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Letter

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Inhibition of Connexin Hemichannels by New Amphiphilic Amino-glycosides without Antibiotic Activity

Madher N. AlFindee[a,b], Yagya P. Subedi[a], Mariana C. Fiori[c], Srinivasan Krishnan[c], Abbey Kjellgren[c,d], Guillermo A. Altenberg[c] and Cheng-Wei T. Chang[a,*]

[a] Department of Chemistry and Biochemistry, Utah State University, 0300 Old Main Hill, Logan, Utah 84322-0300, USA

[b] Department of Pharmaceutical Chemistry, College of Pharmacy, University of Basra, Basra, Iraq

[c] Department of Cell Physiology and Molecular Biophysics, and Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, Texas 79430-6551, USA

[d] Honors College, McClellan Hall, Box 41017, Texas Tech University, Lubbock, Texas 79409-1017, USA

KEYWORDS: aminoglycosides; connexin; gap-junction channel; hemichannel; deafness; ischemia

ABSTRACT: Connexins hemichannels (HCs) from adjacent cells form gap junctional channels that mediate cell-to-cell communication. Abnormal opening of "free" undocked HCs can produce cell damage and participate in the mechanism of disorders such as cardiac infarct, stroke, deafness, skin diseases, and cataracts. Therefore, inhibitors of connexin HCs have great pharmacological potential. Antibiotic aminoglycosides (AGs) have been recently identified as connexin HC inhibitors, but their antibiotic effect is an issue for the treatment of disorders where infections do not play a role. Herein, we synthesized and tested several amphiphilic AGs without antibiotic effect for their inhibition against connexin HCs, using a newly developed cell-based bacterial growth complementation assay. Several leads with superior potency than the parent compound, kanamycin A, were identified. Unlike traditional AGs, these amphiphilic AGs are not bactericidal and are not toxic to mammalian cells, making them better than traditional AGs as HC inhibitors for clinical use and other applications.

Cell-to-cell communication is essential for transporting metabolites, ions, and signal molecules between adjacent cells. This occurs predominantly through gap-junction channels (GJCs) that are formed when two connexin hemichannels (HCs; connexin hexamers), one from each of the adjacent cells, dock head-to-head (Figure 1)[1-2]. There are 21 human connexin isoforms that form a variety of GJCs and HCs that differ in permeability and regulation[1-3]. The importance of GJCs in physiology and pathophysiology is well established from several decades of research, but the role of plasma-membrane undocked HCs is more recent[2,4-5]. Most HCs are closed under normal conditions, but they still participate in autocrine and paracrine signaling through the efflux of ATP, NAD^+, glutamate, prostaglandins and other mediators[6]. HCs also seem to play a role in disorders of the inner ear (deafness), eye (cataracts), heart (infarcts) and brain (cerebrovascular accidents)[2,5]. Abnormal opening of the "large" non-selective HCs can damage cells by depolarization, swelling, alterations in ionic gradients, and concentrations of metabolites and second messengers. There is interest in the finding and development of connexin HC inhibitors since they could offer insights on the role of HCs in normal function and disease, as well as serve as therapeutics in disorders associated with connexin HCs.

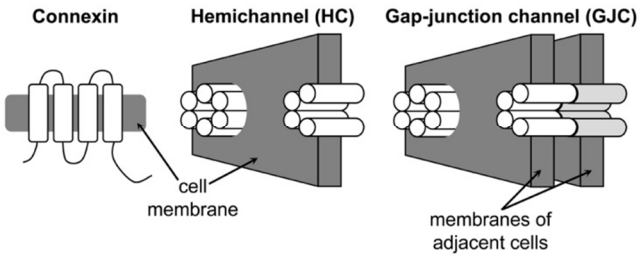
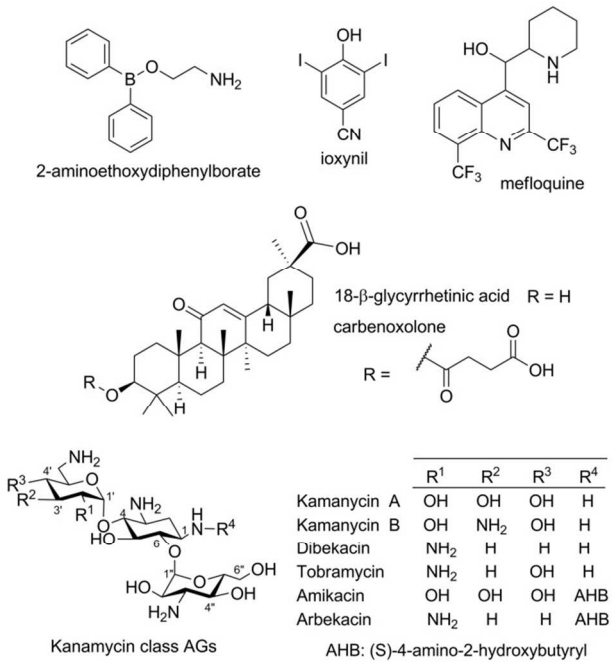


Figure 1. Schematic representation of a connexin monomer, a hemichannel (HC) and a gap-junction channel (GJC). Each cylinder in HC and GJC corresponds to a connexin subunit.

A variety of molecules have been investigated for their inhibitory effect toward connexin HCs, including 2-aminoethoxydiphenyl borate, ioxynil, carbenoxolone, 18-β-glycyrrhetinic acid, antimalarial drugs (mefloquine) and n-alkanols[7] (Figure 2). These cannot be used as starting points for the development of clinically useful HC inhibitors because

they are not selective for connexin HCs and/or they are toxic[7]. Connexin peptide inhibitors are synthetic peptides corresponding to sequences of connexins' extracellular or intracellular loops[7-9]. Some of the connexin peptide inhibitors have been used to treat arrhythmias and to accelerate wound healing[7-9]. Most of these peptides act on GJCs and HCs, but a few seem to selectively inhibit HCs[7]. However, their clinical potential is still unclear[10], and nevertheless, other avenues to develop clinically useful HC inhibitors have been largely unexplored. In this context, aminoglycosides (AGs) such as kanamycin and gentamicin have been recently identified as strong inhibitors of connexin HCs[11-14]. AGs have been used as antibacterial agents for over sixty years, and they are still among the most used antibiotics[15-16]. Although nephrotoxicity and ototoxicity are relatively common complications of AGs treatment, they can be managed[17]. We have discovered that chemically modified AGs, especially amphiphilic kanamycin derivatives, have biological activity, while showing significantly reduced cytotoxicity[18-23]. With this in mind, we began to explore amphiphilic kanamycin derivatives as connexin HC inhibitors, using connexin 26 (Cx26) HCs as test targets.

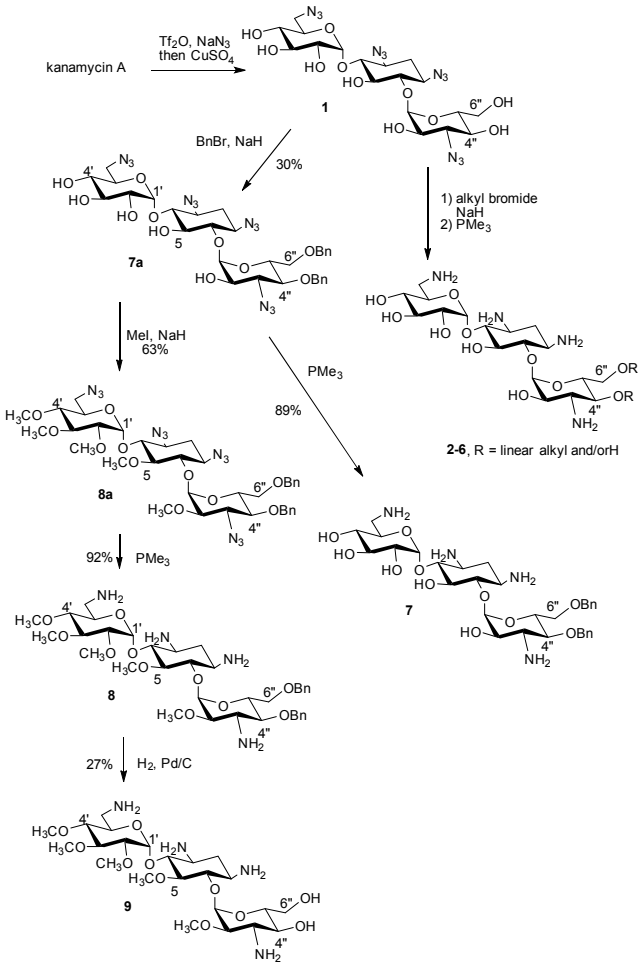


	R^1	R^2	R^3	R^4
Kamanycin A	OH	OH	OH	H
Kamanycin B	OH	NH_2	OH	H
Dibekacin	NH_2	H	H	H
Tobramycin	NH_2	H	OH	H
Amikacin	OH	OH	OH	AHB
Arbekacin	NH_2	H	H	AHB

Figure 2. Examples of inhibitors of connexin HCs.

We have reported a three-step synthesis of amphiphilic kanamycin derivatives bearing linear alkyl chains at the *O*-4″, *O*-6″, or *O*-4″ and *O*-6″ positions of kanamycin A (Scheme 1)[19]. These derivatives show moderate antifungal activity and no antibacterial activity, making them good candidates as Cx26 HC inhibitors without antibiotic effect; they could be used without the risk of promoting the generation of AG-resistant bacteria. Five amphiphilic kanamycin A derivatives were selected randomly for testing. To provide more information in structure-activity relationship (SAR), we also synthesized three additional derivatives: one with a benzyl (Bn) group attached at *O*-4″ and *O*-6″ positions (compound **7**); and two with or without a Bn group attached at *O*-4″ and *O*-6″ positions, and a methyl (Me) group at other hydroxyl groups (5, 2′, 3′, 4′ and 2″) (compounds **8** and **9**, respectively). These derivatives were prepared by sequential benzylation and methylation followed by reduction of azido groups or hydrogenolysis of the Bn. To elucidate the role of amino groups, compound **1** (Scheme 1) was also tested. Please see Supplementary Information for details on the synthesis and characterization of the new kanamycin derivatives, including the use of acetylation and ^{1}H-^{1}H COSY for the confirmation of the sites of modifications.

Scheme 1



Connexin HC inhibition was conducted using a bacteria-based assay of HC function[14]. In this assay, Cx26 HCs are expressed in *E. coli* LB2003 cells, which lack three major K^+ uptake systems (Kdp, Kup and Trk), and as a result they cannot grow in low-[K^+] media[14,24,25]. However, growth of this *E. coli* strain in low-[K^+] medium can be rescued by expression of K^+-permeable channels such as connexin HCs[13,14,26]. Under the conditions of the assay (LB2003 cells expressing HCs and grown in low-K^+ medium), inhibition of the HCs reduces or abolishes growth[13,14,26]. This is a simple assay where bacterial growth in multi-well plates can be followed by measuring the absorbance at 600 nm (OD_{600})[14]. Although not formally needed for most of the AGs tested here (they do not have antibiotic effect), we routinely transform the *E. coli* LB2003 with plas-

mid that codes for the AG modifying enzyme aminoglycoside 3'-phosphotransferase, which makes the cells resistant to kanamycin A and its derivatives[14].

The kanamycin derivative without free amino group (compound **1**) was inactive, suggesting an essential role of this group. One of the di-substituted derivatives (compound **3**) displayed a lower IC_{50} for inhibition of growth dependent on Cx26 than the mono-substituted compound **4** (Table 1). Compared to compound **3** (hexyl), the potency was reduced when the alkyl chain length was shortened to butyl in compound **2** or extended to octyl (compound **5**) or nonyl (compound **6**). The potency of compounds **7** and **8** was similar, implying that the presence of hydroxyl groups, or the associated hydrogen bond interactions, is not necessary for the AG effect on HCs. However, the lack of inhibitory activity of compound **9** demonstrates that having hydrophobic groups at *O*-4″ and/or *O*-6″ positions is essential in the absence of hydroxyl groups (compare compounds **8** and **9**). The maximal inhibition of growth dependent on Cx26 was similar to that of kanamycin A, and was not statistically different among the new compounds with inhibitory effect (compounds **2**-**8**), averaging 99 ± 3%.

From the data in Table 1, compounds **3**, **7** and **8** were the most potent inhibitors. However, derivatization based on the hexyl group scaffold of compound **3** is difficult. Because of the ease of the chemical synthesis and purification process, we decided to focus on the derivatization bearing aryl-based substituents. Compounds **7** and **8**, with Bn groups at *O*-4″ and *O*-6″, can be readily modified with variations of substituent on the benzene ring, or shape/size of the aromatic motif. Considering the simpler reaction scheme and the flexibility on the groups accepted at the *O*-4″ and *O*-6″ positions, and in the absence of an advantage of compound **8** over compound **7**, we selected the latter for further development.

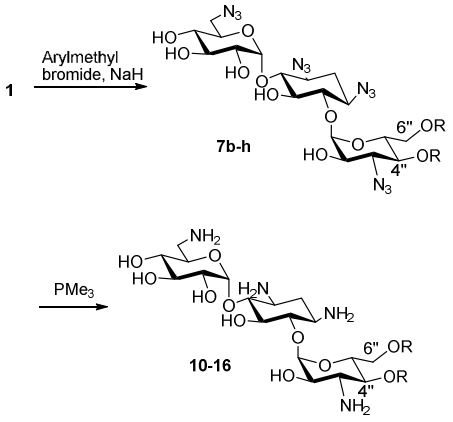
Table 1. Inhibition of Cx26-dependent cell growth by kanamycin A and synthetic AGs.

Compound	Modifications	IC_{50} (μM)
Kanamycin A	-	9.4 ± 1.1
1	-	No effect
2	butyl at *O*-4″ and *O*-6″	19.0 ± 2.8*
3	hexyl at *O*-4″ and *O*-6″	6.2 ± 1.4
4	hexyl at *O*-4″	13.5 ± 1.8*
5	octyl at *O*-4″ and *O*-6″	13.6 ± 0.5*
6	nonyl at *O*-4″ and *O*-6″	18.8 ± 1.3*
7	Bn at *O*-4″ and *O*-6″	7.6 ± 1.2
8	Me at 5, 2′, 3′, 4′ and 2″, Bn at *O*-4″ and *O*-6″	8.4 ± 1.4
9	Me at 5, 2′, 3′, 4′ and 2″	No effect

No effect: absence of inhibition at 100 μM. * denotes $P < 0.01$ *vs*. kanamycin A.

A library of kanamycin derivatives bearing arylmethyl substituents was synthesized to investigate two factors related to SAR (see Supplementary Information): the electronic and steric (size) effects of the aryl group; see Scheme 2 and Table 2, and an expanded Table under Supplementary Information (Supplementary Table 1). We selected four benzene derivatives with electron-donating and withdrawing substituents (methoxy, methyl, chloro and fluoro) at the para position to explore the first factor. Three arylmethyl groups, 1-naphthalenemethyl, 2-naphthalenemethyl, and bi-phenyl, were selected for studying the second factor. The synthesis was conducted as described previously[19], using commercially available arylmethyl bromides. When alkylating compound **1,** we observed a significant amount of mono-substituted adducts bearing arylmethyl groups at *O*-4″ or *O*-6″ positions. Attempts to improve the yields for di-substituted adducts by varying the equivalents of arylmethyl bromides or separating two mono-substituted adducts using flash chromatography were unsuccessful. Therefore, only the di-substituted adducts were isolated to proceed toward the completed synthesis. The IC_{50} values for inhibition by these derivatives are summarized in Table 2. It appears that the relationship between the electronic effect and inhibitory potency is not strong, but rather subtle. Compounds with a moderate electron-donating group (CH_3-, compound **11**) or moderate electron-withdrawing group (Cl-, compound **12**) showed better activities than those with a strong electron-donating (CH_3O-) group (compound **10**) or a strong electron-withdrawing (F) group (compound **13**). Compounds **11** and **12** had superior inhibitory potency than kanamycin A and the parent compound **7**, which has no substituent on the benzene ring. The inhibitory potency of the derivative with a bi-cyclic aromatic ring (compound **14**) was slightly higher than that of compounds **15** and **16**, but the differences were not major. Combining these results, it seems that compounds **11**, **12** and **14** can all serve as leads for further development. The maximal inhibition of growth dependent on Cx26 was similar for compounds **10**-**16**, and averaged 94 ± 2%. Examples of growth inhibition by three of the new aminoglycosides, including compound **12**, are shown in the Supplementary Information (Supplementary Figure 1).

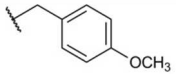
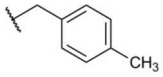
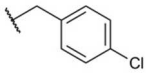
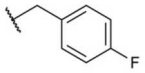
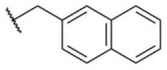
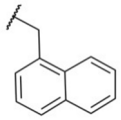
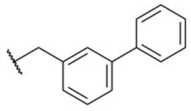
Scheme 2



All of the kanamycin derivatives were examined for their antibacterial activity against *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC25923). Except for compounds **15** and **16**, which have minimum inhibitory concentrations (MICs) of 64 and 16 μg/mL against *S. aureus*, respectively, the derivatives did not show significant antibacterial activity,

with MICs ranging from 128 to >256 μg/mL against both bacteria. The cytotoxicity of the newly synthesized di-substituted kanamycin derivatives on HeLa cells was also examined and is presented in the Supplementary Information (Supplementary Figure 2). Compounds **13**, **14**, and **16** showed moderate cytotoxicity (40-60% reduction of cell viability) at 100 μg/mL, which is at least 20-fold higher than the corresponding IC_{50} for Cx26 HC inhibition. No significant reduction of cell viability was observed for the rest of the compounds at concentrations up to 100 μg/mL. The low cytotoxicity is advantageous for potential uses as therapeutics.

Table 2. Second generation synthetic AGs.

Compound	R	IC_{50} (μM)
10		13.0 ± 0.7**
11		**4.3 ± 0.4***
12		**2.5 ± 0.6****
13		8.1 ± 0.5
14		**4.9 ± 0.2***
15		6.6 ± 0.5
16		6.2 ± 0.7

* and ** denote $P < 0.05$ and $P < 0.01$ *vs*. compound 7, respectively.

In conclusion, we have discovered a new application for amphiphilic kanamycin derivatives as connexin HC inhibitors. Previous studies have shown that the AG gentamicin inhibits HC, but not GJCs,[12] although indirect GJC inhibition, probably through the generation of free radicals, has also been reported[27]. Therefore, the issue of selective inhibition of HC *vs*. GJC by AGs remains to be clarified. In any case, the new amphiphilic kanamycin derivatives presented here represent a novel class of HC inhibitors that have the advantages of not having antibacterial activity and displaying low cytotoxicity. We have identified the preferred structural motifs for improving HC inhibitory potency. Development of suitable connexin HC inhibitors may pave the way for better understanding the mechanism of HC inhibition and the role of connexin HCs in human disorders.

ASSOCIATED CONTENT

Supporting Information

Supporting Information in a PDF file is available free of charge on the ACS Publications website.

AUTHOR INFORMATION

Corresponding Author

* To whom correspondence should be addressed at E-mail: tom.chang@usu.edu.

Author Contributions

Synthesis and characterization of the new compounds was performed by M.N.A. and Y.P.S. The HC inhibition assays were performed and analyzed by M.C.F, A.K. and S.K, and Y.P.S. performed and analyzed the HeLa cytotoxicity assays. G.A.A. and C.-W.T.C. designed and supervised the studies. The manuscript was written through contributions of all authors, and was edited by G.A.A. and C.-W.T.C. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

AG, aminoglycoside; Bn, benzyl; GJC, gap-junction channel; HC, hemichannel; IC_{50}, concentration that produces 50% inhibition; Me, methyl; MIC, minimum inhibitory concentrations; SAR, structure-activity relationship.

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