AlkylGuanidino Ureas, from a serendipitous discovery to a rational design: molecular simplification approach and membrane-based MoA investigation

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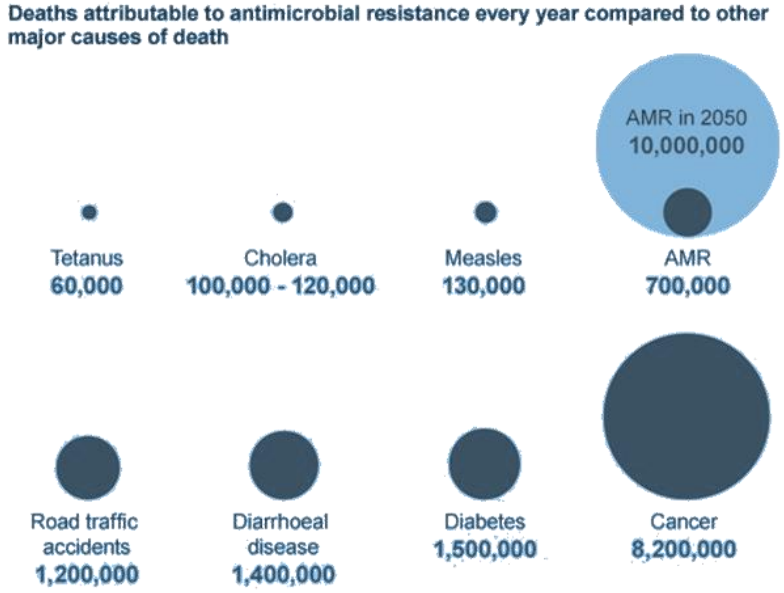
Bacterial Resistance: a worrisome scenario

Antimicrobial resistance (AMR) to currently available antibiotics represents a major global Public Health challenge

accounting for 35,000 and 32,000 deaths every year in the USA and Europe, respectively



Unless action is taken, the scenario is going to be worse by 2050

GLOBAL **A failure to address the problem of antibiotic resistance could result in:**

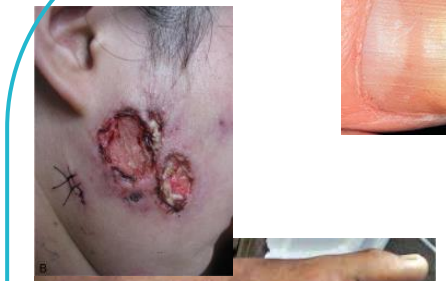
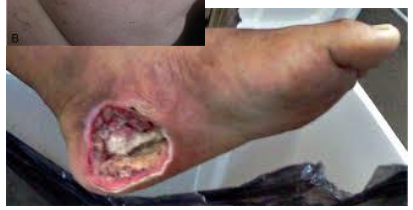
10m deaths by 2050

Costing £66 trillion

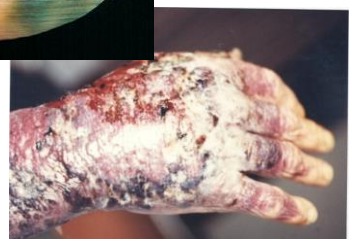
CDC, Antibiotic Resistance Threats in the United States, 2019, U.S. Departmentof Health and Human Services, CDC, Atlanta, GA, 2019, https://doi.org/10.15620/cdc:82532

J. O'Neill, Tackling Drug-Resistant Infections Globally: Final Report and Rec-ommendations the Review on Antimicrobial Resistance, Rev. Antimicrob.Resist., 2016

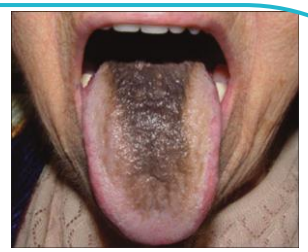
Bacterial Resistance: a worrisome scenario

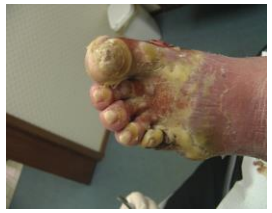
A. baumanii

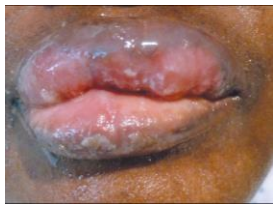
P. aeruginosa



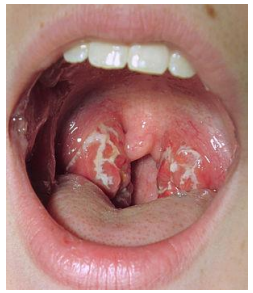
E. cloacae



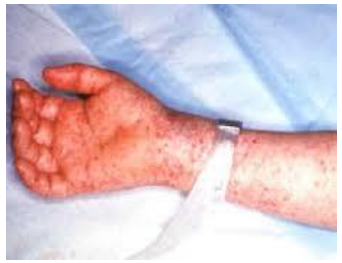
VRE



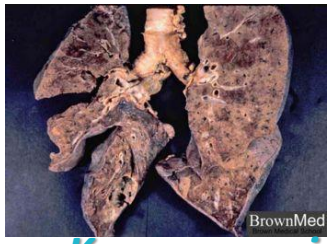
S. maltophilia



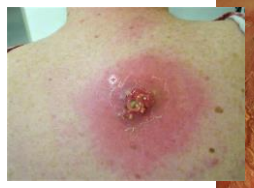
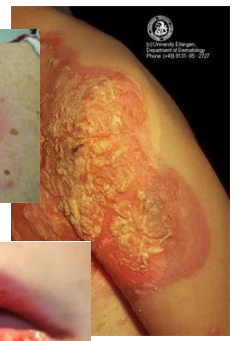
S. pyogenes



B. cepacia



K. pneumoniae


MRSA

Some effects on our bodies

*Health emergency of **COVID-19** pandemic contributed to dramatically increase AMR phenomena*

- high rate of antibiotics prescriptions in hospitalised patients
- overuse of sanitisers and biocides during COVID-19 containment campaigns

Pulingam T, Parumasivam T, Gazzali AM, et al. Antimicrobial resistance: prevalence, economic burden, mechanisms of resistance and strategies to overcome. Eur J Phar Sci 2022;170:106103.

Baghdadi JD, Coffey KC, Adediran T, et al. Antibiotic use and bacterial infection among inpatients in the first wave of COVID-19: a Retrospective Cohort Study of 64,691 patients. Antimicrob Agents Chemother 2021;65:PMC8522758

Rizvi SG, Ahammad SZ. COVID-19 and antimicrobial resistance: a cross-study. Sci Total Environ 2022;807:150873

Bacterial Resistance: a worrisome scenario

The World's Next Big Health Emergency Is Already Here

Covid-19 has claimed nearly 6 million lives. Antimicrobial resistance may claim 10 million annually by 2050 — and that figure is starting to look low.

Antimicrobial multidrug resistance in the era of COVID-19: a forgotten plight?

Eric Pelfrene, Radu Botgros & Marco Cavaleri

Antimicrobial Resistance & Infection Control **10**, Article number: 21 (2021) | Cite this article

14k Accesses | **22** Citations | **47** Altmetric | Metrics

Hidden pandemic of antibiotic-resistant infections, health officials warn

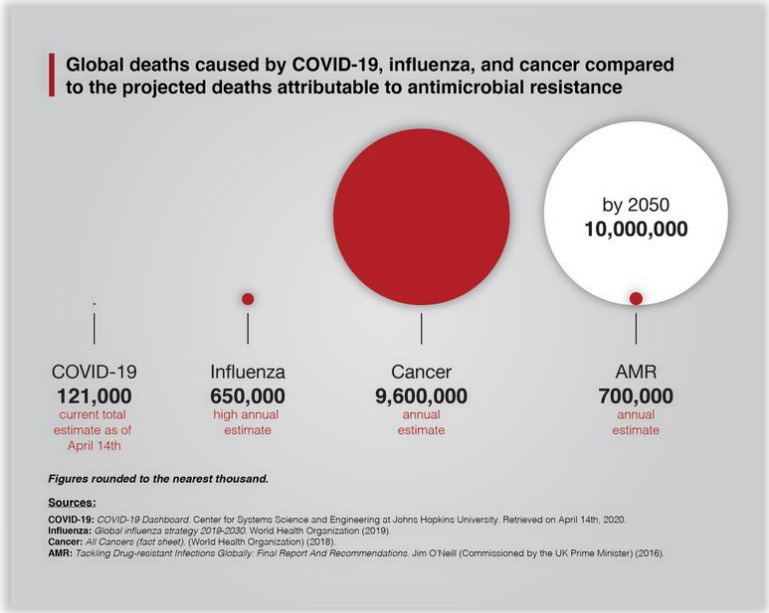
NEWS HEALTH & MEDICINE

Antimicrobial resistance is a leading cause of death globally

The staggering death toll of drug-resistant bacteria

Global survey shows that in 2019, antimicrobial resistance killed more people than HIV/AIDS or malaria.

*Health emergency of **COVID-19** pandemic contributed to dramatically increase AMR phenomena*



high rate of antibiotics prescriptions in hospitalised patients

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Pulingam T, Parumasivam T, Gazzali AM, et al. Antimicrobial resistance: prevalence, economic burden, mechanisms of resistance and strategies to overcome. Eur J Phar Sci 2022;170:106103.

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Rizvi SG, Ahammad SZ. COVID-19 and antimicrobial resistance: a cross-study. Sci Total Environ 2022;807:150873

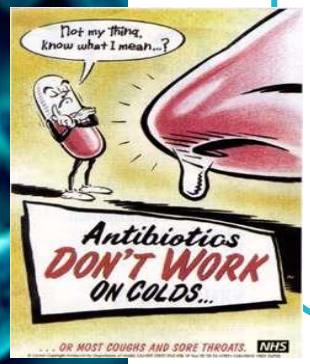
Bacterial Resistance: a worrisome scenario

Use, misuse and overuse of drugs

overprescription, self medication, environmental contamination, uncontrolled use in livestock

misconception of antibiotics as panacea medicine… also for flu!

…and, recently, Online Pharmacy



Innovation gap in Antibiotics R&D of 40 years

Last FDA approval Cefiderocol, November 2019

The only one antibiotic with innovative chemical scaffold is Lefamulin

Few therapeutic options are still available for carbapenem-resistant A. baumannii and P. aeruginosa infections.

NEW ANTIBIOTICS URGENTLY NEEDED!!!

FDA Approves New Antibacterial Drug to Treat Complicated Urinary Tract Infections as Part of Ongoing Efforts to Address Antimicrobial Resistance.https://www.fda.gov/news-events/press-announcements/fda-approves-new-antibacterial-drug-treat-complicated-urinary-tract-infections-part-ongoing-efforts

M.P. Veve, J.L. Wagner, Lefamulin: review of a promising novel pleuromutilinantibiotic, Pharmacotherapy 38 (2018) 935e946, https://doi.org/10.1002/phar.2166

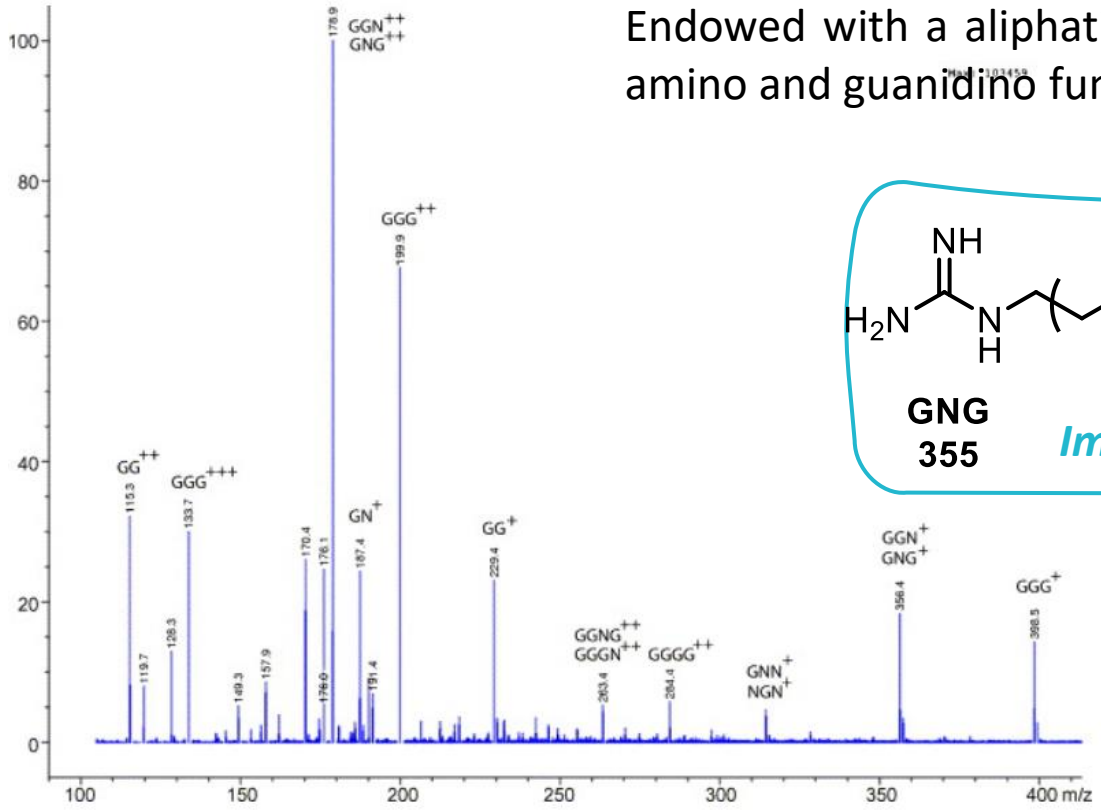
Antibacterial Agents in Clinical Development: an Analysis of the AntibacterialClinical Development Pipeline, 2019. https://www.who.int/publications/i/item/9789240000193

A serendipitous discovery: the AGU class

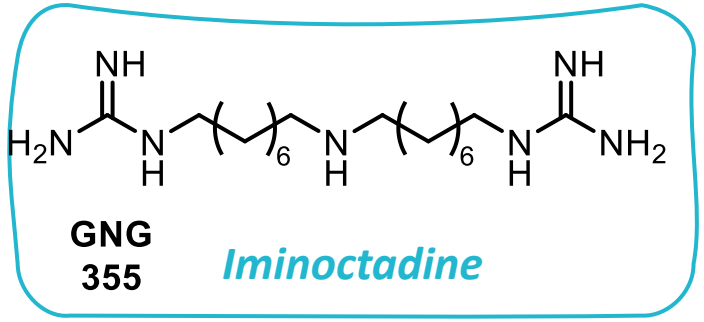
LC–MS analysis of **Guazatine** led to the identification of the components of the mixture

Guazatine Acetate

a non-systemic fungicide used in agriculture chemically a mixture of reaction products from polyamines



Endowed with a aliphatic linear scaffold bearing amino and guanidino functions



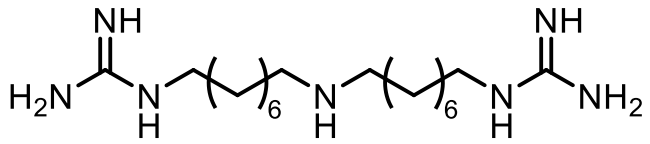
GNG 355 ***Iminoctadine***

MS spectrum for direct injection of a sample of Guazatine

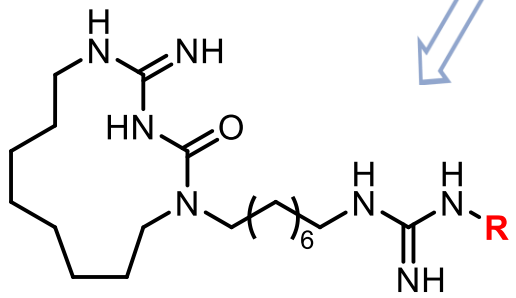
Dreassi E, Zizzari AT, D'Arezzo S, Visca P, Botta M. Analysis of guazatine mixture by LC and LC-MS and antimycotic activity determination of principal components. J Pharm Biomed Anal. 2007 Mar 12;43(4):1499-506. doi: 10.1016/j.jpba.2006.10.029

A serendipitous discovery: the AGU class

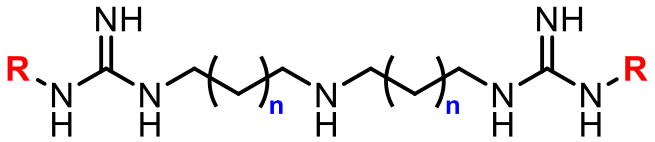
Two families of Guazatine derivatives were developed with different medicinal chemistry aims



GNG 355 **Iminoctadine**



cyclic derivatives as antifungals

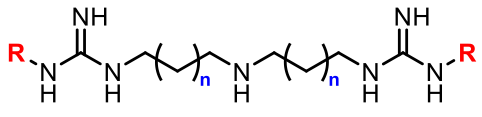


linear derivatives as inhibitors of PAOs

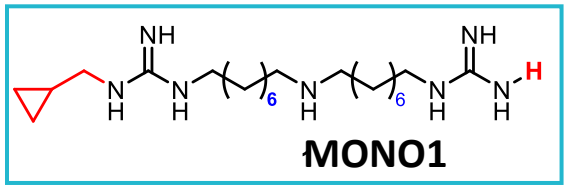
Dreassi E, Zizzari AT, D'Arezzo S, Visca P, Botta M. Analysis of guazatine mixture by LC and LC-MS and antimycotic activity determination of principal components. J Pharm Biomed Anal. 2007 Mar 12;43(4):1499-506. doi: 10.1016/j.jpba.2006.10.029

A serendipitous discovery: the AGU class

Later, the antibacterial susceptibility of the synthetic Guazatine derivatives was investigated



linear derivatives



MONO1

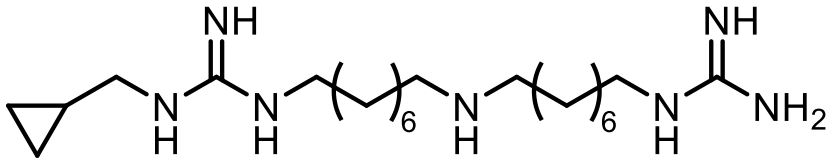
Bacterial strains	MIC (µg/mL)							
	MONO1	other derivatives						
A. baumannii ATCC 17978	4	>256	64	128	>256	>256	64	32
A. hydrophila ATCC 7966	8	>256	64	256	>256	>256	-	64
E.meningoseptica CCUG 4310	32	>256	-	>256	>256	>256	-	128
E. coli CCUG^{T}	0.5	128	8	16	>256	>256	-	64
K. pneumoniae ATCC 13833	1	256	16	32	>256	256	128	32
P. aeruginosa ATCC 27853	8	>256	64	256	>256	>256	>256	256
B. subtilis ATCC 6633	0.5	64	4	4	>256	>256	4	4
E. faecalis ATCC 19433	1	256	16	16	>256	16	32	8
S. epidermidis ATCC 14990	0.5	64	4	4	>256	256	8	4
S. pyogenes ATCC 12344	<0.125	16	1	2	>256	32	32	2

revealing only one compound (**MONO1**) to be active with Minimal Inhibitory Concentrations (MICs) range of 0.125-32 ug/mL on a panel of Gram-positive and Gram-negative bacterial species, including some relevant drug-resistant strains and clinical isolates

Maccari, G.; Sanfilippo, S.; De Luca, F.; Deodato, D.; Casian, A.; Dasso Lang, M. C.; Zamperini, C.; Dreassi, E.; Rossolini, G. M.; Docquier, J. D.; et al. Synthesis of Linear and Cyclic Guazatine Derivatives Endowed with Antibacterial Activity. *Bioorganic Med. Chem. Lett.* **2014**, *24* (23), 5525–5529.

A serendipitous discovery: the AGU class

However, new batches of that compound were found inactive…



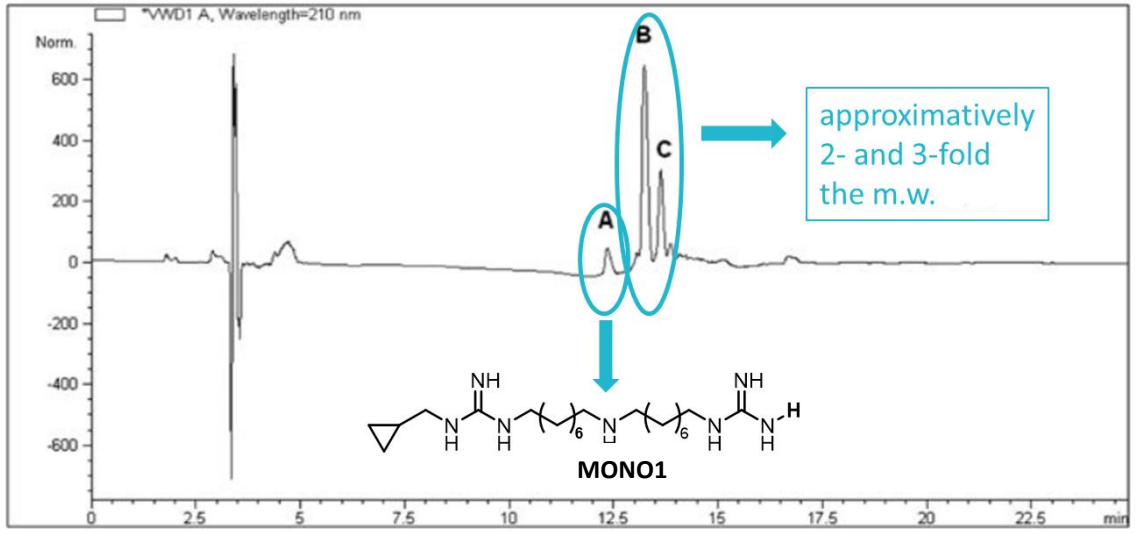
MONO1

Test samples	MIC (µg/mL)[a]							
	E. coli CCUG^T	*K. Pneumoniae ATCC 13833*	*P. aeruginosa ATCC 27853*	*A. baumannii ATCC 17978*	*S. epidermidis ATCC 14990*	*E. faecalis ATCC 29212*	*B. subtilis ATCC 6633*	*S. aureus ATCC 25923*
Old batch	0.5	1	8	4	0.5	1	0.5	4
Freshly synthesized	64	> 64	> 64	> 64	> 64	> 64	64	> 64

Zamperini, C.; Maccari, G.; Deodato, D.; Pasero, C.; D'Agostino, I.; Orofino, F.; D'Agostino, I.; Orofino, F.; De Luca, F.; Dreassi, E.; et al. Identification , Synthesis and Biological Activity of Alkyl- Guanidine Oligomers as Potent Antibacterial Agents. *Sci. Rep.* **2017**, *7* (July), 1–11.

A serendipitous discovery: the AGU class

*The comparison of the HPLC-UV-MS chromatographic fingerprinting of the old and the new batches revealed that the former was a mixture of oligomers (eluate A, B, and C), self-generated in the fridge-stored DMSO stock solution of a **MONO 1** sample*



Chromatographic profile obtained after blank substraction of a sample of the first batch (10 mg/mL) by LC–MS: **A** (t_R = 12.40 min) monomer (**1**); **B** (t_R = 13.34 min) = dimer; **C** (t_R = 13.74 min) = trimer. Chromatographic method and conditions are reported in "Methods - *Chromatographic separation*".

Test samples	MIC (μg/mL)[a]							
	E. coli CCUGT.	*K. pneumonia* ATCC 13833	*P. aeruginosa* ATCC 27853	*A. baumannii* ATCC 17978	*S. epidermidis* ATCC 14990	*E. faecalis* ATCC 29212	*B. subtilis* ATCC 6633	*S. aureus* ATCC 25923
Initial mixture[b]	0.5	1	8	4	0.5	1	0.5	4
Eluate **A**	64	> 64	64	> 64	64	> 64	64	> 64
Eluates **B+C**	1	2	16	16	0.5	1	0.5	-
1 – monomer[c]	64	> 64	> 64	> 64	> 64	> 64	64	> 64

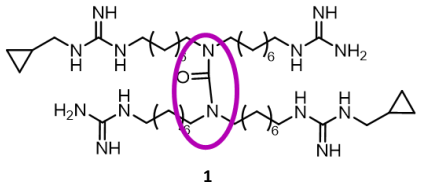
Semipreparative HPLC and biological evaluation allowed us to find the actual responsible for the antibacterial activity of the old batch.

Zamperini, C.; Maccari, G.; Deodato, D.; Pasero, C.; D'Agostino, I.; Orofino, F.; D'Agostino, I.; Orofino, F.; De Luca, F.; Dreassi, E.; et al. Identification , Synthesis and Biological Activity of Alkyl- Guanidine Oligomers as Potent Antibacterial Agents. *Sci. Rep.* **2017**, *7* (July), 1–11.

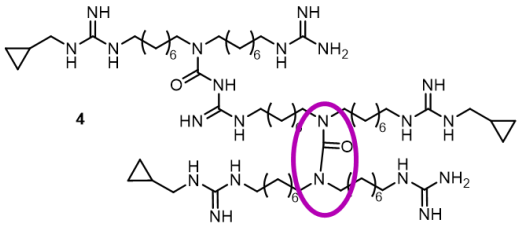
A serendipitous discovery: the AGU class

In-depth MS studies led us to the design and synthesis of the *AlkylGuanidino Urea (**AGU**) Oligomers belonging to the Symmetrics' and the Asymmetrics' series*

The Symmetrics



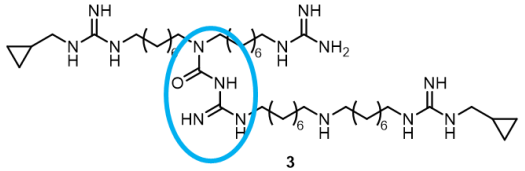
1



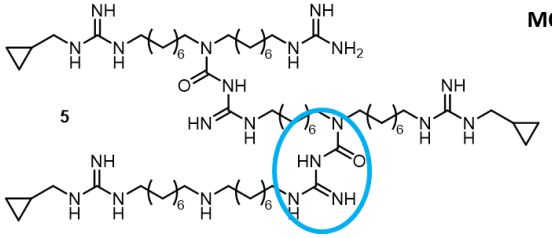
4

Urea or
Amidinourea
moiety

The Asymmetrics



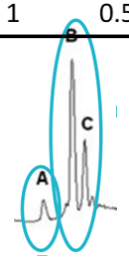
3



5

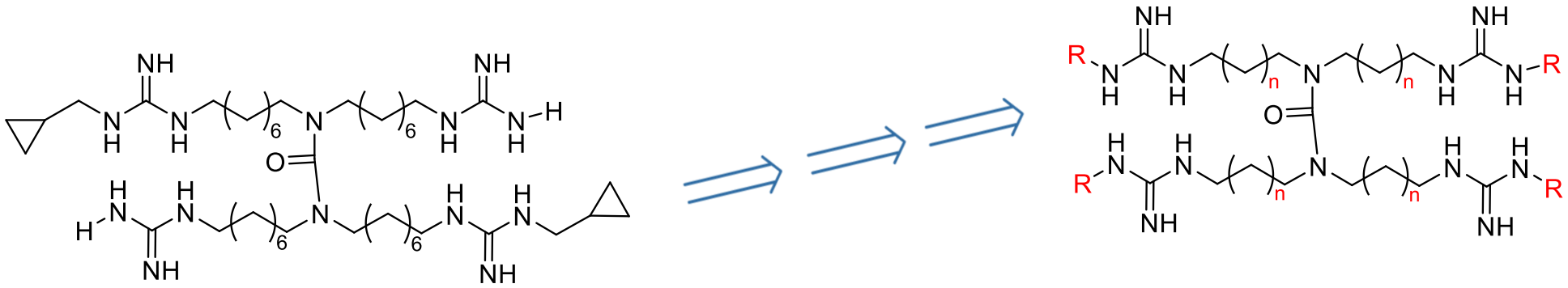
Test samples	MIC (µg/mL)[a]							
	E. coli CCUGT.	*K. pneumonia* ATCC 13833	*P. aeruginosa* ATCC 27853	*A. baumannii* ATCC 17978	*S. epidermidis* ATCC 14990	*E. faecalis* ATCC 29212	*B. subtilis* ATCC 6633	*S. aureus* ATCC 25923
Initial mixture[b]	0.5	1	8	4	0.5	1	0.5	4
Eluate **A**	64	> 64	64	> 64	64	> 64	64	> 64
Eluates **B+C**	1	2	16	16	0.5	1	0.5	-
MONO1 – monomer[c]	64	> 64	> 64	> 64	> 64	> 64	64	> 64
1 – sym. dimer	2	2	8	8	1	2	0.5	2
3 – asym. dimer	>64	>64	>64	>64	8	8	<0.125	4
4 – sym. trimer	4	4	16	8	4	4	4	8
5 – asym. trimer	8	8	32	32	4	<0.125	8	8
Colistin	0.5	0.5	0.5	1	-	-	-	-
Vancomycin	-	-	-	-	2	1	0.5	1
Daptomycin	-	-	-	-	0.25	1	1	0.5

*…and Compound **1** tourned out to be the eluate **B** andowed with high antibacterial potency*



Zamperini, C.; Maccari, G.; Deodato, D.; Pasero, C.; D'Agostino, I.; Orofino, F.; D'Agostino, I.; Orofino, F.; De Luca, F.; Dreassi, E.; et al. Identification , Synthesis and Biological Activity of Alkyl- Guanidine Oligomers as Potent Antibacterial Agents. *Sci. Rep.* **2017**, *7* (July), 1–11.

The development of the AGU class



Investigated features

n: methylene lenght of the urea-guanidino spacer(s)

R: guanidino substituent(s)

Preliminary SAR data

The length of the alkyl chain (*n*) affects the activity of the compounds

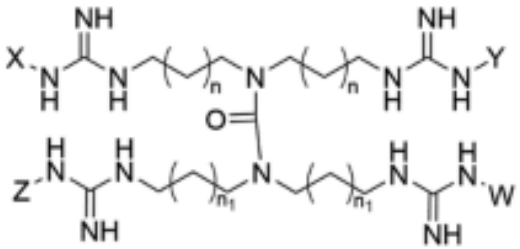
The nature (lipophilicity) and the number of guanidine substituents (*R*) do not influence significantly the antibacterial profile

Botta, M.; Maccari G.; Sanfilippo S.; De Luca F.; Docquier J.D.; Deodato D. Linear Guanidine Derivatives, Methods Of Preparation And Uses Thereof. Int. Patent Appl. WO2016/055644 A1, 14 April 2016.

Pasero, C.; D'Agostino, I.; De Luca, F.; Zamperini, C.; Deodato, D.; Truglio, G. I.; Sannio, F.; Del Prete, R.; Ferraro, T.; Visaggio, D.; et al. Alkyl-Guanidine Compounds as Potent Broad-Spectrum Antibacterial Agents: Chemical Library Extension and Biological Characterization. J. Med. Chem. 2018, acs.jmedchem.8b00619.

The development of the AGU class

MICs of the Dimer Chemical Library and Control Antibiotics on Representative Gram-Negative and Gram-Positive Bacteria



nXY*/n$_1$ZWs

-: not determined. MICs (µg/mL) are expressed as median values calculated from experiments performed at least in triplicate.
Derivatives identity code. XYZW: C, cyclopropylmethyl; H, hydrogen; E, ethyl; M, methyl; B, benzyl; U, CONHCH3. n, n1: 4,6,8. *: carbamoyl intermediate (data regarding synthetic pathways). s: trifluoroacetate salt.

Modification	Cpd	Cpd Code	MIC (µg/mL)[a]								
			E. coli CCUG^T	*K. pneumoniae* ATCC 13833	*P. aeruginosa* ATCC 27853	*A. baumannii* ATCC 17978	*S. pyogenes* ATCC 12344	*E. faecalis* ATCC 19433	*B. subtilis* ATCC 6633	*S. epidermidis* ATCC 14990	*S. aureus* ATCC 25923 SEP
Hit compound	1	8CH/8CHs	1	1	4	4	1	<0.125	0.5	2	2
Lenght of linker	6	10CH*/10CHs	16	8	64	64	4	4	2	-	8
	33	7CH*/7CHs	8	8	32	32	2	8	2	-	4
	11	6CH*/6CHs	16	32	>64	>64	2	32	4	1	-
	34	8CH*/6CHs	8	4	16	16	0.5	4	2	-	1
	35	7CH*/6CHs	16	8	32	32	1	4	2	-	4
	12	6CH*/8CHs	8	4	16	16	0.5	4	2	1	-
	13	8CC*/6CCs	4	4	>64	64	1	8	4	2	-
N-substituent	7	8EH*/8EHs	2	2	16	16	0.5	1	1	-	1
	8	8MH*/8MHs	1	4	8	8	0.5	1	0.5	-	0.5
	9	8EE*/8EEs	2	4	32	16	0.25	1	0.5	-	0.5
	10	8CC*/8CCs	4	4	64	8	2	4	8	-	16
	14	8BH*/8BHs	4	2	8	8	-	2	2	-	-
	15	8BB*/8BBs	4	4	8	16	1	4	4	8	-
	16	8HH*/8HHs	4	32	32	16	1	2	32	-	-
	36	6EE*/6EHs	64	>64	>64	64	4	64	16	-	32
	37	8CH*/8CCs	8	4	16	8	1	2	2	-	2
	17	8CC*/8HHs	4	4	16	16	4	8	2	8	-
	18	6CC*/6CHs	8	16	>64	64	2	16	4	4	-
References		Colistin	0.5	0.5	0.5	1	-	-	-	-	-
		Vancomycin	-	-	-	-	0.5	1	0.5	1	1
		Daptomycin	-	-	-	-	0.125	1	1	1	0.5

Pasero, C.; D'Agostino, I.; De Luca, F.; Zamperini, C.; Deodato, D.; Truglio, G. I.; Sannio, F.; Del Prete, R.; Ferraro, T.; Visaggio, D.; et al. Alkyl-Guanidine Compounds as Potent Broad-Spectrum Antibacterial Agents: Chemical Library Extension and Biological Characterization. J. Med. Chem. 2018, acs.jmedchem.8b00619.

The Molecular Simplification on the AGU class

Molecular Simplification Approach

Reduction of molecular obesity via a step-by-step dissection of the original structure and testing the simplified derivatives

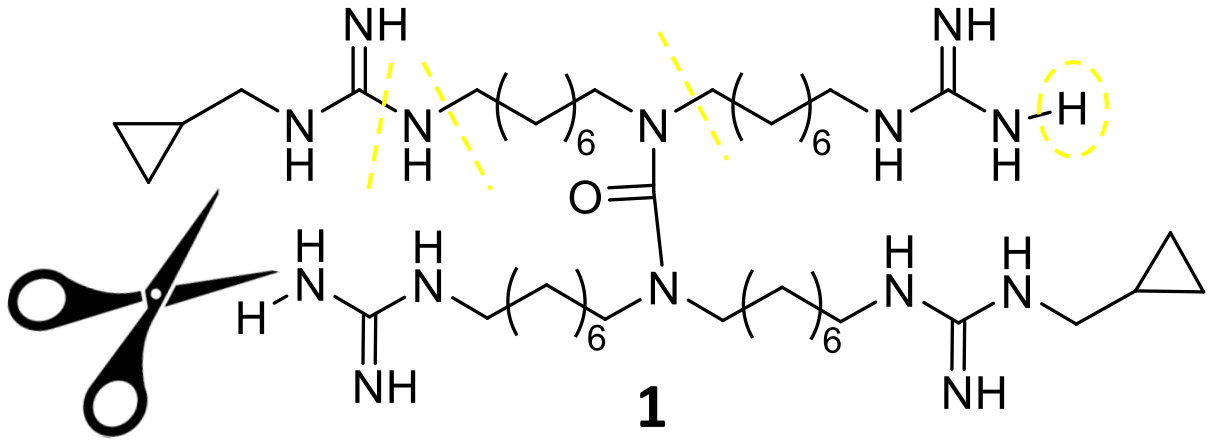
- more accessible syntheses
- improved drug-like properties

Arm(s) – Urea *N*-substituent(s) – removal

Amidine(s) cutting off and free primary amine(s)

Guanidine(s) turning off

Traditional derivatives

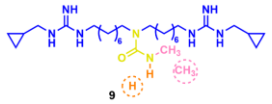
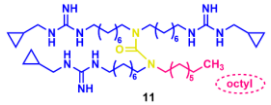
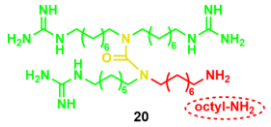
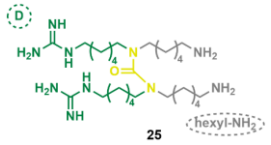


1

Pasero, C.; D'Agostino, I.; De Luca, F.; Zamperini, C.; Deodato, D.; Truglio, G. I.; Sannio, F.; Del Prete, R.; Ferraro, T.; Visaggio, D.; et al. Alkyl-Guanidine Compounds as Potent Broad-Spectrum Antibacterial Agents: Chemical Library Extension and Biological Characterization. J. Med. Chem. 2018, acs.jmedchem.8b00619.

The Molecular Simplification on the AGU class

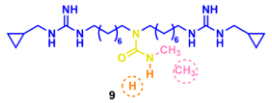
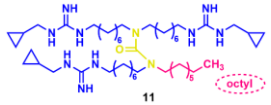
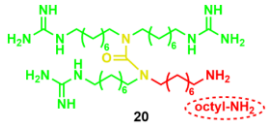
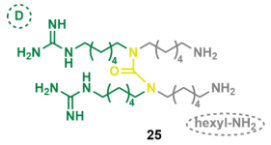
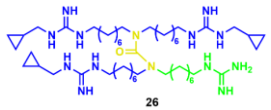
Derivatives series presented in this work (compounds **5-27**) designed by molecular simplification (**5-25**) or by SAR-guided design (**26** and **27**) and MICs on representative Gram-positive and Gram-negative bacterial species.

Series	*Parent cpd*	*Representative Molecular Structures*	Cpd	*Urea Substitution* R^1	R^2	R^3	R^4
Arm-Removed	2	 9	5	A	A	A	H
			6	A	H	A	H
			7	A	A	H	H
			8	A	H	H	H
			9	A	A	H	CH_3
			10	A	H	H	CH_3
Guanidino-Turned Off	2	 11	11	A	A	A	octyl
			12	A	octyl	A	octyl
			13	A	A	octyl	octyl
			14	A	octyl	octyl	octyl
			15	octyl	octyl	octyl	octyl
	11	 19	16	A	A	A	hexyl
			17	A	A	C	octyl
			18	C	C	A	octyl
			19	A	C	C	hexyl
Amidino-Cut Off	3	 20	20	B	B	B	octyl-NH_2
			21	B	octyl-NH_2	B	octyl-NH_2
			22	B	B	octyl-NH_2	octyl-NH_2
			23	B	octyl-NH_2	octyl-NH_2	octyl-NH_2
			24	octyl-NH_2	octyl-NH_2	octyl-NH_2	octyl-NH_2
	21	 25	25	D	hexyl-NH_2	D	hexyl-NH_2
Traditional	1	 26	26	A	A	A	B
			27	A	B	B	B

Series	Cpd	MIC [μg/mL][a] Bsu	Efa	Spy	Sau	Eco	Kpn	Aba	Pae
Arm-Removed	5	1	8	1	4	8	8	64	64
	6	> 128	64	8	128	64	> 128	-	> 128
	7	8	16	2	16	32	32	128	128
	8	1	1	0.5	1	2	1	16	16
	9	32	64	8	128	128	128	> 128	> 128
	10	64	64	128	32	128	128	> 128	> 128
Guanidino-Turned Off	11	2	2	1	16	4	4	4	8
	12	128	32	8	> 128	> 128	128	> 128	> 128
	13	128	128	4	> 128	128	64	128	> 128
	14	64	128	16	> 128	> 128	> 128	> 128	> 128
	15	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128
	16	2	1	1	2	4	8	8	16
	17	2	2	1	1	2	2	4	8
	18	0.5	0.5	1	2	4	4	16	16
	19	1	2	1	2	4	8	32	64
Amidino-Cut Off	20	1	4	0.25	1	2	8	16	16
	21	0.5	8	0.25	0.5	2	16	16	32
	22	0.5	8	0.25	0.5	2	16	16	32
	23	4	32	16	2	32	64	64	128
	24	4	16	4	8	32	16	16	32
	25	32	> 128	128	128	> 128	> 128	> 128	> 128
Tradi-tional	26	2	2	1	2	8	4	8	16
	27	0.5	1	0.25	0.5	0.5	1	4	8
Refe-rences	COL	-	-	-	-	0.5	0.5	1	0.5
	VAN	0.5	1	1	0.5	-	-	-	-
	DAP	1	1	0.5	0.12	-	-	-	-

I. D'Agostino, C. Ardino, G. Poli *et al.*, Antibacterial Alkylguanidino Ureas: Molecular Simplification Approach, Searching for Membrane-Based MoA. *Eur. J. Med. Chem*. **2022**, 114158.

The Molecular Simplification on the AGU class

Series	*Parent cpd*	*Representative Molecular Structures*	Cpd	*Urea Substitution* R¹	R²	R³	R⁴
Arm-Removed	2		5	A	A	A	H
			6	A	H	A	H
			7	A	A	H	H
			8	A	H	H	H
			9	A	A	H	CH_3
			10	A	H	H	CH_3
Guanidino-Turned Off	2		11	A	A	A	octyl
			12	A	octyl	A	octyl
			13	A	A	octyl	octyl
			14	A	octyl	octyl	octyl
	11		15	octyl	octyl	octyl	octyl
			16	A	A	A	hexyl
			17	A	A	C	octyl
			18	C	C	A	octyl
			19	A	C	C	hexyl
Amidino-Cut Off	3		20	B	B	B	octyl-NH_2
			21	B	octyl-NH_2	B	octyl-NH_2
			22	B	B	octyl-NH_2	octyl-NH_2
			23	B	octyl-NH_2	octyl-NH_2	octyl-NH_2
			24	octyl-NH_2	octyl-NH_2	octyl-NH_2	octyl-NH_2
	21		25	D	hexyl-NH_2	D	hexyl-NH_2
Traditional	1		26	A	A	A	B
			27	A	B	B	B

MICs of Selected Compounds on Gram-Negative Antibiotic-Resistant Clinical Isolates

Cpd	*E. cloacae* VA-417/02		*K. pneumoniae* SI-081Rb[a]		*A. baumannii* AC-54/97	
	MIC [μg/mL]	MBC [μg/mL]	MIC [μg/mL]	MBC [μg/mL]	MIC [μg/mL]	MBC [μg/mL]
8	4	8	4	4	16	16
18	8	8	8	8	8	8
20	8	8	16	16	8	8
26	8	8	8	16	8	16
27	2	2	2	2	8	8

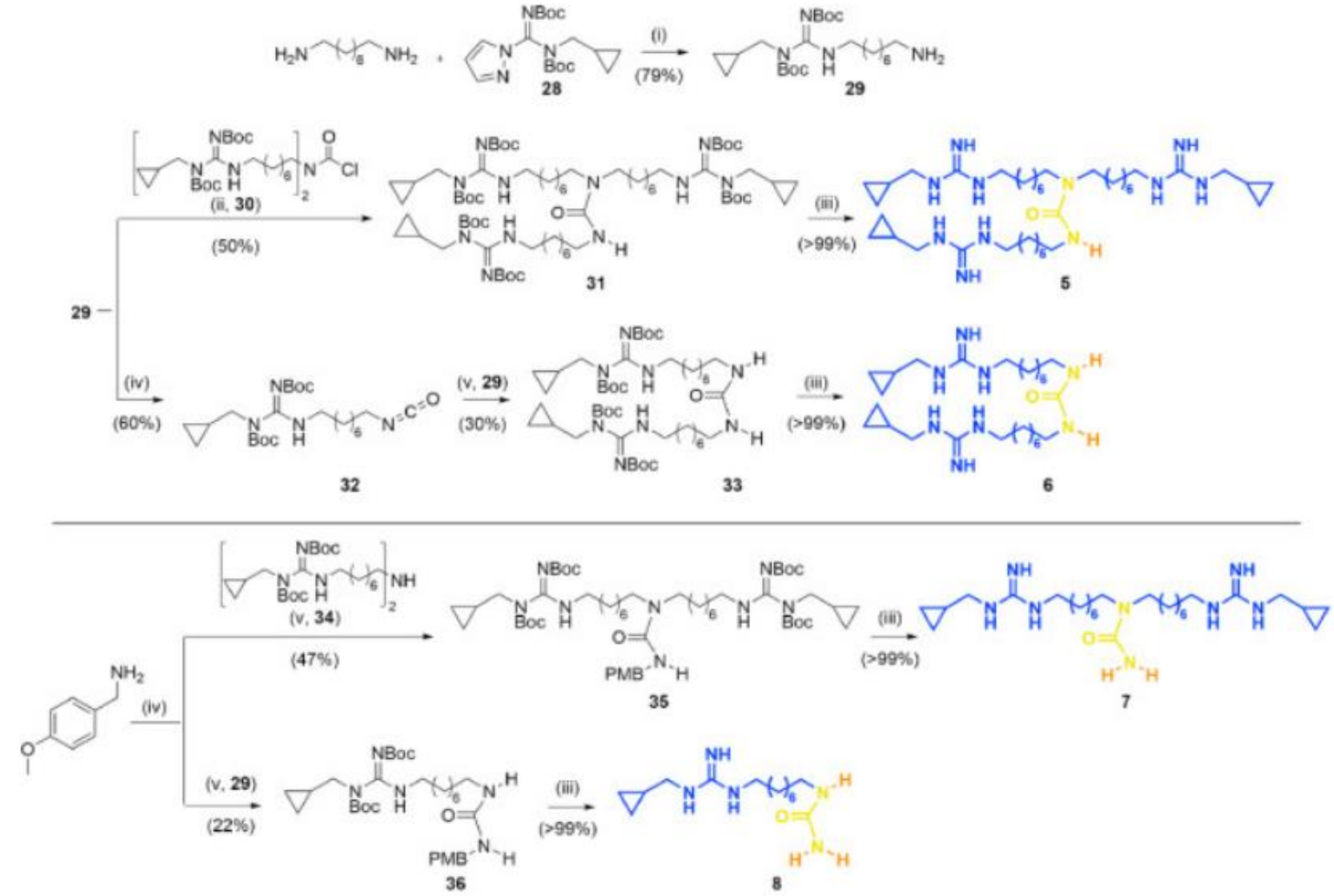
[a]Clinical isolate with pan drug-resistant phenotype. MIC and MBC values (μg/mL) are expressed as median values calculated from experiments performed at least in triplicate.

I. D'Agostino, C. Ardino, G. Poli *et al.*, Antibacterial Alkylguanidino Ureas: Molecular Simplification Approach, Searching for Membrane-Based MoA. *Eur. J. Med. Chem*. **2022**, 114158.

The Molecular Simplification on the AGU class

An example of synthetic scheme for

Guanidino Turned-Off Series



Reagents and conditions: (i) THF/MeOH 10/1, r.t., 16 h, N_2; (ii) cpd reported on the reaction arrow, DIPEA, NaI, dry DCM, sealed tube, ref., 48 h, N_2; (iii) TFA 20%, dry DCM, sealed flask, r.t., 2-4 h; (iv) Triphosgene, dry DIPEA, dry DCM, 0 °C to r.t., 0.5-1.5 h, N_2; (v) cpd reported on the reaction arrow, dry DIPEA, dry DCM, sealed tube, ref., 16 h, N_2.

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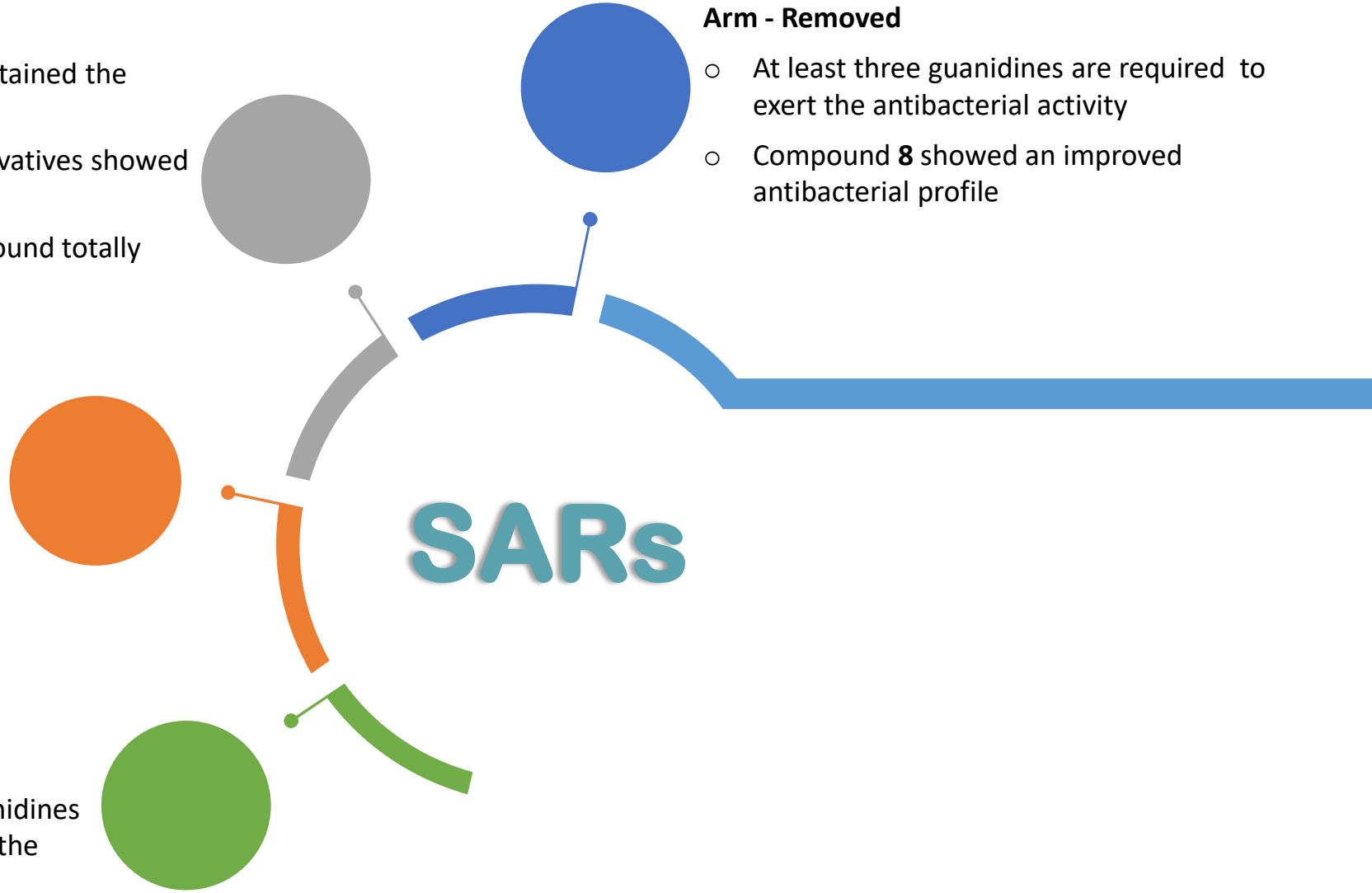
The Molecular Simplification on the AGU class

Guanidino - Turned Off

- Tri-guanidino derivatives retained the biological activity
- Bi- or Mono-guanidino derivatives showed lower potency
- Neutral compounds were found totally inactive

Arm - Removed

- At least three guanidines are required to exert the antibacterial activity
- Compound **8** showed an improved antibacterial profile



Amidino – Cut Off

- Mono or diamino 8-membered ureas are tolerated with an improved potency on specific strains
- 6-membered diamino ureas we found inactive
- Polyamino ureas were found inactive

Traditional

- Three unsubstituted guanidines seem to slightly improve the antibacterial profile

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The Molecular Simplification on the AGU class

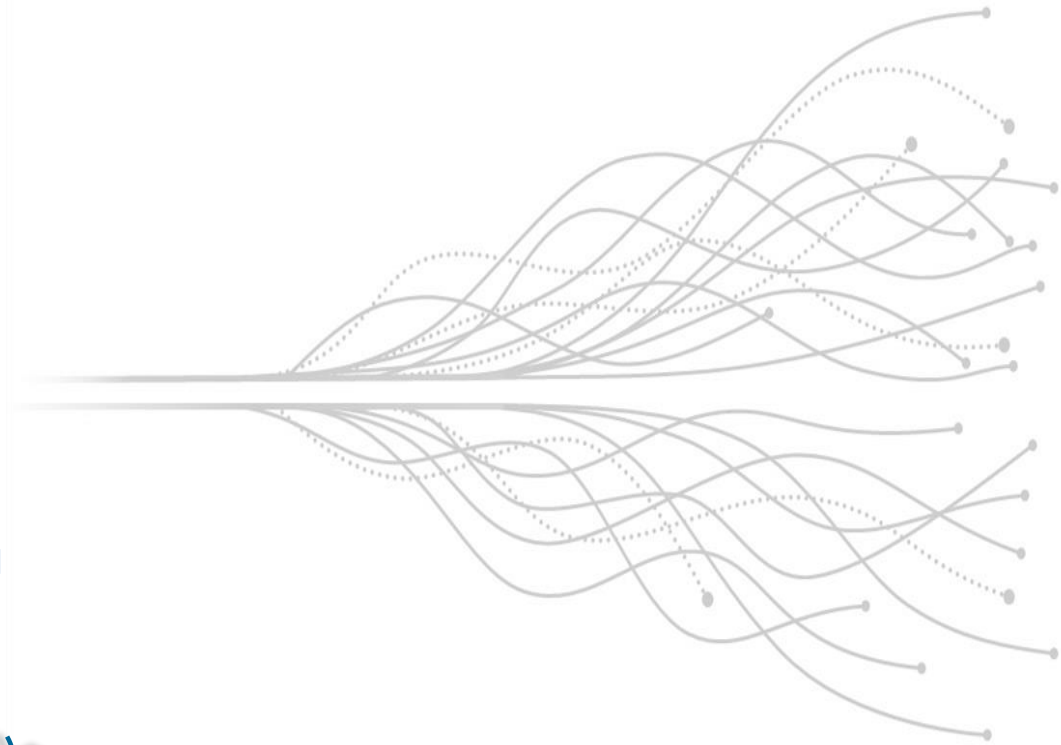
Divergent synthesis for new derivatives

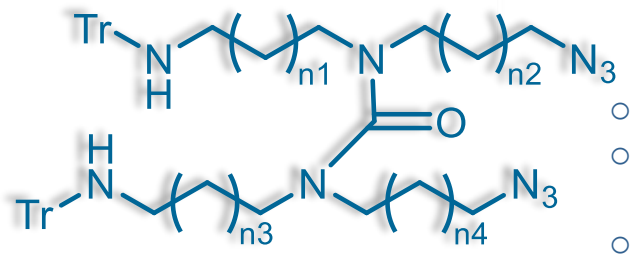
Ready available commercial starting materials

High yielding synthetic steps

Key Urea

Orthogonally protected key urea intermediate





n_{1-4} are numbers ranging from 4 to 7

- Protecting groups selectively cleavable;
- Guanylation steps occurring at the end of the synthetic pathways;
- Reduction in side-products formation;
- Scalable synthesis.

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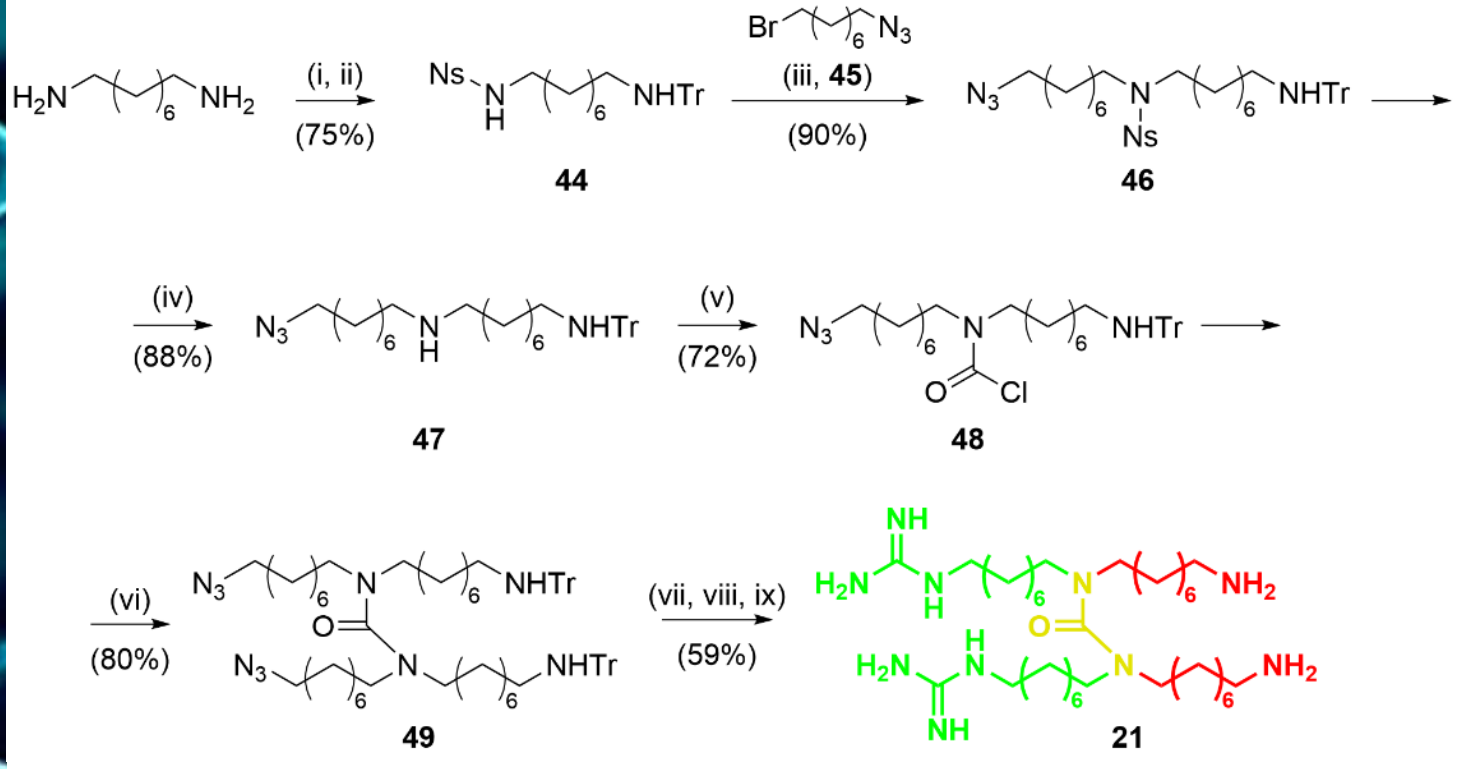
The Molecular Simplification on the AGU class

Synthesis of the key urea intermediate **49** and amino derivative **21**



Reagents and conditions: (i) TrCl, dry DCM, 0 °C to r.t., 6 h, N_2; (ii) NsCl, freshly distilled TEA, dry DCM, r.t., 2 h, N_2; (iii) **45**reported on the reaction arrow, KI, K_2CO_3, dry DMF, 95 °C, 16 h, N_2; (iv) thiophenol, degas. dry DMF, r.t., 2 h, N_2; (v) Triphosgene, dry DIPEA, dry DCM, 0 °C to r.t., 1.5 h, N_2; (vi) **47**, dry DIPEA, NaI, dry DCM, sealed tube, 40 °C, 16 h, N_2; (vii) triphenylphosphine, H_2O, THF, r.t., 5 h; (viii) *N,N'*-Di-Boc-1*H*-pyrazole-1-carboxamidine, DIPEA, THF, r.t., 16 h; (ix) TFA 20%, dry DCM, sealed flask, r.t., 3-5 h.

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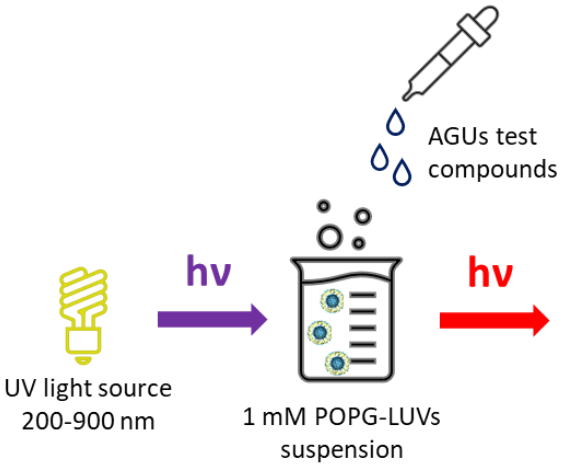
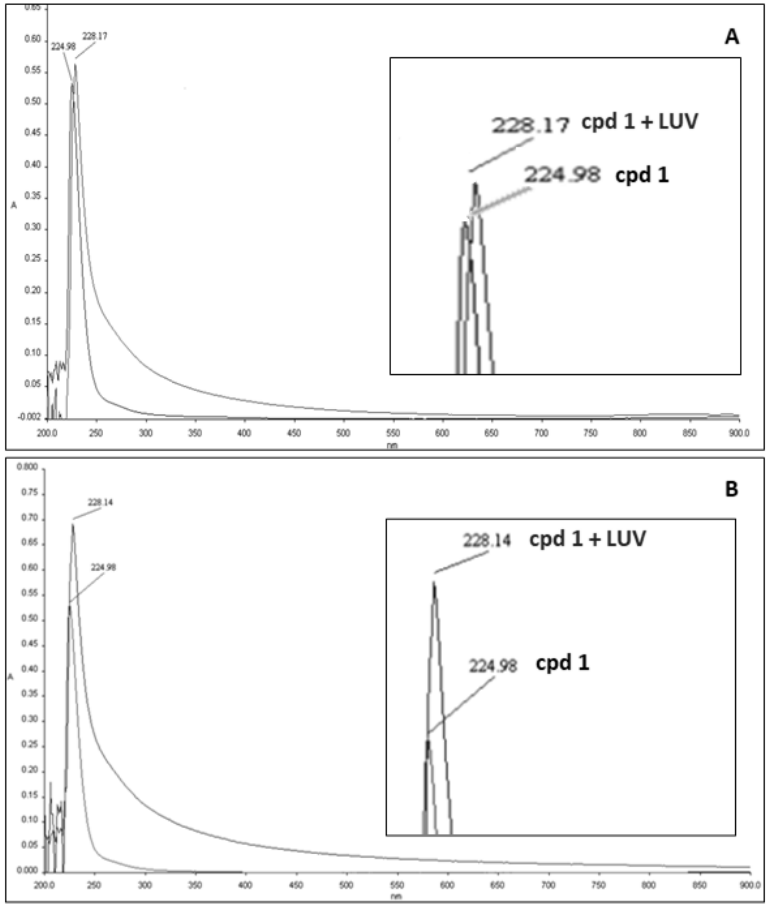
MOA investigation for the AGU class

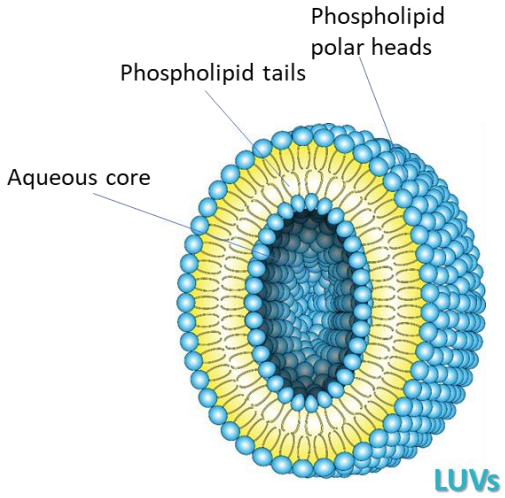
Advantages

Similar size to living prokaryotic cells
Could be used to mimic several cell types bilayers
Easy preparation
Wide variety of phospholipid mixtures to be employed to model membranes
Use of POPG to simulate Gram-positive membranes





LUV-compound interaction assay



UV-Vis spectra of compound **1** in absence or in presence of a POPG-LUVs suspension at t=0 (A) and t=1h (B)

Bathochromic (red) shift
Hyperchromic effect
- inability of cpd to entirely insert into the bilayer
- significant partition fraction into a more hydrophilic microenvironment (bilayer)

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MOA investigation for the AGU class

Traditional protocol

Parallel Artificial Membrane Permeability Assays (PAMPAs) were performed on bilayers simulating mammalian (PC), and Gram-positive (POPG) and Gram-negative (POPE/POPG mixture) species membranes to evaluate the membrane permeability of the test compounds

Results

Scarse passive diffusion of the compounds

Modified protocol

Two non-permeable reference compounds (caffeine and chloramphenicol) were used as probes on the bilayers to evaluate disruptions of the membrane integrity by the test compounds

Results

Increase in the probe permeability

Traditional and modified PAMPAs

Cpd	P_{app} [10^{-6} cm/sec] (MR%)		
	PC-phospholipids	pure POPG	POPE/POPG 6:4
1	1.60[b]	1.88 (46.1)	0.74 (42.4)
8	3.17 (7.7)	6.10 (16.0)	3.48 (3.2)
18	4.79 (0)	0.18 (63.0)	0.13 (65.2)
21	0.21 (36.3)	0.19 (52.4)	0.18 (47.3)
27	1.85 (0)	0.26 (56.0)	0.30 (40.8)
Chloramphenicol	0.54	0.06	1.12
Chloramphenicol[a] + 1	6.12	2.18	1.39
Chloramphenicol[a] + 8	0.04	0.08	1.67
Chloramphenicol[a] + 18	8.73	3.39	6.64
Chloramphenicol[a] + 21	3.24	3.14	6.11
Chloramphenicol[a] + 27	4.67	3.78	6.98
Caffeine	1.84 (3.2)	1.95 (4.5)	1.98 (3.3)
Caffeine[a] + 1	3.54 (12.6)	2.49 (13.5)	3.03 (12.6)
Caffeine[a] + 8	2.04 (12.8)	2.09 (13.4)	2.10 (17.5)
Caffeine[a] + 18	13.19 (0)	5.65 (14.7)	4.98 (16.7)
Caffeine[a] + 21	5.00 (15.8)	5.04 (17.1)	4.97 (16.5)
Caffeine[a] + 27	12.03 (8.1)	6.13 (14.6)	4.28 (19.0)

Values are reported as the mean of at least two experiments. [a]P_{app} values and MR% are referred to the probe.
[b]Data already reported

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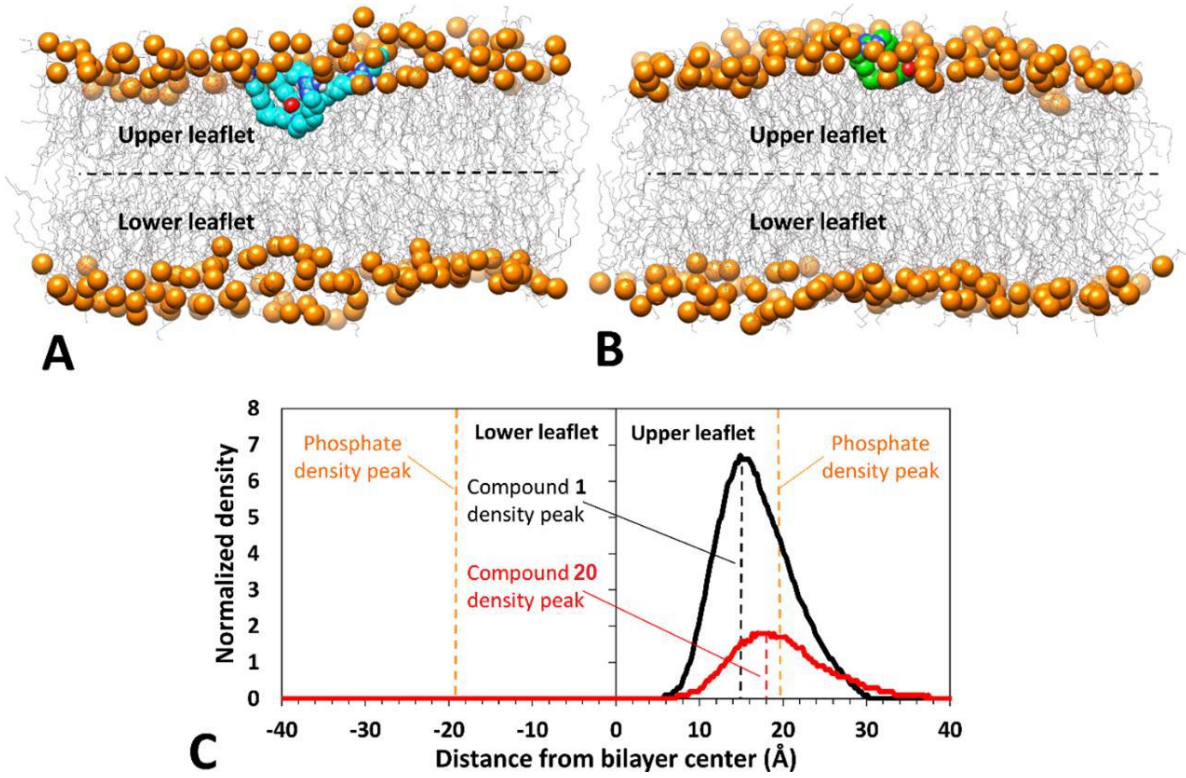
MOA investigation for the AGU class

In silico simulation membranes



Representative snapshots extracted after 300 ns from the MD simulation of the pure POPG membrane in presence of selected compounds. Compounds **1** (A) and (B) **8** are shown as cyan and green spheres, respectively, while the phospholipids are represented as gray wires, and their phosphorous atoms are highlighted as orange spheres. C) Electron density profile of compounds **1** and **8** with the corresponding peaks highlighted by dashed lines (black and red, respectively). Whereas, the density peaks of the lipid phosphate groups are indicated with orange dashed lines.

Molecular dynamics simulations were employed to validate the results obtained by LUVs and PAMPA experiments, by building up both POPG and POPE/POPG bilayers

Results

1 shows a carpet like behavior towards POPG membranes, laying of the surface of the membrane with the urea protruding to the center of the bilayer

Interactions of **8** with the membrane resulted weaker and less stable, as demonstrated by its fluctuations in the solvent layer in the proximity of the membrane

I. D'Agostino, C. Ardino, G. Poli *et al.*, Antibacterial Alkylguanidino Ureas: Molecular Simplification Approach, Searching for Membrane-Based MoA. *Eur. J. Med. Chem*. **2022**, 114158.

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The dyes

SYTO9 and PI selected as dyes for the permeabilization assay:

- SYTO 9 enters all bacterial cells
- PI enters only upon membrane damages

Fluorescence emission upon DNA-binding

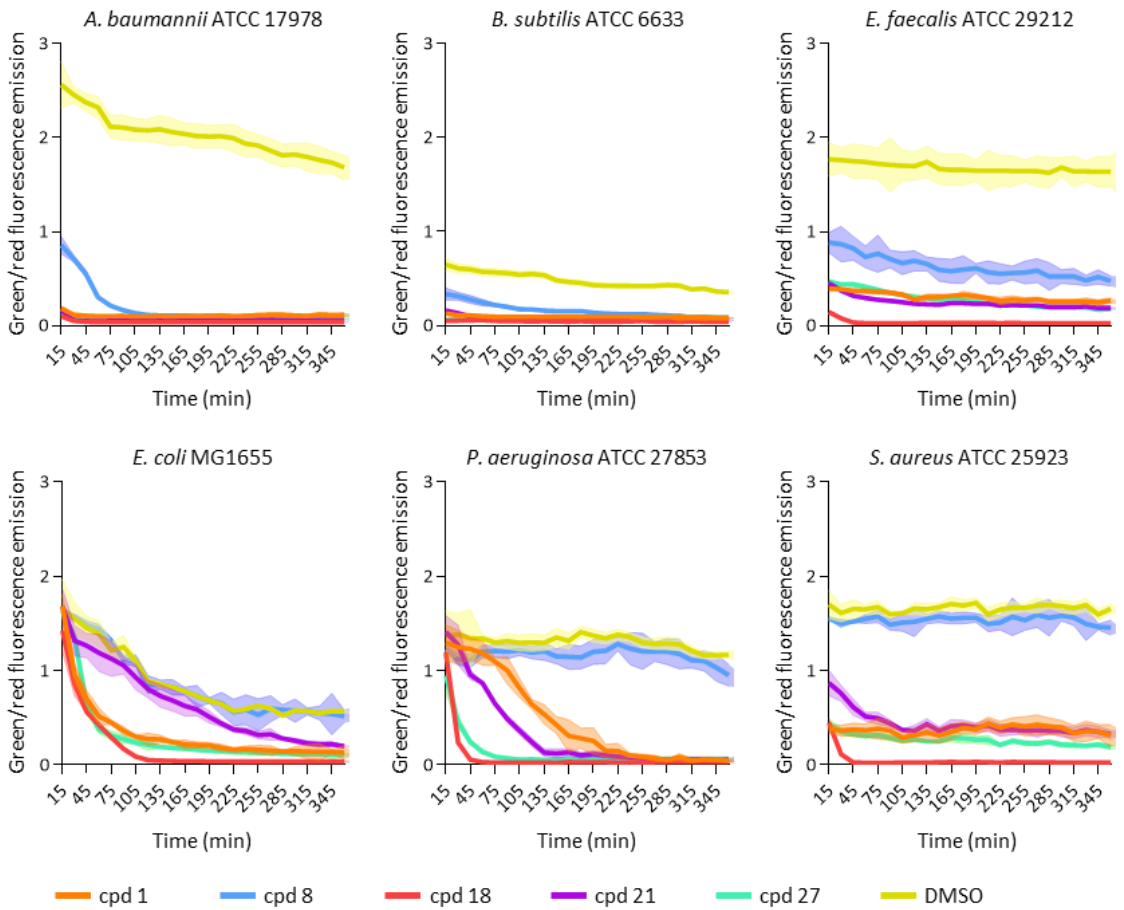
Results

All the tested compounds increased membrane permeability, although in time- and strain-dependent manner

Additional Assays

Confocal laser scanning microscopy (CLSM) imaging of bacterial cells treated for 1 h at 37 °C with 16 µg/mL individual AGUs, prior to SYTO 9 and PI staining confirmed the results

In cellulo assays



Bacterial cells from 16-h cultures in Luria-Bertani broth (LB) were washed with PBS and suspended at OD600= 0.1 in PBS supplemented with SYTO 9 (6 µM), PI (30 µM), and tested compound (16 µg/mL in DMSO, 16 mg/mL stock concentration). An equal concentration of DMSO (0.1% v/v) was used in the untreated control (yellow plot). Bacterial suspensions were dispensed in 96-well microtiter plates, and the fluorescence emission at 498 and 617 nm wavelength (emission max of SYTO 9 and PI, respectively) was recorded every 15 min for 6 h at 37 °C in a Spark 10M (Tecan) microplate reader. The ratio between SYTO 9 (green) and PI (red) fluorescence emissions denotes membrane integrity. Data are the mean of three independent experiments ± standard deviation, indicated as shaded area.

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Toxicity

Hemolytic activity of representative compounds on human erythrocytes from healthy 0 Rh-negative donors

Cpd	**Hemolysis** (%) **at cpd concentration** [µg/mL]							
	0[a]	**1**	**2**	**4**	**8**	**16**	**32**	**64**
1	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.4 ± 0.2	0.8 ± 0.3	1.2 ± 0.5	2.0 ± 0.8	3.4 ± 0.5
8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.1	1.2 ± 0.5	2.4 ± 0.1	8.4 ± 2.5	46.4 ± 5.2
21	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2	1.6 ± 0.6	2.0 ± 0.3
27	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.1	3.3 ± 0.2	8.1 ± 0.6

[a] 0.4 % (v/v) DMSO, equivalent to the maximum test concentration with 64 µg/mL of tested compounds

Results

Any or weak dose-dependent hemolytic activity (<8.1% at 64 µg/mL) for all test compounds

No hemolysis observed for compound **8**

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Conclusions

It’s time to work hard.

No action today means no cure tomorrow.

(Geneva, World Health Day 2011, Antimicrobial Resistance)

According to the predictions, in the time you spent to read this presentation, 400 people could have been dead due to AMR in 2050.

Conclusions

- From a serendipitous discovery to a rational design of derivatives

- **Multidisciplinarity** and **teams-collaboration** were fundamental to reach relevant results in both the serendpitous discovery (in depth HPLC-MS studies, design and synthesis, and biological evaluation) and MoA investigation (analytical, in silico and in cellulo assays)

- Chemically-innovative AGU class was developed as antibacterial agents
- New divergent synthetic pathways including orthogonally-protecting groups strategy
- Molecular simplification approach helped to gain relevant information on the chemical features essential for the biological activity
- New AGU compounds, including some simplified derivatives, were found highly potent broad-spectrum antibacterial agents, still active again drug-resistant clinical isolates
- Innovative analytical and in silico techniques aimed at membrane-based MoA investigation were performed and fully validated

Teams working on AGUs

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"A teacher affects eternity; he can never tell where his influence stops" Henry B. Adams