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- 4 Cyclophostin and Cyclipostins analogs, new promising molecules to treat mycobacterial-

5 related diseases

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26 ABSTRACT

27 The progression of mycobacterial diseases requires the development of new therapeutics. Here, 28 we evaluated the efficacy and selectivity of a panel of Cyclophostin and Cyclipostins analogs 29 (CyCs) against various bacteria and mycobacteria. The activity of these 26 CyCs was first 30 assayed using the agar plate method. Compounds exhibiting a 50-100% growth inhibition rate 31 were then selected to determine their MIC using the REMA assay. The best drug candidate was 32 further tested against mycobacterial clinical isolates and bacteria responsible for nosocomial 33 infections. These included 6 Gram-negative, 5 Gram-positive bacteria, 29 rapid-growing 34 mycobacteria belonging to the M. chelonae-abscessus clade and 3 slow-growing mycobacteria 35 (Mycobacterium marinum, Mycobacterium bovis BCG, Mycobacterium tuberculosis). Among 36 the 26 CyCs tested, 10 were active and their inhibitory activity was exclusively restricted to 37 mycobacteria. The best candidate, CyC_{17} , further tested on the 26 clinical strains showed a high 38 selectivity for mycobacteria with MIC values (<2 up to 40 µg/mL) comparable to those of most 39 classical antibiotics used to treat M. abscessus infections. Together, these results support the 40 fact that such CyCs represent a new family of potent and selective inhibitors against 41 mycobacteria. This is of particular interest for future chemotherapeutic developments against 42 mycobacterial-associated infections, especially against M. abscessus the most drug-resistant 43 mycobacterial species.

44

45 KEYWORDS

46 Cyclipostins, Cyclophostin, anti-mycobacterial agents, drug susceptibility, *Mycobacterium*47 *abscessus*.

49 **1. Introduction**

50 The Mycobacterium genus comprises more than 200 species classified mainly on their 51 pathogenicity and growth rates. Mycobacteria can be separated into rapidly-growing 52 mycobacteria (RGM) that include saprophytic species and opportunistic species such as the M. abscessus complex, M. chelonae and M. fortuitum; and slow-growing mycobacteria (SGM) 53 54 comprising strict and opportunistic pathogens, such as the *M. tuberculosis* complex and the *M.* 55 avium complex, respectively [1]. Treatment of mycobacterial infections remains challenging, 56 essentially because of the presence of a complex lipid-rich cell wall [2], whose general composition and architecture is shared by both RGM and SGM and which contributes to its low 57 58 permeability to many antibiotics, thus limiting the therapeutic options. In addition, the treatment 59 duration is long, ranging from 6 to 9 months for tuberculosis and for up to 2 years for several 60 mycobacterial lung infections due to atypical mycobacteria, such as M. kansasii or M. 61 abscessus. The emergence of multi-drug resistant mycobacteria such as M. abscessus, or M. 62 tuberculosis isolates (responsible for MDR-TB) strongly impacts the treatment success rates with an increased incidence of treatment failure and death [3]. Another major issue relates to 63 64 the emergence of RGM-caused infections [4]. Among them, the difficult-to-manage M. abscessus complex represents one of the most drug-resistant for which standardized 65 66 chemotherapeutic regimens are still lacking [5]. Therefore, more efficient anti-mycobacterial 67 agents are needed.

Previously, we evaluated analogues of natural Cyclophostin and Cyclipostins (**CyCs**) for their activity against *M. tuberculosis* and demonstrated that they efficiently inhibited *M. tuberculosis* growing either extracellularly or within macrophages [6]. Of major importance, no cytotoxicity towards the host mammalian cells has been observed for these **CyC** compounds at concentrations up to 100 μ M (**Table 2**) [6]. Using competitive labelling/enrichment assays and mass spectrometry, 23 putative **CyC** targets were identified, all belonging to the serine/cysteine hydrolase family proteins known to play a role in the lifecycle and/or
pathogenesis of *M. tuberculosis* [7].

Herein, we examined the selectivity and activity of a set of 26 **CyC** analogs (**Figure S1**) against a variety of Gram-positive and Gram-negative bacteria. We also included a large panel of mycobacterial clinical isolates. Our results strongly support the potent and specific activity of **CyCs** against mycobacteria. These molecules constitute an unexploited chemical class of active compounds with promising translational development possibilities for the treatment of mycobacterial infections.

82

83 2. Materials and Methods

84 2.1. Synthesis of Cyclipostins and Cyclophostin (CyC) analogs.

The 26 **CyC** analogs were previously synthesized and obtained at 98% purity as reported in [8-10]. Stock solutions (4 mg/mL) of each **CyC** were prepared in dimethyl sulfoxide (DMSO) prior to susceptibility testing.

88 2.2. Bacterial strains and growth condition.

89 Six Gram-negative, 5 Gram-positive bacteria, 29 RGM (mainly clinical isolates) and 3 90 SGM were included in this study (Table 1). The clinical isolates were collected during the 91 epidemiological study performed in 2004 [11] and include representatives of the *M. chelonae*-92 abscessus clade (10 M. abscessus, 4 M. massiliense, 2 M. bolletii, and 10 M. chelonae strains) 93 obtained from either cystic fibrosis patients (CF isolates) or non-cystic fibrosis patients (non-CF isolates). M. smegmatis mc²155 strain was routinely grown in Middlebrook 7H9 broth (BD 94 95 Difco) supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma-Aldrich) (7H9-S). M. marinum ATCC BAA-535/M, M. bovis BCG Pasteur, M. abscessus CIP104536^T with either a 96 smooth (S) or rough (R) morphotype, and M. tuberculosis mc²6230 (H37Rv *ARD1 ApanCD* 97 98 [12]) strains were grown in this 7H9-S medium supplemented with 10% oleic acid, albumin,

dextrose, catalase (OADC enrichment; BD Difco) (7H9-S^{OADC}). In the case of *M. tuberculosis* 99 mc²6230, 24 µg/mL D-panthothenate (Sigma-Aldrich) was also added in the 7H9-S^{OADC} 100 medium. All cultures were kept at 37°C without shaking, except M. marinum which was grown 101 102 at 32°C. The 5 Gram-positive strains (i.e., Staphylococcus aureus, Enterococcus faecalis, 103 Streptococcus pneumoniae, Staphylococcus epidermidis, Enterococcus faecium) and the 6 104 Gram-negative strains (i.e., Escherichia coli, Pseudomonas aeruginosa, Enterobacter 105 aerogenes, Serratia marcescens, Stenotrophomonas maltophilia, Burkholderia cepacia 106 complex) were grown at 37°C in LB Broth Base medium (ThermoFisher Scientific).

107 2.3. Drug susceptibility testing on solid medium.

108 This was performed in 24-well suspension culture plates (Greiner bio-one) as described 109 in [13]. E. coli and P. aeruginosa were grown on LB agar medium at 37°C. All mycobacteria 110 were grown at either 32°C (*M. marinum*) or 37°C (*M. smegmatis*, *M. bovis* BCG, *M. abscessus* 111 S and R variants, and *M. tuberculosis* mc²6230) on Middlebrook 7H10 agar (BD Difco) 112 supplemented with 10% OADC and 24 μ g/mL D-panthothenate (*M. tuberculosis* mc²6230). 113 The wells were filled with 1 mL of the appropriate medium containing each of the CyC analogs 114 at a single 30 µM final concentration. Each screening plate contained negative (DMSO) and 115 positive (50 µM antibiotics) controls, as well as one well for sterility control (i.e., medium 116 alone). For the 100% inhibition control we used 50 μ M kanamycin (Sigma Aldrich) for M. 117 marinum, M. abscessus, M. smegmatis, M. bovis BCG, M. tuberculosis and E. coli; and 50 µM 118 carbenicillin (Sigma Aldrich) for P. aeruginosa. Each well was spotted with 10 µL of a bacterial culture at 5×10^5 cells/mL. Incubation time varied from 1 day to 2 weeks depending on the 119 120 strain tested. The CyC compounds leading to a minimum of 50% growth inhibition were 121 selected for subsequent minimal inhibitory concentrations (MIC) determination using the 122 REMA assay.

123 2.4. Resazurin microtiter assay (REMA) for MIC determination.

124 Susceptibility testing was performed using the Middlebrook 7H9 broth microdilution 125 method. All assays for each strain were carried out at least in triplicate. MICs of the CyCs, 126 selected from solid medium screening against the various bacterial strains, were determined in 127 96-well flat-bottom Nunclon Delta Surface microplates with lid (ThermoFisher Scientific, ref. 128 167008) using the resazurin microtiter assay (REMA [14, 15]). Briefly, log-phase bacteria were 129 diluted to a cell density of 5×10^6 cells/mL in 7H9-S^{OADC} (7H9 broth + 10% OADC + 0.2% 130 glycerol + 0.05% Tween 80, and 24 μ g/mL D-panthothenate when needed). Then 100 μ L of the above inoculum (*i.e.*, 5×10^5 cells per well) was added to each well containing 100 µL 7H9-131 S^{OADC} medium, serial two-fold dilutions of the selected \mbox{CyC} analog or controls to a final 132 133 volume of 200 μ L. Growth controls containing no inhibitor (*i.e.*, bacteria only = *B*), inhibition 134 controls containing 50 μ g/mL kanamycin and sterility controls (*i.e.*, medium only = M) without 135 inoculation were also included. Plates were incubated at 37°C (32°C for M. marinum) in a 136 humidity chamber [16] to prevent evaporation for either 3-5 days (*M. smegmatis*, *M. abscessus*) or 10-14 days (M. marinum, M. bovis BCG, M. tuberculosis mc²6230). Then, 20 µL of a 0.025% 137 138 (w/v) resazurin solution was added to each well, and the plates were incubated at 37°C for color 139 change from blue to pink or violet and for a reading of fluorescence units (FU). Fluorescence 140 corresponding to the resazurin reduction to its metabolite resorufin was quantified using a 141 Tecan Spark 10M multimode microplate reader (Tecan Group Ltd, France) with excitation at 142 530 nm and emission at 590 nm. For fluorometric MIC determinations, a background 143 subtraction was performed on all wells with a mean of M wells. Relative fluorescence units 144 were defined as: RFU% = (test well FU/mean FU of B wells) \times 100. MIC values were 145 determined by fitting the RFU% sigmoidal dose-response curves [15] in Kaleidagraph 4.2 146 software (Synergy Software). The lowest drug concentrations inhibiting 90% of growth were defined as the MIC₉₀. Isoniazid (INH), amikacin (AMK), imipenem (IPM) and cefoxitin (FOX) 147 148 were used as reference drugs.

149 **3. Results and Discussion**

150 3.1. Screen of CyCs susceptibility testing on solid medium

151 To explore the efficacy and the antibacterial spectrum of the CyC analogues, a 152 preliminary screen on solid medium was first performed with the each of the 26 CyCs at a fixed 153 30 µM final concentration using a selected panel consisting of 6 mycobacterial species (*i.e.*, *M*. 154 smegmatis, M. marinum, M. abscessus R and S, M. bovis BCG and M. tuberculosis mc²6230) 155 and 2 Gram-negative bacteria (i.e., Escherichia coli and Pseudomonas aeruginosa). After a 156 period of incubation (from 1 day to 2 weeks), 10 out of the 26 CyCs were found to inhibit 157 growth in the range of 50-100% relative to the positive growth control (i.e., bacteria without 158 antibiotics). Remarkably, this effect was restricted to mycobacteria only (see Table S1), while 159 under the same conditions, the growth of E. coli and P. aeruginosa was not impacted.

160

161 *3.2. MIC determination*

162 The potency of the selected $CyC_{7(\alpha,\beta)}$, $CyC_{8(\alpha,\beta)}$, $CyC_{9(\beta)}$, $CyC_{10(\alpha)}$, CyC_{11} , CyC_{17} and 163 $CyC_{17(\alpha,\beta)}$ was next confirmed by determining their MICs towards each respective 164 mycobacterial strain, using the REMA assay [14, 15] (Table 2). Nearly all selected CyCs were 165 active against *M. marinum* and *M. bovis* BCG growth with moderate (4.2-25.9 µg/mL) to good $(0.6-2.2 \,\mu g/mL)$ MIC₉₀. Moreover, the obtained MICs against *M. tuberculosis* mc²6230 were 166 167 consistent with those recently reported against *M. tuberculosis* H37Rv extracellular growth [6]. 168 Among the 10 CyCs tested, only CyC_{18(β)} and CyC₁₇ inhibited the growth of all mycobacteria 169 investigated with MICs ranging from 0.18 to 11.2 µg/mL (Table 2). Of importance, CyC₁₇ was 170 also highly active against both R and S variants of M. abscessus with a lower MIC (0.18 µg/mL 171 and 6.4 µg/mL against *M. abscessus* R and S, respectively) than AMK, IMP and FOX used as 172 reference drugs (**Table 2**) as well as most conventional antibiotics used in clinical settings [17].

174 3.3. Potency and selectivity of the best CyC_{17} inhibitor against clinical isolates.

175 The **CyC**₁₇ efficiency/selectivity was investigated deeper by testing its activity towards 176 26 clinical isolates belonging to the M. chelonae-abscessus clade and several Gram-negative 177 and Gram-positive bacterial species (Table 3). As anticipated, CyC17 was only active against 178 M. abscessus and M. chelonae isolates (Table 3). Of interest, MIC₅₀ values for M. abscessus 179 and *M. chelonae* (10 and 40 μ g/mL, respectively) were comparable to those of amikacin (12.5 180 μg/mL) [18], cefoxitin (32 μg/mL) or imipenem (16 μg/mL) [19]. M. abscessus complex 181 isolates were also more sensitive than M. chelonae isolates, with M. bolletii appearing as the 182 most susceptible organism (Table 3).

Overall, these results confirm the potential of CyCs as promising anti-mycobacterial candidates. This selective activity against mycobacteria might be related to the cell envelope composition [2] which is unique, and/or to the increased ability of these hydrophobic compounds to cross this lipid-rich cell wall barrier. This hypothesis is relevant with the recent identification of potential targets for CyC_{17} which are mostly involved in *M. tuberculosis* lipid metabolism and/or in cell wall biosynthesis, such as the Antigen 85 complex playing key role in mycolic acid metabolism, which is restricted to mycobacteria [6].

190

191 4. Conclusion

Taking into account all these results, we thus propose the **CyCs** analogs to be considered as selective inhibitors of mycobacteria and attractive candidates to be further exploited in the fight against mycobacterial-related diseases. Additionally, **CyC**₁₇ was found to be active against the multi-resistant species of the *M. abscessus* complex. Further work to decipher the physiological target(s) of **CyC**₁₇ and to elucidate its mode of action in *M. abscessus* is currently in progress.

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211	Competing interest
212	None.
213	
214	Ethical approval
215	Not required.
216	
217	Supplementary data
218	Supplementary data associated with this article can be found in the online version.
219	
220	

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Table 1.

280 Bacterial strains used in the study.

Species	Bacterial strains	Source
Gram negative bacteria		
Escherichia coli DH10B	Reference strain	
Escherichia coli	Clinical isolate	[11]
Pseudomonas aeruginosa PA01	Reference strain	
Pseudomonas aeruginosa	Clinical isolate	[11]
Enterobacter aerogenes	Clinical isolate	[11]
Serratia marcescens	Clinical isolate	[11]
Stenotrophomonas maltophilia	Clinical isolate	[11]
Burkholderia cepacia complex	Clinical isolate	[11]
Gram positive bacteria		
Staphylococcus aureus	Clinical isolate	[11]
Enterococcus faecalis	Clinical isolate	[11]
Streptococcus pneumoniae	Clinical isolate	[11]
Staphylococcus epidermidis	Clinical isolate	[11]
Enterococcus faecium	Clinical isolate	[11]
Mycobacteria		
<i>M. bovis</i> BCG Pasteur	Reference strain	
<i>M. tuberculosis</i> $mc^{2}6230$	Reference strain	[12]
M. marinum M	Reference strain	[]
M. smegmatis mc ² 155	Reference strain	
<i>M. abscessus</i> CIP 104536^{T}	Reference strain	
M. abscessus (RPC95)	CF isolate	[11]
M. abscessus (RPC96)	CF isolate	[11]
M. abscessus (RPC98)	CF isolate	[11]
M. abscessus (RPC101)	CF isolate	[11]
M. abscessus (RPC102)	CF isolate	[11]
M. abscessus (RPC104)	CF isolate	[11]
M. abscessus (RPC105)	CF isolate	[11]
M. abscessus (RPC106)	CF isolate	[11]
M. abscessus (RPC109)	CF isolate	[11]
M. abscessus (RPC110)	CF isolate	[11]
M. massiliense (RPC99)	CF isolate	[11]
M. massiliense (RPC100)	CF isolate	[11]
M. massiliense (RPC107)	CF isolate	[11]
M. massiliense (RPC103)	CF isolate	[11]
M. bolletii (RPC97)	CF isolate	[11]
M. bolletii (RPC108)	CF isolate	[11]
M. chelonae (RPC128)	CF isolate	[11]
M. chelonae (RPC129)	CF isolate	[11]
M. chelonae (RPC130)	CF isolate	[11]
M. chelonae (RPC131)	Non-CF isolate	[11]
M. chelonae (RPC132)	Non-CF isolate	[11]
M. chelonae (RPC057)	Non-CF isolate	[11]

M. chelonae (RPC059)	Non-CF isolate	[11]
M. chelonae (RPC061)	Non-CF isolate	[11]
M. chelonae (RPC063)	Non-CF isolate	[11]
M. chelonae (RPC066)	Non-CF isolate	[11]

Table 2.
Antibacterial activities of the selected active CyC compounds as compared to four standard
antimicrobial agents against a first panel of mycobacterial strains
287

	$MIC_{90} (\mu g/mL)^{a}$						
Compounds	M. smegmatis	<i>M. abscessus</i> CIP 104536 ^T		M. marinum	M. bovis BCG	M. tuberculosis	$\frac{\text{CC}_{50}}{(\mu g/\text{mL})}$
	mc ⁻ 155	Smooth	Rough			IIIC 0250	
CyC _{7(a)}				11.8	25.9		>40
$CyC_{7(\beta)}$				2.2	17.7	13.9	>40
$CyC_{8(\alpha)}$				0.63	8.5	18.6	>40
$CyC_{8(\beta)}$				4.2	9.3		>10
$CyC_{9(\beta)}$				7.8	26.2		>45
$CyC_{10(\alpha)}$				11.4			>50
CyC ₁₁				9.0			>45
CyC ₁₇	0.81	6.4	0.18	0.74	0.58	1.2	>45
$CyC_{18(\alpha)}$				7.8	9.6	19.4	>45
$CyC_{18(\beta)}$	6.7	6.0	4.9	11.2	11.5	2.6	>45
INH	5.6			9.3	0.10	0.15	>20
AMK		4.7	7.9	1.7	0.48	0.63	
IPM		1.9	8.9				
FOX		4.0	12.0				

^{*a*} MIC₉₀ corresponding to the concentration leading to 90% growth inhibition as determined by the REMA assay, are expressed as mean values of two independent assays performed in triplicate (CV% < 5%). ^{*b*} CC₅₀: compound concentration leading to 50% cell toxicity, determined on murine (raw264.7) macrophages – data from [6]. INH: isoniazid; AMK: amikacin; IPM: imipenem; FOX: cefoxitin.

293

296 **Table 3**.

- 297 MICs (in µg/mL) of CyC17 on a panel of *M. abscessus* and *M. chelonae* clinical strains isolated
- 298 from cystic fibrosis and non-cystic fibrosis patients ^{*a*}
- 299

Clinical isolates	N° of strains with indicated MIC							
(number of strains)	< 2	5	10	20	40	80	MIC ₅₀	MIC ₉₀
Mycobacteria								
All (26)	2	2	8	5	4	5	10	80
M. abscessus (10)	1	2	3	3	1		10	40
M. massiliense (4)			2		2		10	10
M. bolletii (2)	1		1				<2	10
M. chelonae (10)			2	2	1	5	40	80
Gram-negative bacteria								
Escherichia coli							>820	
Pseudomonas aeruginosa							>820	
Serratia marcescens							>820	
Stenotrophomonas maltophilia							>820	
Burkholderia cepacia complex							>820	
Gram-positive bacteria								
Staphylococcus aureus							>820	
Staphylococcus epidermidis							>820	
Streptococcus pneumoniae							>820	
Enterobacter aerogenes							>820	
Enterococcus faecalis							>820	
Enterococcus faecium							>820	

300 ^a MICs are expressed as mean values of triplicate. Respective MIC₅₀ / MIC₉₀ values of standard

301 antimicrobial agents against the *M. chelonae* – *M. abscessus* clinical isolates: imipenem $16/32 \mu g/mL$

302 and cefoxitin $32 / 64 \mu g/mL$ [19].