Bioorganic & Medicinal Chemistry 25 (2017) 2917–2925

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Covalently linked kanamycin – Ciprofloxacin hybrid antibiotics as a tool to fight bacterial resistance

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ARTICLE INFO

Article history:
Received 15 January 2017
Accepted 3 February 2017
Available online 16 March 2017

Keywords:
Aminoglycosides
Fluoroquinolones
Hybrid antibiotics
Antibiotic resistance
Delay of resistance

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.[7–10] 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]

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

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http://dx.doi.org/10.1016/j.bmc.2017.02.068
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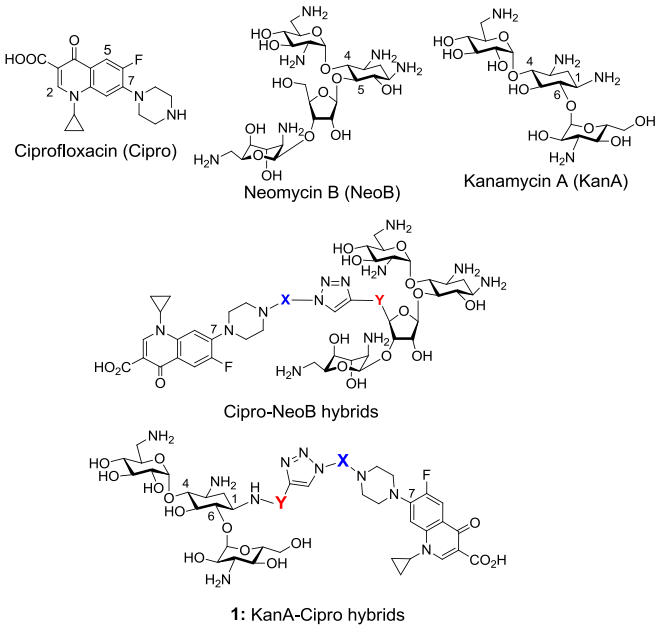


Fig. 1. Structures of ciprofloxacin, neomycin B, kanamycin A, Cipro-Neo B hybrids and the designed KanA-Cipro hybrids.

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.

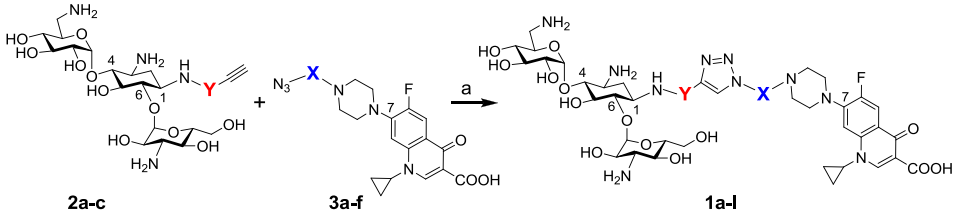
To test this hypothesis, we have designed, synthesized and biologically evaluated new variants of the previously studied Cipro-NeoB hybrid structures, in which the NeoB moiety is replaced by another common AG antibiotic, kanamycin A (KanA). We report that the resulted KanA-Cipro hybrids (**1**, Fig. 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 intermediatederivative 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,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



Scheme 1. Synthetic strategy for the assembly of KanA-Cipro Hybrids **1a-l**. [a] (a) $[(CH_3CN)_4Cu]PF_6$, 7% Et_3N in water, microwave 40 s.