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Research paper

Amphiphilic aminoglycosides with increased selectivity for inhibition of connexin 43 (Cx43) hemichannels

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ABSTRACT

Gap junction channels formed by the association of connexin hemichannels play a crucial role in intercellular communication. Connexin 43 (Cx43) is expressed in a variety of tissues and organs, including heart and brain, and abnormal sustained opening of undocked "free" hemichannels contributes to the cell damage in cardiac infarcts and stroke. Selective inhibitors of Cx43 hemichannels for clinical use are then desirable. Here, we synthesized and tested new aminoglycosides for their connexin inhibitory activity towards Cx26 and Cx43 hemichannels. The lead compounds displayed enhanced Cx43/Cx26 selectivity for hemichannel inhibition when compared to the parent kanamycin A and other commercially available aminoglycosides. These lead compounds are not cytotoxic to mammalian cells and show promise for the treatment of ischemic damage of the heart, brain, and kidneys. We identified a new compound as a promising lead based on its good selectivity for Cx43 hemichannels inhibition and the simplicity and affordability of its production.

1. Introduction

Gap junction channels are formed by the head-to-head association of two connexin hemichannels (HCs), one from each of two adjacent cells [1,2]. Gap junction channels coalesce into junctional plaques, which allow the intercellular exchange of molecules of up to 600–800 kDa, including inorganic ions, metabolites and second messengers, coupling neighboring cells metabolically and electrically [3,4]. Besides their participation in intercellular communication by forming gap junction channels, HCs mediate autocrine and paracrine signaling through the efflux of compounds such as ATP, NAD^+, glutamate, prostaglandins, and other signaling molecules [5,6]. Undocked "free" HCs at the plasma membrane are mostly closed, but abnormal and sustained increase in their opening occurs under conditions characterized by hypoxia, reduced intracellular redox potential, and changes in phosphorylation, among many [7–14]. The increased HCs activity under such conditions contributes to the cell damage in a variety of disorders, including cardiac infarcts, stroke, neurodegenerative diseases and some forms of deafness [9,10,13,15–18]. Therefore, connexin HCs are potentially valuable pharmacological targets. From the 21 human connexin isoforms, Cx26 and Cx43 belong to different subgroups and are among the most dissimilar based on their sequence, phylogenetic relationship within the connexin family, size, permeability, and presence of the regulatory C-terminal domain only in Cx43 [2–4]. While the critical role for Cx26 in the normal function of the inner ear and deafness is well established [9,18], the abnormal opening of Cx43 HCs in the heart, brain, and kidneys contributes to the ischemic damage of these organs [7,10,12–15,19]. Because of the frequency of cardiac infarcts and strokes, selective Cx43 HC inhibitors are highly desirable.

Common HC inhibitors such as 2-aminoethoxydiphenyl borate, carbenoxolone, 18-β-glycyrrhetinic acid, and n-alkanols are not good starting points for the development of clinically useful drugs because they are not selective and/or they are toxic [20–23]. Connexin peptide inhibitors, synthetic peptides corresponding to sequences of extracellular or intracellular loops of connexins, are more promising [20,21,24].Most of these peptides act on gap junction channels and HCs, but a few seem to be selective HC inhibitors [20]. Some have been used to treat arrhythmias and to

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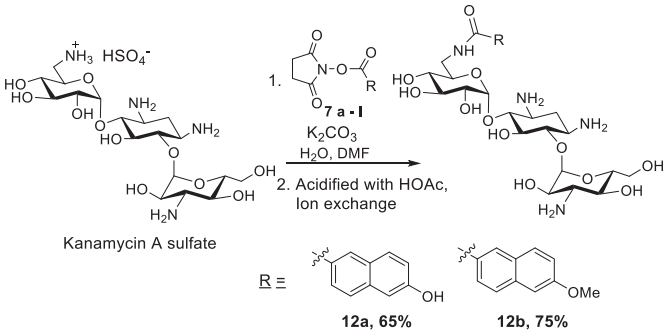
accelerate wound healing, but their clinical potential is still unclear [20,21,24,25]. Nevertheless, other approaches for the design and development of HC inhibitors have been largely unexplored.

A new paradigm in therapeutic development is to "re-purpose" drugs for different applications. In this context, antibacterial aminoglycosides (AGs) have been shown to inhibit connexin HCs, but not gap junction channels [26–30]. AG antibiotics have been used for more than 70 years, although they have nephrotoxic and ototoxic effects, these side effects can be managed and AGs are still among the most used antibiotics [31,32]. In a previous study, we found that commercially-available AGs inhibit HCs formed by Cx26 and Cx43 [30]. Defining selectivity as Cx26 IC_{50}/Cx43 IC_{50}, the tested commercially available AGs showed selectivity for Cx26 HC inhibition, with Cx26 IC_{50}/Cx43 IC_{50} ratios <0.25 [33]. In a study aimed at developing AGs HC inhibitors without antibiotic effect, we synthesized and characterized 4″,6″ diarylmethyl kanamycin derivatives as Cx26 HC inhibitors [33]. These amphiphilic kanamycins (AKs) inhibit Cx26 HCs without the antibacterial activity of the parent compound kanamycin, and display low toxicity towards human cells [33]. However, the need to use the hazardous sodium azide for the synthesis of the precursor, azidokanamycin, and the cumbersome column purification steps that limit large scale synthesis, complicate the use of these compounds as leads. We have recently reported the facile and low-cost synthesis of 6″- and 6′-modified AKs with diverse structural variations [34,35]. These AKs also have hydrophobic group conjugated to kanamycin core like 4″,6″ diarylmethyl kanamycin. Here, we determined the inhibition of Cx43 HC by the 4″,6″ diarylmethyl kanamycin derivatives previously reported as inhibitors of Cx26 HCs [33], and explored the selectivity for inhibition of Cx43 *vs* Cx26 HCs of new AKs bearing aryl and alkyl substituents.

2. Results and discussion

2.1. Synthesis and design of 6″-modified amphiphilic kanamycins

We have previously reported the synthesis of 6″-modified AKs *via* a concise route with the tosylate precursor (compound **9**) that could be synthesized in a large scale (90 g) as the key intermediate [34]. As the aryl entities are the main structural feature of the previously reported 4″,6″ disubstituted kanamycin derivatives, the attempt of incorporating aryl groups at 6″ position was implemented. The attempts of nucleophilic substitution using aryl alcohols and amines were examined and only aryl alcohols (substituted phenols) furnished the desired products (Scheme 1). Using



Scheme 2. Synthesis 6′-modified kanamycin derivatives.

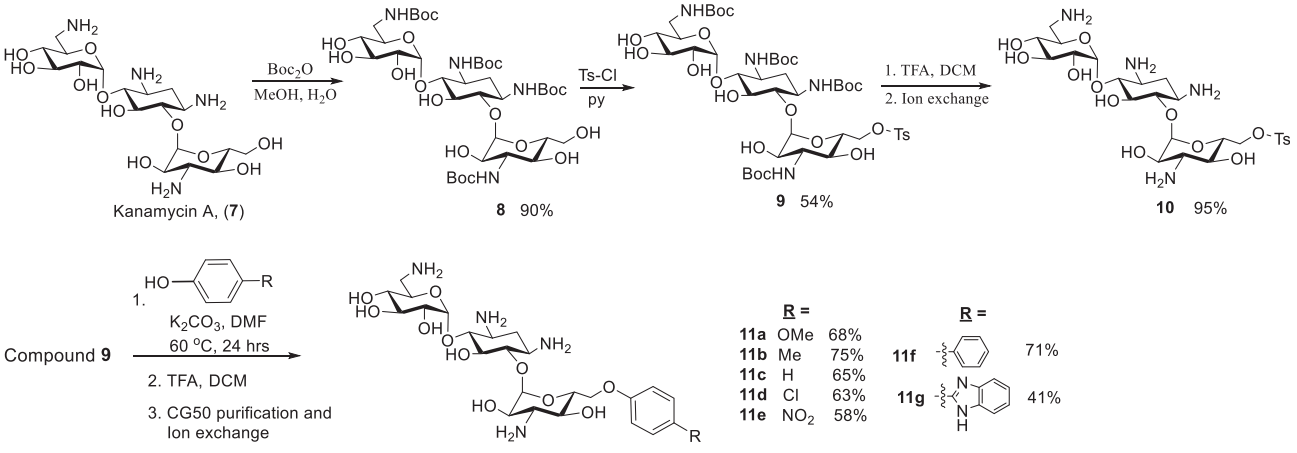
compound **9** as the starting material, compounds **11a-g** were synthesized *via* a nucleophilic substitution followed by the deprotection of Boc groups. These AKs have structural variations on the electronic effect on the substituents attached to the phenyl group as well as the steric effect, as in the case of compounds **11f** and **11g**.

2.2. Synthesis and design of 6′-modified amphiphilic kanamycins

The 6′-modified AKs were produced from kanamycin in one step with excellent overall yield [35]. Following this method, we prepared compounds **12a,b**, which contain an aryl group (Scheme 2). In addition to the newly synthesized AKs, we also selected several members that have been prepared previously to cover more structural features (Fig. 1).

2.3. Inhibition of connexin HCs by amphiphilic kanamycins

To determine the inhibition of human Cx26 and Cx43 HCs, we used a growth complementation assay based on the use of LB2003 *E. coli* cells [36]. In these cells, the K^+ uptake systems Kdp, Kup, and Trk are knocked out, and as a result the cells grow normally in high-[K^+] medium but cannot grow in low-[K^+] medium [36]. HCs expressed in LB2003 cells can reverse the no-grow phenotype in low-[K^+] medium due to the permeability of HCs to K^+, allowing K^+ uptake and cell growth [36]. All the growth complementation studies were performed on kanamycin-resistant cells (transformed with pREP4, a kanamycin-resistance plasmid). Most of the AKs did not inhibit growth by themselves in these cells at concentrations of at least 5 times the IC_{50} values for Cx43-dependent growth complementation (see Supplementary Information). Although we



Scheme 1. Synthesis of 6″-modified kanamycin A derivatives.

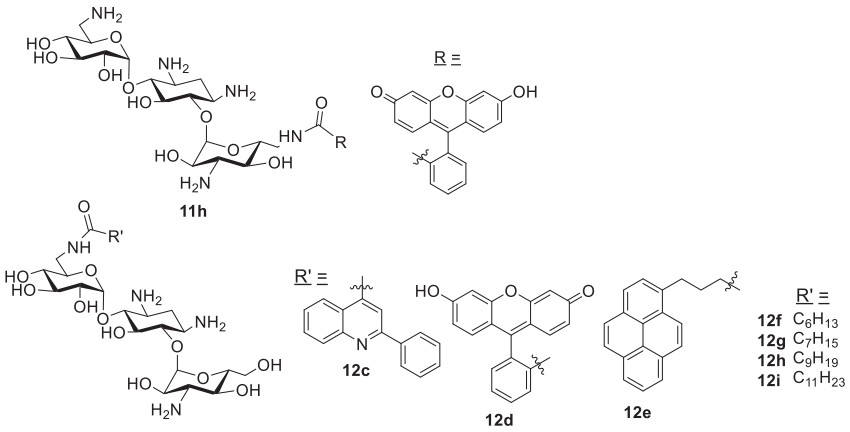
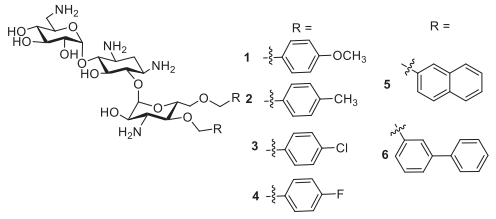


Fig. 1. Structures of previously reported AKs.

Table 1
IC_{50} values of 4″–6″ benzyl substituted kanamycin towards Cx43 and Cx43/Cx26 selectivity.



Compound	IC_{50} Cx43 (μM)	Cx43/Cx26 selectivity[a]
1	4.1 ± 1.5	3.17
2	3.1 ± 0.9	1.39
3	17.1 ± 1.7	0.15
4	26.6 ± 7.8	0.30
5	6.2 ± 4.4	1.07
6	5.5 ± 2.0	1.23

[a] The Cx43/Cx26 selectivity was calculated as IC_{50} Cx26/IC_{50} Cx43, using Cx26 IC_{50} values previously published[33] and the new data on Cx43 presented here. Data are means ± SEM from 6 to 12 replicates *per* independent experiment (compound **1**, n = 2; compound **2**, n = 5; compound **3**, n = 2; compound **4**, n = 3; compound **5**, n = 2; compound **6**, n = 2).

use the term HC inhibition frequently in the text, we measured HC function indirectly through the HC-dependent growth complementation, an indicator of HC permeability [29,36].

As mentioned above, we have characterized 4″,6″ diarylmethyl kanamycin derivatives as Cx26 HC inhibitors, and identified a lead compound (compound **3** in Table 1) that is a four times more potent inhibitor than the parent kanamycin [33]. Here, we tested several of the 4″,6″ diarylmethyl kanamycin derivatives as Cx43 HC inhibitors. Table 1 shows that several of these compounds display selectivity towards Cx43 HCs (selectivity >1; compounds **1**, **2**, **5** and **6**) when compared to commercial AGs, which have selectivities <0.25 [30]. However, there was no apparent trend in the structure activity relationship (SAR) that explains the variation of selectivity (see Table 1).

We then explored 6′- and 6″-modified AKs for their inhibitory activity against Cx26 and Cx43 HCs. For the initial screening, we tested the compounds at two concentrations, 15 and 50 μM. These concentrations were chosen because they are close to the IC_{50} values for inhibition of Cx26 (~10 μM) and Cx43 HCs (~50 μM) [30] by kanamycin. The HC inhibition by 6″- and 6′-modified AKs are shown in Tables 2 and 3, respectively. The compounds that seemed more potent inhibitors of Cx43 HCs (>50% inhibition at 50 μM) were selected for determination of IC_{50} values (Table 4). Examples of the concentration dependence of the inhibition of Cx26-and Cx43-dependent growth complementation are shown in Supplementary Figure 1. The three selected 6″-modified AKs had similar potency against Cx43 HCs, with IC_{50} values in the low micromolar level. Of these, compounds **11b** and **11d** were more selective against Cx43 HCs, whereas compound **11g** displayed selectivity against Cx26 with decreases in IC_{50} for inhibition of Cx26 and Cx43 HCs (Table 4). When compared to kanamycin, compound **11g** increased the potency for inhibition of both Cx26 and Cx43 HCs,

Table 2
Inhibition of Cx26 and Cx43-dependent growth complementation by 6″-modified kanamycins.[a]

Compound	% Inhibition Cx26		% Inhibition of Cx43		cLogD (pH = 7)
	15 μM	50 μM	15 μM	50 μM	
10	26.8 ± 1.7	49.2 ± 5.5	38.1 ± 2.8	51.0 ± 8.8	−12.02
11a	15.9 ± 4.2	36.5 ± 4.7	20.0 ± 7.4	34.8 ± 7.8	−12.52
11b	19.3 ± 2.2	28.2 ± 4.5	**63.8 ± 6.8**	76.8 ± 5.8	−11.80
11c	14.6 ± 4.3	69.4 ± 4.2	36.0 ± 3.9	46.7 ± 7.8	−12.27
11d	44.4 ± 7.0	69.5 ± 4.5	**50.3 ± 1.0**	68.3 ± 4.6	−11.75
11e	35.9 ± 2.9	62.6 ± 9.2	36.5 ± 0.6	49.3 ± 2.2	−12.31
11f	46.3 ± 8.6	67.8 ± 6.0	27.3 ± 4.1	54.3 ± 8.9	−10.58
11g	**83.1 ± 2.1**	85.5 ± 1.7	**43.2 ± 7.3**	71.7 ± 3.5	−10.88
11h	37.1 ± 13.5	81.0 ± 1.7	45.3 ± 2.7	55.1 ± 5.8	−10.73

[a] Data are means ± SEM from 3 to 5 independent experiments with 6–12 replicates *per* experiment. Data in bold indicate values larger than those of kanamycin A at 15 μM (compounds selected for the more detailed inhibition studies in Table 4).

Table 3
Inhibition of Cx26 and Cx43-dependent growth complementation by 6′-modified kanamycins.[a]

Compound	% Inhibition of Cx26		% Inhibition of Cx43		cLogD (pH = 7)
	15 μM	50 μM	15 μM	50 μM	
12a	4.7 ± 1.6	32.8 ± 11.1	34.1 ± 6.0	37.1 ± 7.4	−10.27
12b	15.1 ± 4.9	27.9 ± 2.6	42.1 ± 1.5	45.1 ± 1.5	−10.24
12c	8.0 ± 2.0	22.8 ± 2.2	25.5 ± 7.1	51.0 ± 2.5	−8.81
12d	**22.3 ± 6.9**	79.6 ± 3.5	**56.8 ± 8.8**	83.4 ± 1.1	−8.97
12e	**36.9 ± 3.9**	79.2 ± 7.5	**52.2 ± 8.8**	69.6 ± 7.5	−7.75
12f	22.5 ± 1.9	34.3 ± 2.6	18.5 ± 0.7	36.0 ± 6.1	−10.90
12g	14.0 ± 2.1	25.2 ± 2.3	17.0 ± 5.2	37.2 ± 7.6	−10.11
12h	16.1 ± 3.2	33.6 ± 3.8	44.4 ± 5.3	53.5 ± 1.9	−9.31
12i	**16.4 ± 2.3**	56.2 ± 6.8	**53.6 ± 4.6**	70.9 ± 2.3	−8.52

[a] Data are means ± SEM from 3 to 5 independent experiments with 6–12 replicates *per* experiment. Data in bold indicate values larger than those of kanamycin A at 15 μM (compounds selected for the more detailed inhibition studies in Table 4).

Table 4
IC_{50} of AKs toward Cx26 and Cx43.[a]

Class of AKs	Compound	IC_{50} Cx26	IC_{50} Cx43	Cx43/Cx26 selectivity[b]	cLogD (pH 7)
6″-modified	**11b**	49.4 ± 9.3	6.2 ± 1.4	7.97	−11.80
	11d	17.2 ± 3.2	8.9 ± 1.6	1.93	−11.75
	11g	6.0 ± 1.2	17.7 ± 5.6	0.34	−10.88
6′-modified	**12d**	12.7 ± 2.2	8.9 ± 3.7	1.42	−8.97
	12e	18.6 ± 2.1	8.6 ± 3.0	2.16	−7.75
	12i	66.7 ± 6.9	9.7 ± 1.8	6.88	−8.52
Kanamycin A		11.5 ± 1.8	48.0 ± 2.0	0.24	N.D.[c]

[a] Data are means ± SEM from 3 to 4 independent experiments with 6−12 replicates *per* experiment. IC_{50} values were calculated from fits of the Hill's equation to the data (see Supplementary Figure 1 for examples).
[b] The Cx43/Cx26 selectivity was calculated as IC_{50} Cx26/IC_{50} Cx43.
[c] N.D.: not determined.

but did not affect much the selectivity (0.34 vs. 0.24) [30]. Compound **11b** was particularly interesting because it showed a Cx26 HC IC_{50} ~10-fold higher than the parental compound, whereas its Cx43 HC IC_{50} was ~8-fold lower than that of kanamycin A.

Different from the 6″-modified AKs, the three selected 6′-modified AKs showed selectivity for Cx43 HC inhibition (Table 4). Compound **12i** was the most selective with a Cx26 IC_{50}/Cx43 IC_{50} of 7, a value ~30-folds higher than kanamycin A (0.24) [30]. Albeit with the limited number of compounds for which IC_{50} values were determined, the AK's with 6″ modification show a correlation between cLogD and selectivity, more selective towards Cx43 (higher ratio of Cx26/Cx43 IC_{50} values) with increase in hydrophobicity (higher cLogD), whereas the three AK's with 6′ modification are all among the more hydrophobic 6′-modified AKs. None of the 6″-(compounds **11b**, **11d** and **11g**) or 6′-modified AKs (compounds **12d**, **12e** and **12i**) selected displayed cytotoxicity on HeLa cells at a concentration of up to 100 μM (Supplementary Figure 2).

Since the inhibitory studies suggested a potential relationship between AG hydrophobicity and HC inhibition, we plotted the inhibition of Cx26-and Cx43-dependent growth complementation as a function of cLogD (Fig. 2). We used the inhibition of HC-dependent growth complementation at 15 μM, except for inhibition by compounds **12a** and **12c**, for which we used the 50 μM values due to the poor inhibition of Cx26-dependent growth complementation at 15 μM (see Table 3). In all cases, the inhibition was normalized to the value of kanamycin at the corresponding concentration. One obvious conclusion from Fig. 2 is that all the 6″- and 6′-modified AKs display increased Cx43 HC inhibitory potency (squares) with increased cLogD, except for the 6″-modified compounds **11f**, **11g** and **11h**. For the 6″-modified AKs (Fig. 2A) specifically, there was an increase in the potency for Cx43 HC inhibition for the compounds with *p*-substituted phenyl groups (**11a-e**) and toluenesulfonyl (**10**) as hydrophobicity increased (higher cLogD). The line fit in Fig. 2A does not include compounds **11f**, **11g** and **11h** and yielded an r^2 of 0.82. However, when these compounds were included in the fit r^2 dropped progressively to 0.03, whereas removal of any of the other compounds had little effect on the correlation. Interestingly, compounds **11f**, **11g** and **11h** comprise substituents with at least two aromatic rings in contrast to other 6′-modified AKs. Therefore, steric hindrance is probably the cause for the relationship dropped off for compounds **11f-h**. A weaker correlation between the inhibitory potency against Cx43 HC and the cLogD was observed for the 6′-modified AKs, (Fig. 2B; $r^2 = 0.45$). Different from the 6″-modified AKs, the correlation seems to be maintained from the compounds with linear alkyl groups to those with aryl moieties, so an increase in steric hindrance does not seem to affect Cx43 HC inhibition by the 6′-modified AKs. In general, the inhibition of both the 6″- and 6′-modified AKs against Cx26 HC was lower and did not show correlation with the change of cLogD (Fig. 2; circles). Unfortunately, the limited structural information on Cx43 precludes a mechanistic interpretation of the relationship between compound structure and Cx43/Cx26 selectivity.

3. Summary and conclusions

We prepared two libraries of AKs (6″- and 6′- modified) and investigated their inhibitory activity against Cx26 and Cx43 HCs, as evaluated by an *E. coli*-based growth complementation assay. The inhibitory effect towards Cx43 HCs by the 6″-modified AKs was enhanced with increased hydrophobicity (cLogD) and decreased with steric hindrance. We found a similar correlation between the inhibitory effect on Cx43 HCs by the 6′-modified AKs and cLogD. However, an increase in steric hindrance did not affect Cx43 HC inhibition in these compounds. No distinct SAR could be derived for the activity against Cx26, but the potency for Cx26 HC inhibition was reduced for most compounds. In summary, we identified several AKs bearing 6′ and of 6″ modifications that display an enhanced selectivity for inhibition of Cx43 *vs* Cx26 HCs, displaying

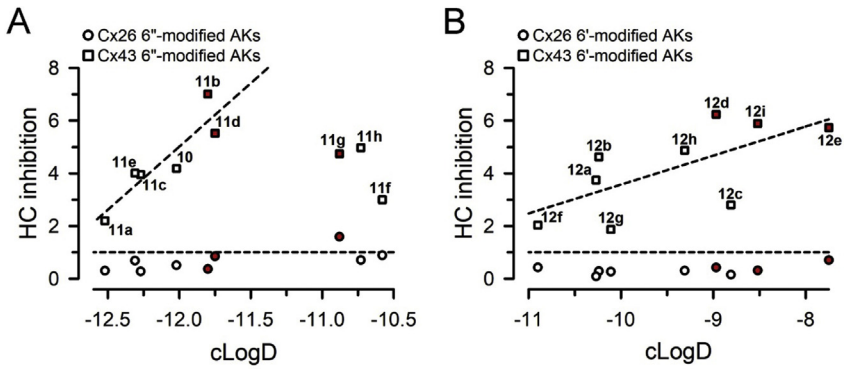


Fig. 2. Correlation between the inhibition of HC-dependent growth complementation by AKs and cLogD. The values corresponding to the compounds in Table 4 are shown in red.

a selectivity that is opposite to that of the parental compound kanamycin. The results show the importance of substitutions at positions 6′ and 6″ of kanamycin for the differential inhibition of Cx26 and Cx43 HCs. Based on the correlation between Cx43 HC inhibitory potency and hydrophobicity, it seems possible to enhance the selectivity of AKs toward Cx43 by increasing the hydrophobicity at position 6″ without inducing steric hindrance, and at position 6′ by increasing hydrophobicity. Compound **12i** seems the most promising lead as Cx43 HC inhibitor because of its good selectivity, and the simplicity and affordability of its production, since it can be synthesized on a large scale without a need for column purifications.

4. Experiment

4.1. General procedure for the synthesis of compounds 11a-g

Two equiv (0.4 mmol) of substituted phenol and 0.055 g (0.4 mmol, 2 equiv) of K_2CO_3 were added to 0.20 g (0.2 mmol, 1 equiv) of compound **9** dissolved in anhydrous dimethylformamide (DMF) and the mixture was heated at 60 °C for 24 h with vigorous stirring. The solvent was removed under reduced pressure and the residue was washed with 3 × 20 mL water. The washed reaction mixture was dried and treated with 2 mL of trifluoroacetic acid using 10 mL anhydrous dichloromethane (DCM) as solvent. The reaction was stirred at room temperature for 6 h and the solvent was removed by blowing air inside a hood. Ten mL of water was added and the mixture was stirred for 15 min at room temperature. The reaction mixture in water was filtered through a 0.2-μm syringe filter and the water from the filtrate was removed by air blowing. Then, the compound was purified using a CG50 cation exchange resin (Amberlite), and the pure compound, obtained in acetate form after the CG50-based purification, was converted to the chloride form using IR410 (Cl^- form) resin.

4.2. 6″-O-(4-methoxyphenyl)kanamycin A (11a)

This compound was synthesized as a white solid following the general procedure. ^{1}H NMR (500 MHz, D_2O) δ 6.9–7.0 (m, 4H), 5.51 (d, J = 3.5 Hz, 1H), 5.05 (d, J = 3.0 Hz, 1H), 4.1–4.2 (m, 3H), 3.9–4.0 (m, 2H), 3.8–3.9 (m, 2H), 3.6–3.8 (m, 7H), 3.3–3.5 (m, 5H), 3.1–3.2 (m, 1H), 2.3–2.5 (m, 1H), 1.7–1.8 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 153.63, 152.32, 116.06 (2C), 115.17 (2C), 100.51, 96.82, 84.39, 79.60, 73.00, 72.15, 71.15, 70.87, 70.72, 68.59, 68.20, 66.91, 65.50, 55.87, 54.96, 50.07, 48.00, 40.27, 28.88. ESI/APCI calculated for $C_{25}H_{43}N_4O_{12}^+$ $[MH]^+$: 591.2877; measured m/e: 591.2879.

4.3. 6″-O-(4-methylphenyl)kanamycin A (11b)

The compound was synthesized as a light-brown solid following the general procedure. ^{1}H NMR (500 MHz, D_2O) δ 7.08 (d, J = 8.5 Hz, 1H), 6.83 (d, J = 8.5 Hz, 1H), 5.50 (d, J = 3.5 Hz, 1H), 5.05 (d, J = 3.5 Hz, 1H), 3.8–4.0 (m, 5H), 3.6–3.8 (m, 2H), 3.4–3.6 (m, 4H), 3.2–3.4 (m, 2H), 3.0–3.1 (m, 1H), 2.4–2.5 (m, 1H), 2.14 (s, 1H) 1.8–1.9 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 155.73, 131.57, 130.20 (2C), 114.78 (2C), 100.59, 96.24, 83.83, 78.15, 72.65, 72.07, 71.18, 70.79, 70.77, 68.64, 68.12, 66.35, 65.47, 54.94, 49.90, 47.84, 40.36, 27.63, 19.53. ESI/APCI calculated for $C_{24}H_{47}N_4O_{12}^+$ $[MH]^+$: 575.2928; measured m/e: 575.2927.

4.4. 6″-O-phenylkanamycin A (11c)

This compound was synthesized as a white solid following the general procedure. ^{1}H NMR (500 MHz, D_2O) δ 7.2–7.3 (m, 2H), 6.9–7.0 (m, 3H), 5.49 (d, J = 4.0 Hz, 1H), 5.05 (d, J = 4.0 Hz, 1H), 4.1–4.2 (m, 3H), 3.7–4.0 (m, 5H), 3.6–3.7 (m, 2H), 3.4–3.6 (m, 4H), 3.2–3.4 (m, 2H), 3.0–3.1 (m, 1H), 2.4–2.5 (m, 1H), 1.8–1.9 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 157.87, 129.92 (2C), 121.80, 114.79, 100.57, 96.33, 83.91, 78.37, 72.70, 72.06, 71.11, 70.80, 70.77, 68.61, 68.13, 66.09, 65.48, 54.93, 49.93, 47.88, 40.35, 27.84. ESI/APCI calculated for $C_{24}H_{41}N_4O_{11}^+$ $[MH]^+$: 561.2772; measured m/e: 561.2764.

4.5. 6″-O-(4-chlorophenyl)kanamycin A (11d)

This compound was synthesized as a light-brown solid following the general procedure. ^{1}H NMR (500 MHz, D_2O) δ 7.29 (d, J = 8.5 Hz, 2H), 6.93 (d, J = 9.0 Hz, 2H), 5.52 (d, J = 3.5 Hz, 1H), 5.07 (d, J = 3.0 Hz, 1H), 4.1–4.2 (m, 3H), 3.8–4.0 (m, 5H), 3.6–3.8 (m, 3H), 3.4–3.6 (m, 3H), 3.3–3.4 (m, 2H), 3.0–3.2 (m, 1H), 2.4–2.5 (m, 1H), 2.14 (s, 1H) 1.8–1.9 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 156.64, 129.50 (2C), 125.96, 116.18 (2C), 100.65, 96.67, 83.99, 78.78, 72.81, 72.09, 71.07, 70.80, 70.67, 68.65, 68.15, 66.35, 65.40, 54.91, 49.97, 47.90, 40.26, 28.03. ESI/APCI calculated for $C_{24}H_{40}N_4O_{11}^+$ $[MH]^+$: 595.2382; measured m/e: 595.2377.

4.6. 6″-O-(4-nitrophenyl)kanamycin A (11e)

This compound was synthesized as a light-yellow solid following the general procedure. ^{1}H NMR (500 MHz, D_2O) δ 8.19 (d, J = 9.0 Hz, 2H), 7.07 (d, J = 9.0 Hz, 2H), 5.52 (d, J = 3.0 Hz, 1H), 5.05 (d, J = 3.5 Hz, 1H), 4.3–4.4 (b, 2H), 4.2–4.3 (m, 1H), 3.6–4.0 (m, 9H), 3.3–3.6 (m, 5H), 3.1–3.2 (m, 1H), 2.3–2.5 (m, 1H), 1.7–1.8 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 163.52, 141.52, 126.21 (2C), 114.83 (2C), 100.59, 97.26, 84.39, 79.78, 73.19, 72.11, 70.92, 70.83, 70.72, 68.60, 68.22, 66.59, 65.47, 54.91, 50.16, 48.16, 40.27, 29.01. ESI/APCI calculated for $C_{24}H_{40}N_5O_{13}^+$ $[MH]^+$: 606.2623; measured m/e: 606.2634.

4.7. 6″-O-(4-(1,1′-biphenyl))kanamycin A (11f)

This compound was synthesized as a white solid following the general procedure. ^{1}H NMR (500 MHz, D_2O) δ 7.4–7.5 (m, 4H), 7.2–7.4 (m, 3H), 6.94 (d, J = 8.5 Hz, 2H), 5.51 (d, J = 4.0 Hz, 1H), 5.06 (d, J = 3.5 Hz, 1H), 4.1–4.3 (m, 3H), 3.8–4.0 (m, 5H), 3.6–3.8 (m, 2H), 3.4–3.6 (m, 4H), 3.2–3.4 (m, 2H), 3.0–3.1 (m, 1H), 2.4–2.5 (m, 1H), 2.14 (s, 1H) 1.8–1.9 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 157.57, 139.74, 133.73, 129.09 (2C), 128.11 (2C), 127.19, 126.43 (2C), 115.19 (2C), 100.56, 96.67, 83.92, 78.33, 73.03, 72.04, 71.17, 70.86, 70.80, 68.63, 68.14, 66.30, 65.59, 54.98, 49.90, 48.02, 40.38, 27.96. ESI/APCI calculated for $C_{30}H_{45}N_4O_{11}^+$ $[MH]^+$: 637.3085; measured m/e: 637.3085.

4.8. 6″-O-(4-(1H-benzo[d]imidazole-2-yl)phenyl)kanamycin A (11g)

4-(1*H*-benzo[*d*]imidazole-2-yl)phenol was synthesized by refluxing 1,2-diaminobenzene with 4-hydroxybenzaldehyde in the presence of sodium metabisulfite [37]. For this, 0.475 g (0.25 mmol, 1 equiv) of sodium metabisulfite was added to a solution of 0.27 g (0.25 mmol, 1 equiv) 1,2-diaminobenzene and 0.30 g (0.25 mmol, 1 equiv) of 4-hydroxybenzaldehyde in 10 mL DMF. The reaction mixture was refluxed at 150 °C for 24 h, and the solvent was removed under reduced pressure. The residue was washed with 2 × 10 mL water followed by 2 × 5 mL DCM. Compound 11g was synthesized as a white solid following the general procedure using 4-(1*H*-benzo[*d*]imidazole-2-yl)phenol as the substituted phenol. ^{1}H NMR (500 MHz, D_2O) δ 7.83 (d, J = 8.0 Hz, 2H), 7.5–7.7 (m, 2H), 7.3–7.4 (m, 2H), 7.09 (d, J = 8.5 Hz, 2H), 5.58 (d, J = 4.0 Hz, 1H), 5.11 (d, J = 3.5 Hz, 1H), 4.2–4.3 (m, 3H), 3.8–4.0 (m, 5H), 3.7–3.8 (m,

2H), 3.5–3.7 (m, 4H), 3.3–3.4 (m, 2H), 3.1–3.2 (m, 1H), 2.4–2.5 (m, 1H), 1.8–1.9 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 161.66, 149.22, 132.19 (2C), 129.28 (2C), 125.59 (2C), 116.16, 115.64 (2C), 113.58 (2C), 100.79, 97.16, 83.85, 78.46, 73.12, 72.01, 71.00, 70.88, 70.72, 68.72, 68.17, 66.23, 65.43, 54.93, 49.95, 48.05, 40.30, 27.72. ESI/APCI calculated for $C_{31}H_{45}N_6O_{11}^+$ $[MH]^+$: 677.3146; measured m/e: 677.3148.

4.9. 6′-N-(6-hydroxy-2-naphthoyl)kanamycin A (12a)

This compound was synthesized as a white solid essentially as reported [35]. ^{1}H NMR (500 MHz, D_2O) δ 8.09 (s, 1H), 7.82 (d, J = 9.0 Hz, 1H), 7.70 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 8.5 Hz, 1H), 7.18 (s, 1H), 7.14 (d, J = 8.5 Hz, 1H), 5.34 (d, J = 3.5 Hz, 1H), 4.86 (d, J = 3.0 Hz, 1H), 3.6–4.0 (m, 8H), 3.4–3.6 (m, 7H), 3.3–3.4 (m, 2H), 2.3–2.4 (m, 1H), 1.7–1.8 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 171.29, 155.39, 136.33, 131.15, 128.19, 127.89, 127.32, 126.92, 124.05, 119.04, 109.19, 99.87, 97.45, 83.55, 79.74, 72.66, 72.47, 72.36, 71.47, 71.14, 70.86, 68.07, 65.49, 59.76, 54.86, 49.57, 48.09, 40.56, 27.99. ESI/APCI calculated for $C_{29}H_{43}N_5O_{13}^+$ $[MH]^+$: 655.2860; measured m/e: 655.2825.

4.10. 6′-N-(6-methoxy-2-naphthoyl)kanamycin A (12b)

This compound was synthesized essentially as described in a previous report [35]. ^{1}H NMR (500 MHz, D_2O) δ 8.04 (s, 1H), 7.76 (d, J = 9.5 Hz, 1H), 7.72 (d, J = 9.0 Hz, 1H), 7.62 (d, J = 8.5 Hz, 1H), 7.20 (d, J = 2.0 Hz, 1H), 7.12 (dd, J = 9.0 Hz, J = 2.5 Hz, 1H), 5.37 (d, J = 4.0 Hz, 1H), 4.89 (d, J = 3.5 Hz, 1H), 3.8–4.0 (m, 5H), 3.4–3.8 (m, 13H), 3.3–3.4 (m, 2H), 2.4–2.5 (m, 1H), 1.7–1.8 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 171.13, 158.50, 136.17, 130.66, 128.34, 127.67, 127.64, 127.32, 124.04, 119.23, 106.14, 99.97, 97.38, 83.46, 79.30, 72.71, 72.45, 72.31, 71.53, 71.11, 70.78, 68.07, 65.46, 59.77, 55.43, 54.86, 49.56, 48.08, 40.49, 27.69. ESI/APCI calculated for $C_{30}H_{45}N_4O_{13}^+$ $[MH]^+$: 669.2983; measured m/e: 669.2985.

Procedure for large scale synthesis of 6′-*N*-dodecylkanamycin A (12i).

To synthesize this compound, 2.76 g (2 equiv, 20 mmol) of anhydrous potassium carbonate was added to 5.82 g (1 equiv, 10 mmol) of kanamycin A sulfate dissolved in 50 mL of water. The mixture was stirred for 15 min and then, 4.46 g (2 equiv, 20 mmol) of succinimidyl laurate dissolved in 50 mL of DMF was added slowly in four portions, with a 15-min interval between additions. After 48 h of stirring at room temperature the solvent was removed by blowing compressed air. The solid residue obtained was washed with 3 × 50 mL of water, and the white powder collected as a residue was extracted with 5% acetic acid in water to obtain the product. After ion-exchange on IR410 (Cl^- form), 6.36 g (8.2 mmol, 82%) of the desired product were obtained as a white powder. The proton NMR spectra of the product matched the previously reported spectra [35].

4.11. Procedure to assess connexin hemichannel inhibition

We used LB2003 cells to assess HC function. LB2003 is a strain of *E. coli* that is defective K^+ uptake [29,36]. Since K^+ is necessary for cell growth and its uptake by the LB2003 cells is limited, these cells cannot grow in low-[K^+] medium [29,36]. The assay is based on the demonstration that expression of human connexin HCs reverts the "no-grow" phenotype of LB2003 cells in low-[K^+] medium by mediating sufficient K^+ uptake to allow growth [29,36]. Details of the protocol can be found in a recent publication [36]. Briefly, cells in a minimal medium containing low-[K^+] and isopropyl-β-D-thiogalactopyranoside (IPTG; to induce connexin expression) were seeded in 96-well plates. The assay has been optimized for Cx26 expression using 4 mM K^+ and 500 μM IPTG in the medium, and for Cx43 using 8 mM K^+ and 10 μM IPTG [30,36]. After addition of the cells, incubation proceeded at 30 °C for 18 h, and then cell growth was estimated from the absorbance measured at 600 nm (OD_{600}) in a plate reader. Values were calculated as percentage of inhibition normalized to the maximal growth measured in LB2003 cells in the absence of inhibitors.

One important point of the growth complementation assay is that it is performed on kanamycin-resistant LB2003 (these cells contain the pREP4 plasmid, which confers resistance to kanamycin and neomycin [36]), and as a result, growth inhibition is not expected to be due to an antibiotic effect of the AKs. To test for growth inhibition in the absence of connexin expression (see Supplementary Information), we grew the cells in high-[K^+] medium (115 mM K^+) as described [36]. Under these conditions, growth of LB2203 cells is not limited by the deficiency of K^+ uptake mechanisms. We have previously reported that in our assay system the expression of Cx43 is higher than that of Cx26 [29]. However, binding to HCs is not expected to affect the dependency of inhibitor occupancy of HCs as a function of concentration because the levels of HC expression are very low, the extracellular volume in the assay is much larger than the cell volume, and the kanamycins are amphiphilic and therefore poorly-permeable through membranes and unlikely to accumulate inside cells. Nevertheless, the main point of the present studies is that we could produce compounds that are better to inhibit Cx43 HCs relative to Cx26 HCs when compared to commercially available aminoglycosides and previously synthesized kanamycin derivatives.

5. Calculation of the LogD

Log D value was calculated in Marvin Sketch (version 18.19) keeping 0.1 M concentration of Na^+, K^+, and Cl^- ions. The values presented are at pH = 7.0.

Declaration of competing interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.112602.

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