Cite this: *Med. Chem. Commun.*,
2018, 9, 909

Received 21st March 2018,
Accepted 15th April 2018

DOI: 10.1039/c8md00155c

rsc.li/medchemcomm

Antifungal amphiphilic kanamycins: new life for an old drug

Yagya Prasad Subedi, ^a Madher N. AlFindee, ^a
Jon Y. Takemoto ^b and Cheng-Wei Tom Chang ^{*a}

Classical aminoglycoside antibiotics are obsolete or hampered by the emergence of drug resistant bacteria. Recent discoveries of antifungal amphiphilic kanamycins offer new strategies for reviving and repurposing these old drugs. A simple structural modification turns the clinically obsolete antibacterial kanamycin into an antifungal agent. Structure–activity relationship studies have led to the production of **K20**, an antifungal kanamycin that can be mass-produced for uses in agriculture as well as in animals. This review delineates the path to the discovery of **K20** and other related antifungal amphiphilic kanamycins, determination of its mode of action, and findings in greenhouse and field trials with **K20** that could lead to crop disease protection strategies.

1. Introduction

Produced by *Streptomyces kanamyceticus*, the kanamycins are one of the most successful broad-spectrum antibacterial agents (Fig. 1).¹ Structurally, the kanamycins are a class of aminoglycosides (AGs) having a 4,6-disubstituted 2-deoxystreptamine (2-DOS) core (ring II). Kanamycins exert their antibacterial activity by binding selectively to the A-site

of bacterial 16S rRNA causing interference of protein synthesis that eventually leads to cell death. The emergence of bacterial resistance to the kanamycins, however, has rendered them clinically obsolete.^{2,3} With advancements in knowledge about resistance mechanisms against AGs^{4–7} and the molecular details of AG binding to ribosomal targets,^{8–11} some progress on the development and discovery of novel AGs has occurred with the aim of overcoming the AG resistance problem.^{12–16} Examples include the introduction of amikacin,¹⁷ gentamicin,¹⁸ sisomicin,¹⁹ arbekacin²⁰ and plazomicin.²¹ But to date, no major transformational breakthroughs with long-term and broad-spectrum effectiveness against bacterial resistance have emerged. In contrast, recent discoveries of antifungal amphiphilic AG derivatives may

^a Department of Chemistry and Biochemistry, Utah State University, 0300 Old Main Hill, Logan, Utah 84322-0300, USA. E-mail: tom.chang@usu.edu

^b Department of Biology, Utah State University, 5305 Old Main Hill, Logan, Utah 84322-5305, USA

Yagya Prasad Subedi

Yagya Prasad Subedi received his M. Sc. in organic chemistry in 2011 from Tribhuvan University, Nepal. Then he moved to Utah State University in 2013, where he is currently working under Prof. Tom Chang in the synthesis of different analogs of the aminoglycosides. In addition to this, Yagya also work on the synthesis and imaging of the fluorescent molecules.

Madher N. AlFindee

Madher N. AlFindee received his M.Sc. in 2000 from Basra University in IRAQ. In 2002, he started to work as a faculty member in the College of Pharmacy, Basra University. He joined Dr. Tom Chang group at Utah State University as a PhD student in 2013. He is a recipient of scholarship from the Higher Committee for Education Development in Iraq (HCED). His work in Dr. Chang's lab is focused in medicinal organic chemistry.

Fig. 1 Structure of the kanamycin class aminoglycosides.

Fig. 2 Structure of natural aminoglycosides reported for antifungal activity.

open the way for alternative therapeutics uses for AGs including the repurposing of classical AGs such as kanamycin.²² This review summarizes recent developments and investigations of antifungal AGs with emphasis on amphiphilic derivatives of the kanamycin class of AGs.

2. The discovery of antifungal kanamycin, FG08

In 1967, Urabe and Nakama²³ reported antifungal properties of kasugamycin, a naturally occurring AG (Fig. 2). It was not until 2005 that Lee, *et al.*²⁴ then reported on the antifungal activities of additional naturally occurring AGs, including neomycin, paromomycin, ribostamycin, and streptomycin (Fig. 2). Both articles reported modest antifungal activity with minimum inhibitory concentrations (MICs) ranging from 0.01 to 15 mg mL⁻¹ against a panel of fungi. In the meantime, C.-W. T. Chang and his group had been working on the chemical synthesis of AGs creating a library of over a hundred synthetic AGs with many based on the 2-DOS core and kanamycin.^{25–28} Inspired by the earlier antifungal AG reports, Chang and Takemoto began to randomly screen the synthetic

AG library for antifungal properties which led to the identification of a structural analog of kanamycin B, FG08.²⁹ Unlike previously reported aminoglycosides, FG08 is inactive against bacteria and instead is antifungal against a broad array of fungi (Table 1). This serendipitous finding has two significant implications. First, it demonstrates that chemical modification can alter the bioactivity profile of a natural product. Second, it provides an example of re-purposing and reviving the use of an old drug. The re-purposing strategy is applicable to other marketed antimicrobial therapeutics that have declined in utility due to the evolution of antimicrobial resistance.

The synthesis of FG08 involved the preparation of a glycosyl acceptor, 1 from neamine, and a glycosyl donor, 2 from D-glucose (Scheme 1).²⁹ Following a glycosylation, global deprotection and ion exchange, FG08 is prepared as a chloride salt with an estimated overall yield of less than 5%. As a broad-spectrum antifungal agent, FG08 showed particularly strong inhibitory activities against plant pathogens such as *Fusarium graminearum*, the etiological agent of wheat and barley head blight (scab) diseases (Table 1).

Jon Y. Takemoto

Professor Jon Y. Takemoto received his B.A. and Ph.D. (1973) degrees in microbiology from the University of California, Los Angeles, and was an NIH Post-doctoral Fellow at the Biological Laboratories, Harvard University, in 1973–1974. He has been on the faculty of the Department of Biology at Utah State University and with the Utah Agricultural Experiment Station since 1975. His research work includes the physiology of photosynthetic microbes, biology and chemistry of bacterial cyclic lipopeptides, and the discovery of novel natural product antimicrobials and anti-inflammatories. His research activities have involved the mentoring of thirty-seven graduate students (M.S. and Ph.D.) and a dozen postdoctoral associates.

Tom Chang

Prof. Tom Chang received his B. S. degree in 1988 from Tunghai University in Taiwan and Ph.D. degree in organic chemistry from Washington University in St. Louis, Missouri in 1997. Although trained as a synthetic chemist, Prof. Chang's research interest encompasses chemistry, biology, and microbiology. His research team has been engaging in multiple projects in anti-bacterial, antifungal, antiviral, and anticancer areas. His research focuses not only on product development but also on basic science. New projects even include alternative energy development and green chemistry. He has been a faculty member at the Department of Chemistry and Biochemistry, Utah State University since 2000.

Table 1 Minimal inhibitory concentrations (MICs) of FG08 and kanamycin B^a

Organism	Strain	FG08	Kanamycin B
Bacteria	<i>Escherichia coli</i> TG1	125–250	1.95
	<i>Staphylococcus aureus</i> ATC 25923	250	<0.98
	<i>Pseudomonas aeruginosa</i> (ATCC 27853)	250	1.95
	<i>Enterococcus faecalis</i> (ATCC 29212)	125–250	<0.98
	<i>Klebsiella pneumoniae</i> (ATCC 138883)	250	1.95
	<i>Klebsiella pneumoniae</i> (ATCC 700603)	250	1.95
	Fungi	<i>Rhodotorula pilimanae</i>	7.8
<i>Candida albicans</i>		31.3	>250
<i>Saccharomyces cerevisiae</i> W303		31.3	>250
<i>Fusarium graminearum</i>		7.8	>250
<i>Fusarium oxysporum</i>		7.8	>250
<i>Ulocladium</i> spp.		7.8	ND ^b
<i>Pythium irregulare</i>		15.6	ND
<i>Pythium ultimum</i>		15.6	ND
<i>Phytophthora parasitica</i>		15.6	ND
<i>Rhizopus stolonifer</i>		31.3	ND
<i>Cladosporium cladosporioides</i>		31.3	ND
<i>Curvularia brachyspora</i>		31.3	ND
<i>Bortrytis cinerea</i>		31.3	ND
<i>Phoma</i> spp.		31.3	ND

^a Microbroth dilution assays were performed at least twice, and each in triplicate. Unit: $\mu\text{g mL}^{-1}$. ^b Not determined.

3. Structure–activity relationship (SAR) studies of antifungal kanamycin

A distinct structural feature of **FG08** is the presence of a hydrophobic octyl group at the O-4" position making it an amphiphilic compound. Subsequently, two questions regarding SAR arise: will other chain length of the hydrophobic group also exert antifungal activity, and will octylation at other sites of kanamycin yield antifungal activity? Two analogs of **FG08** bearing butyl (C4) and dodecyl (C12) were synthesized,²⁹ and the results showed the presence of octyl group offered the optimal antifungal activity. Following this information, a library

Scheme 1 Synthesis of FG08.

of amphiphilic kanamycin with octyl groups at various sites were prepared using mainly a glycodiversification approach (Fig. 3).³⁰ Although the results revealed that O-4" with an octyl group exerts the best antifungal activity, substitution at O-6" also have comparable antifungal activity (**FG06**) (Fig. 4). These SAR studies inspired us to introduce amphiphilic group at O-6" of kanamycin A to synthesize **K20**, cost-effective the next generation amphiphilic kanamycin which will be discussed below in details.

4. Approaches toward the synthesis of antifungal amphiphilic kanamycin and biological studies

The above-described SAR investigation established the structural criteria for converting antibacterial kanamycin into an antifungal kanamycin, *i.e.* the attachment of hydrophobic groups at 4'' and/or 6'' hydroxyl groups. This finding prompted further expansion of ongoing efforts aimed at the synthesis of novel antifungal amphiphilic kanamycin derivatives.

Two common synthetic approaches for the synthesis of new aminoglycoside derivatives are pursued: 1) direct modification of existing aminoglycosides; and 2) glycosylation strategies on selected cores, such as 2-deoxystreptamine and neamine (rings I and II).³¹ The former provides desired products in fewer synthetic steps while the latter offers more flexibility in structural modifications, especially at various sites of AGs.

The synthesis of FG compounds is achieved through the glycosylation strategy that attaches synthetic ring III to the 2-DOS, an approach which requires complex synthetic steps. Following the identification of pivotal SAR required for the synthesis of amphiphilic antifungal kanamycin derivatives, a more economic approach is to employ the commercially available kanamycin as the starting material *via* a direct modification strategy. Considering the cost of starting material, kanamycin A is the most cost-effective, followed by tobramycin and then kanamycin B.

To conduct regioselective incorporation of hydrophobic functionalities on hydroxyl groups of kanamycin, protection or

Fig. 3 Structure of amphiphilic kanamycin for SAR investigation.

Fig. 4 Summary of MICs ($\mu\text{g mL}^{-1}$) of amphiphilic kanamycin (analogs of FG08) against *F. graminearum*.

masking amino groups is essential. Two types of amino group protection/masking methods have been commonly employed. The first one is to use carbamate types of protecting groups. The second strategy is to mask amino groups as azido groups. Using carbamate type of protecting groups is more cost effective. However, the carbamate protecting groups may undergo intramolecular cyclization with vicinal hydroxyl group and form cyclic carbamate, which could further complicate the synthesis. Masking the amino groups as azido groups has the advantage of better solubility in organic media. Nevertheless, it is not as cost effective as the approach using carbamate-based protecting group, and it requires the use of azides, which raises the safety and environmental concern. Regardless, both amino group protection/masking methods often rely on the regioselective differentiation of 4'' and/or 6'' hydroxyl groups of the kanamycin class AGs.

Without using special reagents or protecting groups, Chang's group discovered that alkylation of tetra-azidokanamycin A can take place regioselectively at of 4'' and/or 6'' hydroxyl groups (Scheme 2).³² Using traditional Williamson reaction and controlling the equivalent of alkyl bromides (from C4 to C16), three types of alkylated tetra-azidokanamycin A can be obtained in one-pot. Following the separation and Staudinger reduction, these libraries of amphiphilic kanamycin derivatives (K46, K4 and K6) were tested against a panel of bacteria and fungi (Table 2). In this study, octyl (C8) along with hexyl (C6) appears to be the optimal chain length for antifungal activity.

Scheme 2

Table 2 Antimicrobial activity of amphiphilic kanamycin A derivatives

Entry	Compound	MIC ($\mu\text{g mL}^{-1}$)			
		A ^a	B ^a	C ^a	D ^a
1	K4604 (R = butyl)	>500	125	≥250	≥250
2	K404 (R = butyl)	>500	≥500	≥250	125
3	K4606 (R = hexyl)	500	15.6	≥250	64
4	K406 (R = hexyl)	>500	31.3	≥250	64
5	K4607 (R = heptyl)	500	15.6	125	125
6	K407 (R = heptyl)	>500	125	≥250	125
7	K4608 (R = octyl)	>500	31.3	≥250	≥250
8	K608 (R = octyl)	>500	125	≥250	≥250
9	K408 (R = octyl)	>500	15.6	≥250	125
10	K4609 (R = nonyl)	500	125	16	8
11	K609 (R = nonyl)	>500	125	32	≥250
12	K409 (R = nonyl)	>500	62.5	≥250	≥250

^a A: *Aspergillus flavus*; B: *F. graminearum*; C: *E. coli* (ATCC 25922); D: *S. aureus* (ATCC 25923).

Protecting the amino group with a carbamate type of protecting group, Garneau-Tsodikova reported the synthesis and bioactivity studies of antifungal amphiphilic tobramycin³³ and kanamycin B³⁴ with hydrophobic groups selectively attached to the 6''-position (Scheme 3). Two libraries of amphiphilic AGs were synthesized first with the protection of amino groups with *t*-butoxycarbonyl (Boc) group using Boc_2O . The selectivity toward 6''-OH is achieved by using 2,4,6-triisopropylbenzenesulfonyl chloride (TIPBSCl), which allows a nucleophilic substitution to enable the attachment of hydrophobic alkanethiols. Removal of Boc using trifluoroacetic (TFA) yielded the amphiphilic AGs ready for biological testing.

Interestingly, the optimal antifungal activities occurred with tetradecyl (C14) and hexadecyl (C16) groups (Tables 3 and 4). In contrast to the FG members, the reported amphiphilic tobramycin and kanamycin B derivatives with an octyl group did not display strong antifungal activity. In addition, several amphiphilic AGs also manifested moderate to strong antibacterial activity, especially against Gram-positive (G+)

Table 3 MICs of amphiphilic kanamycin B^a

	12a (C8)	12b (C10)	12c (C12)	12d (C14)	12e (C16)	14c (C12) ^c	14d (C14) ^c
A ^b	>125	125	31.2	7.8	3.9	31.2	>125
B ^b	>125	125	31.2	7.8	3.9	31.2	>125
C ^b	>125	125	31.2	7.8	3.9	31.2	>125
D ^b	>125	125	31.2	15.6	3.9	62.5	>125
E ^b	>125	62.5	7.8	7.8	3.9	62.5	>125
F ^b	>125	125	31.2	7.8	3.9	62.5	>125
G ^b	>125	125	31.2	7.8	3.9	62.5	>125
H ^b	>125	≤1.95	≤1.95	≤1.95	1.95	3.9	>125

^a Unit: $\mu\text{g mL}^{-1}$. ^b A, *Candida albicans* ATCC 10231; B, *C. albicans* ATCC 64124; C, *C. albicans* ATCC MYA-2876 (S); D, *C. albicans* ATCC 90819 (R); E, *C. albicans* ATCC MYA-2310 (S); F, *C. albicans* ATCC MYA-1237 (R); G, *C. albicans* ATCC MYA-1003 (R); H, *Aspergillus nidulans* ATCC 38163. The S or R designation following a strain name indicates the strain susceptible (S) or resistant (R) to itraconazole (ITC) and fluconazole (FLC). ^c The alkyl groups are attached to AG via SO₂ group.

bacteria. The difference between the reported amphiphilic tobramycin and kanamycin B compounds and FG compounds may be attributed to the presence of 6''-S in place of 6''-O although the details are not yet understood. Another intriguing trend is loss of the fungal-specific property as the hydrophobic chain length is extended from C8 to C14.

Recently, the group of Fridman reported that an increased degree of unsaturation in the lipid of amphiphilic tobramycin derivatives can lead to enhanced selective fungal cell disruption (Scheme 4).³⁵ The synthesis is similar to the method reported by Garneau-Tsodikova. Azide was employed as the nucleophile, which can be reduced to an amine using Staudinger reduction. Unsaturated fatty acids (C18) bearing one, two and three double bonds in both *cis* and *trans* configurations as well as the saturated fatty acid were incorporated via amide linkage. All of these tobramycin derivatives show good antifungal activities against a collection of fungi including the azole-resistant *C. albicans* with MICs ranging from 2–8 $\mu\text{g mL}^{-1}$. Subsequent investigations, including hemolysis tests, viability evaluation of immortalized and primary mammalian cells, and cell membrane permeabilization test, confirm that increase selectivity (activity) toward fungi as the degree of unsaturation (particularly the *cis* double bond) increased.

5. Development of cost effective large-scale synthesis of amphiphilic kanamycin

An essential requirement for practical use of antifungal AGs in human, animals or agriculture is the development of cost effective large-scale synthesis. The industrial scale production of kanamycin, which comes from a fermentation process, yields kanamycin sulfate as a mixture of kanamycin A ($\geq 95\%$) and kanamycin B ($\leq 5\%$), and provides the most cost-effective source of the kanamycin class of AGs. Hence, Chang and Takemoto devoted most of their efforts towards using kanamycin sulfate as the starting material and obtained SAR results that facilitated the development of a cost effective large-scale synthesis of amphiphilic kanamycin.³⁶ Although attaching hydrophobic groups at the O-4'' position provides better antifungal activity, the synthesis often requires multiple chemical steps in the differentiation of 4''-OH from other hydroxyl groups on kanamycin. To simplify the synthesis, and thus reduce the cost of production, the only primary hydroxyl group, 6''-OH, represents the most accessible site for introducing hydrophobic groups without multi-step protection and deprotection to differentiate the hydroxyl groups. A more cost-effective reagent, tosyl chloride (TsCl) is selected

Table 4 MICs of amphiphilic tobramycin^a

	13a (C4)	13b (C6)	13c (C8)	13d (C10)	13e (C12)	13f (C14)
A ^b	>125	>125	>125	31.2	31.2	7.8
B ^b	>125	>125	>125	62.5	31.2	7.8
C ^b	>125	>125	>125	62.5	15.6	7.8
D ^b	>125	>125	>125	125	31.2	7.8
E ^b	>125	>125	>125	125	31.2	7.8
F ^b	>125	>125	>125	125	15.6	7.8
G ^b	>125	>125	>125	125	31.2	7.8
H ^b	31.25	7.8	1.95	15.6	1.95	1.95
I ^b	>125	125	125	62.5	7.8	7.8
J ^b	62.5	31.2	31.2	3.9	3.9	3.9

^a Unit: $\mu\text{g mL}^{-1}$. ^b A, *Candida albicans* ATCC 10231; B, *C. albicans* ATCC 64124; C, *C. albicans* ATCC MYA-2876 (S); D, *C. albicans* ATCC 90819 (R); E, *C. albicans* ATCC MYA-2310 (S); F, *C. albicans* ATCC MYA-1237 (R); G, *C. albicans* ATCC MYA-1003 (R); H, *C. neoformans* MYA-895; I, *Aspergillus nidulans* ATCC 38163; J, *Fusarium graminearum* 053. The S or R designation following a strain name indicates the strain susceptible (S) or resistant (R) to itraconazole (ITC) and fluconazole (FLC).

Scheme 4

instead of a more selective but expensive (TIPBSCl). Following this concept, a library of amphiphilic kanamycin A was synthesized through a four-step process from kanamycin sulfate (Scheme 5). Further shortening of the synthetic steps using reagents such as octanoyl chloride, decanoyl chloride and octanesulfonyl chloride, resulted in a three-step synthesis of the designed amphiphilic kanamycin (Scheme 6).

The purification process is also important. Since AG derivatives are known to be difficult to purify with recrystallization techniques, yield improvements at every chemical step and avoiding flash column chromatography are crucial for scale-up synthesis of amphiphilic kanamycin. After extensive attempts of perfecting the process of synthesis with scale ranging from 10 to 300 g of kanamycin sulfate to begin with, the protocol of **K20** synthesis stands out. **K20** also has all the

Scheme 5

Scheme 6

broad-spectrum fungi-specific activity as **FG08** does (Table 5). More importantly, **K20** can be mass-produced even in Kg scale in academic laboratory. Therefore, **K20** represents the most prominent successor from **FG08**.

6. Mode of action (MOA) and cytotoxicity of amphiphilic kanamycins

The most likely effects of amphiphilic compounds on cellular targets is disturbance of bio-membrane function. Since the amphiphilic AGs show selective inhibitory activities toward fungi or bacteria, it is also likely that their bio-membrane effects involve molecular interactions with higher degrees of complexity and specificity than chaotropic amphiphiles such as small detergents and antiseptic quaternary ammonium compounds. For membrane permeability studies with **FG08**, Chang and Takemoto employed fluorogenic Sytox dye uptake³⁷ and haemolysis of sheep erythrocytes.²⁹ The results showed that **FG08** allowed rapid dye permeation of *C. albicans* cells and *F. graminearum* hyphae (within 10 min) and caused <20% lysis of erythrocytes at levels that were 10 times higher than the antifungal MICs. Therefore, at antifungal MICs, **FG08** appears to bind specific target(s) of the fungal plasma membrane rather than as a non-specific bio-membrane disrupting agent. The MOA of **FG08** is clearly different from that of the traditional AGs. Parallel fungal cell permeability studies were conducted with **K20** and similar results were obtained.³⁸ Additionally, fluorescent calcein trapped within small unilamellar vesicles (SUVs) composed of lipids that mimic those of fungal plasma membranes was rapidly (15 min) and dose-dependently released from vesicles with exposure to **K20**. Finally, **K20** caused cellular uptake by >95% of *C. neoformans* and *F. graminearum* cells of membrane permeability indicator fluorescein isothiocyanate. The combined studies point to direct and rapid plasma membrane pore formation as the MOA of **K20** as with **FG08**, the predecessor of **K20**. The group of Garneau-Tsodikova later confirmed these findings using fluorogenic propidium iodide (PI) dye^{33,34} in cell permeability studies with amphiphilic kanamycin.

A main concern of employing bioactive compounds that target cell membranes to form pores for therapeutic applications is cytotoxicity, *i.e.* whether the compounds will target only the organisms of interest or exert non-selective toxicity including toward mammalian cells. Non-selective amphiphilic compounds have limited uses as in the case of

Table 5 Minimal inhibitory concentrations of **K20** and kanamycin A against bacteria and fungi^a

Organism	Strain	K20	Kanamycin A	ITC ^c	FLC ^c
Bacteria	<i>E. coli</i> TG1	125–250	1.95	—	—
	<i>S. aureus</i> ATCC 25923	250	<0.98	—	—
	<i>Micrococcus luteus</i> ATCC 10240	62.5	1.95	—	—
Fungi or yeasts	<i>Cryptococcus neoformans</i> H99	3.9–7.8	>125	1.56	1.56
	<i>C. neoformans</i> 94-2586	3.9–7.8	>125	0.06	1.56
	<i>C. neoformans</i> 90-26	3.9–7.8	>250	0.37	>0.195
	<i>C. pseudotropicalis</i> YOGI	15.6	>250	0.125–0.8	ND ^d
	<i>C. lusitaniae</i> 95-767	>7.8	>250	0.2	1.56
	<i>C. rugosa</i> 95-967	15.6	>250	0.12	>0.78
	<i>C. tropicalis</i> 95-41	15.6	>250	>25	>25
	<i>C. albicans</i> 10231	15.6	>250	0.75	25
	<i>C. albicans</i> 64124 (R) ^b	31.3	>500	>64	>200
	<i>C. albicans</i> MYA 2876 (S) ^b	15.6	>250	>2	1.56
	<i>C. albicans</i> B-311	>7.8	>250	16–32	>25
	<i>C. albicans</i> 94-2181	>7.8	>250	>8–16	>12.5
	<i>C. parapsilosis</i> (R)	15.6–31.3	>250	0.5	>16
	<i>C. parapsilosis</i> (S)	15.6	>250	0.015	0.12
	<i>F. graminearum</i> B-4-5A	7.8	>125	ND	ND
	<i>F. oxysporum</i>	31.3	>250	ND	ND
	<i>A. flavus</i>	300	>250	0.125	ND
	<i>A. niger</i>	>150	>250	ND	ND
	<i>B. alcala</i>	15.6	ND	ND	ND

^a Unit: $\mu\text{g mL}^{-1}$. ^b (R) Resistant, (S) sensitive. ^c FLC: Fluconazole, ITC: itraconazole. ^d ND: not determined.

quaternary ammonium salts, which can only be used topically as antiseptic agents. All reported antifungal amphiphilic kanamycin derivatives have been screened for hemolysis and cell-based cytotoxicity^{29,32,34} and all show low or no toxicities toward mammalian cells at antifungal MICs. This is a clear indication that the core molecule, kanamycin, also plays a key role in the selectivity toward fungal cells. In addition to the *in vitro* cytotoxicity studies, **K20** has also been examined for efficacy and toxicity *in vivo* using mouse models.³⁸ All results show that **K20** is not toxic and well tolerated.

To address the molecular basis of amphiphilic kanamycin selectivity for pore formation in the fungal membrane *vs.* mammalian and bacterial membranes, the growth susceptibilities of wild-type *vs.* mutant yeasts to **K20** were investigated.³⁸ Yeast sphingolipids, located mainly in plasma membranes, are structurally different from mammalian sphingolipids and bacterial membrane lipids.³⁹ Yeast sphingolipids have C4-hydroxylated sphinganine backbones and most have inositolphosphate-containing head groups (Fig. 5, highlighted in red): features not found in mammalian or bacterial cell lipids (Fig. 5). **K20** growth sensitivity screens were conducted against isogenic yeast mutant strains each

defective in single sphingolipid biosynthetic genes.^{40,41} It was observed that mutant strains with sphingolipids that lack the C4-hydroxyl group on the sphinganine backbone or that contain truncated fatty acid (C22–C26) tails (structures enclosed in the box) were 2 and 4-fold less sensitive to **K20** compared to isogenic wild-type strains. These results suggest that **K20** pore formation depends on properties of the fungal plasma membrane that are promoted by these unique structural features of fungal sphingolipids. Such properties unique to fungi but lacking in mammalian and bacterial cells may, at least in part, explain the selective action of **K20** toward fungi. However, SUVs that lacked sphingolipids were still permeabilized by **K20** showing that with artificial membranes C4 hydroxylated sphingolipids with very long fatty acid tails are not solely required to provide the molecular structural features needed for interaction with **K20**.

7. Synergism of amphiphilic kanamycin derivatives and azoles

Azole-based antifungal agents, including triazoles, diazoles, and thiazoles, are the most commonly used treatments for human fungal infections.^{42–44} As compared to amphotericin B and polyene classes of antifungal agents, azole-based antifungal agents have more favorable solubility characteristics, moderate toxicity, and proven effectiveness in treating human fungal infections. Most of the azole-based antifungal drugs rely on a specific mode of action: inhibition of sterol C14-demethylases, involved in the C14-demethylation step of sterol biosynthesis.^{45,46} Therefore, the MOAs of amphiphilic kanamycin derivatives involving fungal membrane pore formation differ from that of the azole-based antifungal agents.

Fig. 5 Structure of the major yeast plasma membrane sphingolipid mannosyl-diinositolphosphoryl phytoceramide.

Table 6 Examples of *in vitro* susceptibility of *C. albicans* strains to **13e** and azoles alone and in combination

Drug ^a	Strains	MICs of drugs (μg mL ⁻¹)				FICIs ^c (interpretation) ^d
		Drug alone		In combination		
		Azole	13e	Azole	13e	
FLC	A ^b	25	32	1.56	8	0.31 (SYN)
	B ^b	>25	32	0.78	16	0.53 (IND)
	C ^b	>25	16	12.5	8	1 (IND)
	D ^b	>25	32	1.56	2	0.12 (SYN)
	E ^b	>25	16	0.39	1	0.7 (SYN)

^a FLC: Fluconazole. ^b A: *C. albicans* 10231, B: *C. albicans* 64124, C: *C. albicans* 2876, D: *C. albicans* 90819, E: *C. albicans* MYA-2310. ^c FICI = $[A]/(\text{MIC}_A) + [B]/(\text{MIC}_B)$ where [A]: concentration of drug A; MIC_A : the MIC of the testing organism to drug A alone; [B]: concentration of drug B; and MIC_B : the MIC of the testing organism to drug B alone. ^d SYN: synergism; IND: indifference.

Both the Chang and Takemoto⁴⁸ and Garneau-Tsodikova^{33,34,47} groups have reported growth inhibitory synergism between these two classes of compounds by calculating the fractional inhibitory concentration index (FICI) values. In most cases, strong synergies between the tested compounds and various azole-based antifungal agents, such as fluconazole, posaconazole, itraconazole, and voriconazole, against *Candida albicans*, non-*albicans Candida* spp., and *Cryptococcus* spp. were observed (Tables 6 and 7). Such a strong synergism offers new approaches for combating fungal pathogens, especially for drug-resistant strains.

Inspired by the fungal inhibitory synergy between K20 and azole-based antifungal agents and capability for scalable K20 production, studies are underway to examine the efficacies of K20-triazole and strobilurin agrifungicide combinations against crop diseases. Commercial fungicides Caramba (8.6% [wt vol⁻¹] metconazole) (BASF Ag Products), Headline (23.6% [wt vol⁻¹] pyraclostrobin) (BASF Ag Products), Proline 480 SC (41% [wt vol⁻¹] prothioconazole, 6% [vol vol⁻¹] glycerol) (Bayer CropScience), or Propiconazole (41.8% [wt vol⁻¹]) (Tide International USA), in combination with K20 were examined for *in vitro* inhibition of the Fusarium head blight (FHB) pathogen *F. graminearum* (Table 8).⁴⁹ *F. graminearum* was selected for study because of the nearly exclusive reliance on C14-methylase inhibitor triazoles for controlling this pathogen and FHB.⁴⁹ These agrifungicides all showed moderate to strong synergism with K20. Headline and Proline 480 SC were further examined in an FHB greenhouse study and field trials, which will be discussed in more detail below. It is worth mentioning that pyraclostrobin, the

active compound in Headline, belongs to the strobilurin class of fungicides. Strobilurins bind to complex III of the respiratory chain in mitochondria to inhibit electron transport – a different MOA than that of azole-based fungicides. The synergism between K20 and Headline suggests that K20 could possibly exert synergism with a broad range of fungicides having different cellular targets.

8. Greenhouse and field trials of amphiphilic kanamycin derivatives and azoles as an agrofungicide

Azole-based fungicides are commonly used in agriculture for controlling plant fungal disease and predictably with overuse resistance against these fungicides develops.^{50,51} Meanwhile, it is estimated that fungal pathogens destroy annually at least 125 million tons of the top five food crops (rice, wheat, maize, potatoes and soybeans) that can, otherwise, feed more than 600 million people.⁵² As the global human population increases, food shortages or increases in food prices will create serious disturbances to social stability worldwide. Thus, the discovery of new fungicidal chemicals with unique modes of action, like amphiphilic kanamycin derivatives, are valuable for food security and to counter widespread fungal resistance to current fungicides.

Table 8 *In vitro* synergistic activities of K20 and commercial fungicides against growth of *F. graminearum* strain B4-5A

Drug ^a	Strains	MICs of drugs (μg mL ⁻¹)				FICIs (interpretation) ^b
		Drug alone		In combination		
		Azole	K20	Azole	K20	
FLC	A ^b	>200	16	25	4	0.37 (SYN)
	B ^b	>25	16	6.25	4	0.5 (SYN)
	C ^b	>2	16	0.25	4	0.37 (SYN)
	D ^b	>12.5	16	0.195	8	0.5 (SYN)
	E ^b	>25	16	12.5	4	0.7 (IND)

^a Data from ref. 49. ^b Drug interactions were classified as synergistic if the FICI was ≤ 0.5 , indifferent if >0.5 –4, and antagonistic if >4 .

Table 7 Examples of *in vitro* susceptibility of *C. albicans* strains to K20 and azoles alone and in combination

Fungicide	MIC ($\mu\text{g mL}^{-1}$)		FICI
	K20 alone	Fungicide alone	
Caramba	12.5	0.098	0.499
Headline	12.5	6.25	0.281
Proline 480 SC	12.5	0.098–0.39	0.125–0.313
Propiconazole	12.5	3.13–6.25	0.375–0.531

Table 9 Greenhouse study of FHB suppression by K20, Headline and their combinations^a

% disease (spikelet curling or spikelet infected)		Headline		
		0	Half-label rate	Full-label rate
K20	0	51.4	9.8	2.5
(mg L ⁻¹)	360	34.7	4.7	1.5

^a Data from ref. 49.

Following the success of K20 scale-up production (hundred grams scale in the laboratory) and demonstration of *in vitro* inhibition of *F. graminearum* with K20 alone or in combination with agrifungicides (Table 8), Chang and Takemoto and collaborators expanded the studies of K20 to the suppression of *F. graminearum* and FHB in greenhouse and field studies.⁴⁹

Beginning with greenhouse experiments, applying K20 to wheat heads using field spray equipment at a K20 MIC of 360 mg L⁻¹ suppressed FHB an average of 60% (range: 48–71%) compared to the control. FHB disease suppression by combinations of K20 and Headline were also observed. Applying K20 combined with half-label rate Headline provided significant FHB suppression, better than K20 alone (Table 9). The effectiveness of disease suppression resulting from treatment with K20 plus half- rate Headline combination approaches the suppressive effects with Headline alone at full rate. Since Headline has been noted for its toxicity to humans and environmental effects,⁵³ the K20 combination results suggest the possibility of reducing the usage of Headline while still achieving the desired level of crop protection.

An FHB field trial was conducted in 2017 with K20 alone and in combination with Prosaro 421 SC (19% [wt vol⁻¹] prothioconazole, 19% [wt vol⁻¹] tebuconazole) (Bayer CropScience) and Caramba at half-label rates (Table 10).⁴⁹ FHB index was reduced by 80% (Prosaro 421 SC at full label rate) to 90% (K20 at 360 mg mL⁻¹ + Prosaro 421 SC at half label rate) (Table 10). FHB index did not significantly differ (*P* = 0.05)

Table 11 Suppression of DON by fungicides K20, Headline, and Proline 480 SC applied alone and in combinations^a

Treatment ^b	DON (ppm)
Non-treated control	1.2
K20, 360 mg L ⁻¹	1.4
Headline, ½ label rate	1.3
Headline, full label rate	1.2
Proline 480 SC, ½ label rate	1.1
Proline 480 SC, full label rate	0.7
K20, 360 mg L ⁻¹ plus Headline ½ label rate	1.6
K20, 360 mg L ⁻¹ plus Proline 480 SC, ½ label rate	0.3

^a Data from ref. 49. ^b Half and full label rates for Headline: 4.0 and 8.0 ml L⁻¹ = 0.219 and 0.439 L ha⁻¹, respectively; half and full label rates for Proline 480 SC: 2.8 and 5.5 ml L⁻¹ = 0.183 and 0.366 L ha⁻¹, respectively.

among K20 and K20 + Prosaro 421 SC or Caramba treatments but was significantly lower in these treatments than in the check treatment. The results show that spraying K20 alone or in combination with half-rate of Prosaro 421 SC or Caramba reduced FHB incidence, disease index and severity comparable to applying Prosaro 421 SC or Caramba alone at half- or full rate.

In addition to FHB disease, infection of wheat by *F. graminearum* results in contamination of wheat heads and kernels with deoxynivalenol (DON), a secondary metabolite of the fungus and highly toxic to animals and humans. DON contamination levels that exceed 1 to 10 ppm render wheat and barley grain unusable and commercially inviable. In 2016, a wheat FHB field trial was conducted with K20 treatment alone and in combination with Headline and Proline 480 SC. Wheat kernels were harvested and DON concentration measured to determine the effects of K20 and the combinations on levels of this fungal toxin (Table 11).⁴⁹ A combination of K20 and half-label rate Proline 480 SC provided significantly lower DON among all the tests including with Proline 480 SC alone at full-label rate. The synergism between K20 and agrofungicides in reducing FHB disease as well as DON levels suggests the possible use of both chemicals at lower

Table 10 Suppression of FHB in wheat by fungicides K20, Prosaro 421 SC, and Caramba applied alone and in combinations in a 2017 field trial^a

Treatment ^b	FHB incidence (%) ^c	FHB severity (%) ^d	FHB index (%) ^e
Non-treated control	70	75	53
K20, 180 mg L ⁻¹	15	60	9
K20, 360 mg L ⁻¹	14	62	8
Prosaro 421 SC, ½ label rate	12	47	6
Prosaro 421 SC, full label rate	16	66	11
Caramba, ½ label rate	13	69	9
Caramba, full label rate	12	58	7
K20, 180 mg L ⁻¹ plus Prosaro 421 SC, ½ label rate	11	55	6
K20 – 180 mg L ⁻¹ plus Caramba, ½ label rate	15	50	7
K20, 360 mg L ⁻¹ plus Prosaro 421 SC ½ label rate	10	54	5
K20, 360 mg L ⁻¹ plus Caramba, ½ label rate	13	61	8

^a Data from ref. 49. ^b Half and full label rates for Prosaro 421 SC: 0.438 and 0.475 L ha⁻¹; half and full label rates for Caramba: 0.512 and 1.02 L ha⁻¹. ^c Percentage of symptomatic heads in a plot. ^d Percent of symptomatic spikelets on a head, uninfected heads not included. ^e [FHB incidence × FHB severity] × 100.

concentrations and thus reduce costs, toxicities and environmental impacts in crop protection strategies.

Conclusions

Due to the emergence of AG resistant bacteria and toxicity problems in medicine and agriculture classical AGs are becoming “last resort” antibacterial treatments for serious infections or limited to topical applications. Semi-synthetic approaches have offered new AGs but most still fall within the traditional scope of antibacterial applications. Antifungal amphiphilic kanamycin derivatives bring possibilities for new applications and markets. The antifungal synergism of amphiphilic kanamycin derivatives with azoles further augments the possible options for uses in medicine and agriculture. Among these new antifungal amphiphilic derivatives, K20 is one of the most studied in terms of *in vitro* cell-based testing, MOA, scale-up synthesis, *in vivo* animal testing, and crop protection tests. K20 therefore represents a new AG poised for development of applications in medicine and agriculture. The development of antifungal amphiphilic kanamycin represents a model for the strategy of reviving old drugs that otherwise have increasingly limited use due to toxicity or resistance issues.

Conflicts of interest

The authors declare no competing interest.

Notes and references

- U. Hamao and U. Masahiro, U.S. Pat. US2931798 A, 1960.
- J. M. Blair, M. A. Webber, A. J. Baylay, D. O. Ogbolu and L. J. Piddock, *Nat. Rev. Microbiol.*, 2015, 13, 42–51.
- M. S. Ramirez, N. Nikolaidis and M. E. Tolmasky, *Front. Microbiol.*, 2013, 4, 121.
- M.-P. Mingeot-Leclercq, Y. Glupczynski and P. M. Tulkens, *Antimicrob. Agents Chemother.*, 1999, 43, 727–737.
- L. P. Kotra, J. Haddad and S. Mobashery, *Antimicrob. Agents Chemother.*, 2000, 44, 3249–3256.
- G. D. Wright, *Curr. Opin. Microbiol.*, 1999, 2, 499–503.
- S. Garneau-Tsodikova and K. J. Labby, *MedChemComm*, 2016, 7, 11–27.
- J. D. Puglisi, *Science*, 1996, 274, 1367–1371.
- D. Fourmy, M. I. Recht and J. D. Puglisi, *J. Mol. Biol.*, 1998, 277, 347–362.
- Q. Vicens and E. Westhof, *Structure*, 2001, 9, 647–658.
- D. H. Fong and A. M. Berghuis, *EMBO J.*, 2002, 21, 2323–2331.
- H. Umezawa and S. Kondo, in *Aminoglycoside Antibiotics*, Springer, 1982, pp. 267–292.
- P. G. Wang and C. R. Bertozzi, *Glycochemistry: principles, synthesis, and applications*, Marcel Dekker, Inc., 2001.
- D. P. Arya, *Aminoglycoside antibiotics: from chemical biology to drug discovery*, John Wiley & Sons, 2007, pp. 141–180.
- K. D. Green, W. Chen, J. L. Houghton, M. Fridman and S. Garneau-Tsodikova, *ChemBioChem*, 2010, 11, 119–126.
- M.-P. Mingeot-Leclercq and J.-L. Décout, *MedChemComm*, 2016, 7, 586–611.
- H. Kawaguchi, T. Naito, S. Nakagawa and K.-I. Fujisawa, *J. Antibiot.*, 1972, 25, 695–708.
- M. J. Weinstein, G. M. Luedemann, E. M. Oden, G. H. Wagman, J. P. Rosselet, J. A. Marquez, C. T. Coniglio, W. Charney, H. L. Herzog and J. Black, *J. Med. Chem.*, 1963, 6, 463–464.
- M. J. Weinstein, J. A. Marquez, R. T. Testa, G. H. Wagman, E. M. Oden and J. A. Waitz, *J. Antibiot.*, 1970, 23, 551–554.
- Y. Kobayashi, H. Uchida and Y. Kawakami, *Int. J. Antimicrob. Agents*, 1995, 5, 227–230.
- J. B. Aggen, E. S. Armstrong, A. A. Goldblum, P. Dozzo, M. S. Linsell, M. J. Gliedt, D. J. Hildebrandt, L. A. Feeney, A. Kubo and R. D. Matias, *Antimicrob. Agents Chemother.*, 2010, 54, 4636–4642.
- C.-W. Chang and J. Takemoto, *MedChemComm*, 2014, 5, 1048–1057.
- H. Urabe and T. Nakama, *J. Antibiot., Ser. B*, 1967, 20, 424–426.
- H. Lee, Y. Kim, J. Kim, G. Choi, S. H. Park, C. J. Kim and H. Jung, *J. Appl. Microbiol.*, 2005, 99, 836–843.
- C.-W. T. Chang, Y. Hui, B. Elchert, J. Wang, J. Li and R. Rai, *Org. Lett.*, 2002, 4, 4603–4606.
- B. Elchert, J. Li, J. Wang, Y. Hui, R. Rai, R. Ptak, P. Ward, J. Y. Takemoto, M. Bensaci and C.-W. T. Chang, *J. Org. Chem.*, 2004, 69, 1513–1523.
- R. Rai, H.-N. Chen, P. G. Czyryca, J. Li and C.-W. T. Chang, *Org. Lett.*, 2006, 8, 887–889.
- J. Li, F.-I. Chiang, H.-N. Chen and C.-W. T. Chang, *J. Org. Chem.*, 2007, 72, 4055–4066.
- C.-W. T. Chang, M. Y. Fosso, Y. Kawasaki, S. K. Shrestha, M. F. Bensaci, J. Wang, C. K. Evans and J. Y. Takemoto, *J. Antibiot.*, 2010, 63, 667–672.
- M. Fosso, M. N. AlFindee, Q. Zhang, V. d. P. N. Nziko, Y. Kawasaki, S. K. Shrestha, J. Bearss, R. Gregory, J. Y. Takemoto and C.-W. T. Chang, *J. Org. Chem.*, 2015, 80, 4398–4411.
- J. Wang and C. Chang, *Aminoglycoside Antibiotics*, ed. Arya, D. P., John Wiley & Sons, Inc., 2007, pp. 141–180.
- Q. Zhang, M. N. Alfindee, J. P. Shrestha, V. d. P. N. Nziko, Y. Kawasaki, X. Peng, J. Y. Takemoto and C.-W. T. Chang, *J. Org. Chem.*, 2016, 81, 10651–10663.
- S. K. Shrestha, M. Y. Fosso, K. D. Green and S. Garneau-Tsodikova, *Antimicrob. Agents Chemother.*, 2015, 59, 4861–4869.
- M. Y. Fosso, S. K. Shrestha, K. D. Green and S. Garneau-Tsodikova, *J. Med. Chem.*, 2015, 58, 9124–9132.
- K. B. Steinbuch, R. I. Benhamou, L. Levin, R. Stein and M. Fridman, *ACS Infect. Dis.*, 2018, DOI: 10.1021/acsinfecdis.7b00272.
- C.-W. T. Chang and J. Y. Takemoto, U.S. Pat. US 13/316,720, US8865665 B2, 2014.
- S. Shrestha, M. Grilley, M. Y. Fosso, C.-W. T. Chang and J. Y. Takemoto, *PLoS One*, 2013, 8, e73843.
- S. K. Shrestha, C.-W. T. Chang, N. Meissner, J. Oblad, J. P. Shrestha, K. N. Sorensen, M. M. Grilley and J. Y. Takemoto, *Front. Microbiol.*, 2014, 5, 671.

- 39 R. L. Lester and R. C. Dickson, *Adv. Lipid Res.*, 1993, **26**, 253–274.
- 40 M. M. Grilley, S. D. Stock, R. C. Dickson, R. L. Lester and J. Y. Takemoto, *J. Biol. Chem.*, 1998, **273**, 11062–11068.
- 41 S. D. Stock, H. Hama, J. A. Radding, D. A. Young and J. Y. Takemoto, *Antimicrob. Agents Chemother.*, 2000, **44**, 1174–1180.
- 42 S. C. Chen and T. C. Sorrell, *Med. J. Aust.*, 2007, **187**, 404.
- 43 M. K. Kathiravan, A. B. Salake, A. S. Chothe, P. B. Dudhe, R. P. Watode, M. S. Mukta and S. Gadhwe, *Bioorg. Med. Chem.*, 2012, **20**, 5678–5698.
- 44 S. Knight, V. Anthony, A. Brady, A. Greenland, S. P. Heaney, D. Murray, K. Powell, M. Schulz, C. Spinks and P. Worthington, *Annu. Rev. Phytopathol.*, 1997, **35**, 349–372.
- 45 F. C. Odds, A. J. Brown and N. A. Gow, *Trends Microbiol.*, 2003, **11**, 272–279.
- 46 A. Carrillo-Munoz, G. Giusiano, P. Ezkurra and G. Quindós, *Rev. Esp. Quimioter.*, 2006, **19**, 130–139.
- 47 S. K. Shrestha, M. Y. Fosso and S. Garneau-Tsodikova, *Sci. Rep.*, 2015, **5**, 17070.
- 48 S. K. Shrestha, M. Grilley, T. Anderson, C. Dhiman, J. Oblad, C.-W. T. Chang, K. N. Sorensen and J. Y. Takemoto, *Med. Mycol.*, 2015, **53**, 837–844.
- 49 J. Y. Takemoto, S. N. Wegulo, G. Y. Yuen, J. A. Stevens, C. C. Jochum, C.-W. T. Chang, Y. Kawasaki and G. W. Miller, *Fungal Biol.*, 2018, DOI: 10.1016/j.funbio.2017.12.001, in press.
- 50 J. Loeffler and D. A. Stevens, *Clin. Infect. Dis.*, 2003, **36**, S31–S41.
- 51 A. S. Orozco, L. M. Higginbotham, C. A. Hitchcock, T. Parkinson, D. Falconer, A. S. Ibrahim, M. A. Ghannoum and S. G. Filler, *Antimicrob. Agents Chemother.*, 1998, **42**, 2645–2649.
- 52 M. C. Fisher, D. A. Henk, C. J. Briggs, J. S. Brownstein, L. C. Madoff, S. L. McCraw and S. J. Gurr, *Nature*, 2012, **484**, 186–194.
- 53 C. f. D. Control and Prevention, *MMWR: Morbidity and mortality weekly report*, 2008, vol. 56, pp. 1343–1345.