg, 1.61 mmol), 5-hexynoic acid (67.6 μL, 0.69 mmol) and DDC (332.22mg 1.61mmol) were dissolved in DMF (5 mL), and stirred for 1hr at 0ºC. The two solutions were combined at -10ºC, and the resulting mixture was stirred for about 2 hours. The reaction progress was monitored by TLC (MeOH/DCM 1:4), which indicated completion after 2 hours. A solution of $MeNH_2$ (20 ml, 33% methylamine in MeOH) was then added, and the resulting solution was stirred overnight at room temperature. The crude mixture was evaporated to dryness and thenpurified by flash chromatography (silica, MeOH/DCM) to yield compound **7b**. (165 mg, 88%). ^{1}H NMR (500 MHz, CD_3OD/$CDCl_3$) δ_H 2.61-2.73 (m, 1H, CH of triple bond), 3.63-4.08 (m, 2H, -*CH*$_2$-triple bond), 5.05-5.10 (m, 4H, CH_2 of Cbz), 7.25-7.35 (m, 10H, aromatic); ring I δ_H 3.38-3.48 (m, 5H, H-2, H-3, H-4, H-6, H-6'), 3.63-3.81 (m, 1H, H-5), 4.96-5.10 (m, 1H, H-1); ring II δ_H 1.43-1.52 (ddd, J_1=J_2=J_3=9 Hz, 1H, H-2ax), 2.31-2.36 (dt, *J*=4.0, 12.5 Hz, 1H, H-2eq), 2.83-2.86 (m, 1H, H-1), 3.37-3.54 (m, 1H, H-5), 3.63-4.08 (m, 3H, H-3, H-4, H-6); ring III δ_H 3.22-3.26 (dd, *J*=4.0, 13.0 Hz, 1H, H-6), 3.31-3.34 (m, 1H, H-6'), 3.37-3.54 (m, 2H, H-2, H-4), 3.63-4.08 (m, 2H, H-3, H-5), 4.96-5.10 (m, 1H, H-1); ^{13}C NMR (125 MHz, CD_3OD/$CDCl_3$) δ_C 31.2 (C-2), 35.0 (C-6''), 40.9 (C-6'), 50.1, 55.7, 61.2 (triple bond), 63.4, 66.7, 66.8, 67.7, 69.7, 70.4, 71.4, 72.5, 72.8, 73.4, 73.9, 75.2, 84.0, 85.5, 100.41 (C-1'), 101.3 (C-1''), 114.87, 117.2, 127.8, 127.9, 128.0, 128.1, 128.4, 136.1, 136.2, 156.5, 157.9, 158.9, 159.2. MALDI TOFMS calcd for $C_{42}H_{53}F_3N_4O_{17}Na$ ([M+Na]$^+$) *m*/*e* 951.3; measured *m*/*e* 951.4.

Compound **7b** from the previous step (100 mg, 0.113 mmol) was dissolved in 33% solution of $MeNH_2$ in EtOH (10 mL) and the resulting solution was stirred at room temperature for 12 h. The reagent and the solvent were removed by evaporation and the residue was dissolved in acetic acid (2 mL), stirred at 15°C for 10 minutes, after which 30% solution of HBr in AcOH (0.5 mL) was added. Reaction progress was monitored by TLC [CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH) 10:15:6:1], which indicated completion after 2 hours. 1N NaOH solution was added to the reaction mixture until pH became neutral. The crude mixture was evaporated to dryness at room temperature and then purified by flash chromatography (silica, MeOH/$MeNH_2$) to yield compound **2c** (40 mg, 40%). ^{1}H NMR (500 MHz, D_2O, pH=3.20) δ_H 2.44-2.47 (t, *J*=5.0 1H, CH of triple bond), 3.91-3.96 (dd,

J=2.5, 15.5 Hz, 1H, -CH_2-triple bond), 4.09-4.12 (dd, *J*=2.5, 15.5 Hz, 1H, -CH_2-triple bond); ring I δ_H 3.23-3.27 (dd, *J*=8.4, 12.9 Hz, 1H, H-6), 2.54-2.67 (t, *J*=6.8 Hz, 1H, H-4), 3.34-3.37 (dd, *J*=3.5, 9.0 Hz, 1H, H-6'), 3.60-3.76 (m, 1H, H-2), 3.81-3.93 (m, 2H, H-3, H-5), 5.55-5.56 (d, *J*=4.0 Hz, 1H, H-1); ring II δ_H 1.75-1.86 (ddd, J_1=J_2=J_3=13.75 Hz, 1H, H-2ax), 2.32-2.35 (dt, *J*=4.0, 12.5 Hz, 1H, H-2eq), 3.43-3.48 (m, 1H, H-3), 3.63-3.67 (m, 1H, H-5), 3.84-3.88 (m, 3H, H-1, H-4, H-6); ring III δ_H 3.43-3.48 (m, 1H, H-3), 3.56-3.62 (dd, *J*=4.0, 10.5 Hz, 1H, H-4), 3.58-3.62 (m, 2H, H-6, H-6'), 3.81-3.93 (m, 2H, H-2, H-5), 5.22-5.23 (d, *J*=3.0 Hz, 1H, H-1). ^{13}C NMR (125 MHz, D_2O) δ_C 17.6, 24.1 (C-2), 31.0, 35.2 (-CH_2-triple bond), 40.2 (C-6'), 47.5, 50.5, 55.5, 60.1 (C-6"), 65.9, 68.0, 68.8, 70.8, 71.9, 72.8, 73.0, 73.9, 76.9, 79.2, 80.2, 84.8, 95.5, 98.0 (C-1'), 100.3, 115.1, 117.6, 133.0, 162.8, 176.2 (C-1"). MALDI TOFMS calcd for $C_{24}H_{42}N_4O_{12}$ ([M+H]$^+$) *m*/*e* 601.3; measured *m*/*e* 601.1

*3.3. Synthesis of KanA-Cipro hybrids **1a-l**.*

General procedure for the preparation of hybrid structures 1a-l. This procedure was very similar to that previously reported by us for the preparation of Cipro-NeoB hybrids (Fig. 1)[13]. Briefly, a solution of compound **2** (0.06 mmol), ciprofloxacin azido derivative **3** (0.05 mmol)[13], and $[(CH_3CN)_4Cu]PF_6$ (0.025 mmol) in 7% solution of Et_3N in water (5 mL) was placed in a glass vial (25 mL) compatible for working with microwave irradiation. The vial was closed non-hermetically with a stopper and carefully heated in a domestic microwave oven at maximum power, in 5 second runs, for a total of 40 seconds. We note that the volume of the solution (5 mL) and the 40 seconds of heating were found to be optimal for the particular reactions reported here. Reaction progress was monitored by TLC [CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH), 10:15:6:15]. After completion, the mixture was purified on a short column of Amberlite CG-50 (H^+-form). The column was sequentially washed by: MeOH, MeOH/$MeNH_2$ (33% solution in EtOH) 95:5, MeOH/$MeNH_2$ (33% solution in EtOH) 9:1 and MeOH/$MeNH_2$ (33% solution in EtOH) 4:1. Fractions containing the product were combined, evaporated, re-dissolved in water and evaporated again to afford the free amine form of the product. The product was dissolved in water, the pH was adjusted to 3.2 with TFA (0.01 M), and lyophilized to afford the TFA salt of the final product, usually as a white foamy solid. Chemical yields of the resulting hybrids **1a-l** are given in Table 2 and their complete analytical data are given in Supporting Information.

3.4. Antibacterial activity

For the determination of MIC values we used the double-microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS)[37] with two different starting concentrations of the tested compounds: 384 μg/mL and 1.5 μg/mL. All the experiments were performed as duplicates and analogous results were obtained in two to four different experiments. The evolution of resistance was studied in parallel with *E. coli* ATCC 35218 and *B. subtilis* ATCC 6633 strains as we reported earlier[13]. Briefly, the experiments were performed in the presence of Cipro, KanA, Cipro:KanA mixture (1:1 molar ratio), and the hybrids **1b** and **1c**. MICs were determined for 15 passages as follows: for each compound tested, bacteria from the 1/2 MIC well were diluted 100-fold (50 μL of the bacterial growth in the total of 5 mL LB medium) and were grown overnight at 37°C. The OD_{600} of the bacteria was diluted to yield $5x10^5$ cells/ml in LB (determined by using a calibration curve) and used again for MIC determination in the subsequent generation. Note that the MIC evolution during these subcultures was compared concomitantly with each new generation, using bacteria harvested from control wells (wells cultured without antimicrobial agent from the previous generation). The relative MIC was calculated for each experiment from the ratio of MIC obtained for a given subculture to that obtained for first-time exposure.

3.5. Biochemical studies

Resistance conferring plasmids used in this study were obtained as follows. The plasmid pSF815 carrying the AAC(6')-APH(2") gene was kindly provided by Prof. S. Mobashery, University of Notre Dame. The plasmid pETSACG1 carrying the APH(3')-IIIa gene (Gene bank Accession No. V01547) was obtained from Prof. A. Berghuis, McGill University. The plasmid pET9d carrying the APH(3')-Ia gene was from New England Biolabs.

DNA supercoiling and relaxation activities were assayed according to the manufacturer's protocols by following the procedures we described previously[15]. Briefly, DNA supercoiling activity was assayed with relaxed pBR322 DNA as a substrate (TopoGEN, Inc) and the DNA relaxation activity was assayed with supercoiled pBR322 DNA as a substrate (Inspiralis Ltd). The IC_{50} values in both experiments were defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%.

DNA fragments were separated on 1% agarose gel using TAEx1 buffer. Agarose was added to TAE buffer for a 1% (w/v) concentration and heated with a microwave oven until fully dissolved. Etidium Bromide was added to a slightly cooled solution to a final concentration of 0.625µg/ml. Molecular weight was determined with DNA standards λ DNA/HindIII (Fermentas). 6×Loading Dye (Fermentas) were added to each sample (1:1 ratio), loaded on to the gel and ran at 90mV in a Sub-Cell GT Cell (Bio-Rad) until separation (approximately 90min). DNA fragments were examined under UV light (Uvitec).

Prokaryotic *in-vitro* translation inhibition was quantified in quick coupled transcription/translation assays by using *E. coli* S30 extract for circular DNA with the pBEST*luc*™ plasmid (Promega), according to the manufacturer's protocol and the detailed procedure described previously by us[13] with some minor modifications as follows. Translation reactions were performed in a total volume of 10 µL (instead of 25 µL volume reported previously) and the luminescence was measured immediately (into 96-well plates) after the addition of the luciferase assay reagent (4.5 µL of reaction and 45µl dilution reagent; Promega). The IC_{50} values were obtained from fitting concentration-response curves to the data of at least two independent experiments by using Grafit 5 software (Leatherbarrow, R. J. Erithacus Software Ltd.: Horley, U.K., 2001).

Acknowledgments

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at htpp://dx.doi.org/10.1016/j.bmc.2017……

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