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

5 **related diseases**

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24

ABSTRACT

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

KEYWORDS

Cyclipostins, Cyclophostin, anti-mycobacterial agents, drug susceptibility, *Mycobacterium 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.*
53 *abscessus* complex, *M. chelonae* and *M. fortuitum*; and slow-growing mycobacteria (SGM)
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
57 composition and architecture is shared by both RGM and SGM and which contributes to its low
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
63 with an increased incidence of treatment failure and death [3]. Another major issue relates to
64 the emergence of RGM-caused infections [4]. Among them, the difficult-to-manage *M.*
65 *abscessus* complex represents one of the most drug-resistant for which standardized
66 chemotherapeutic regimens are still lacking [5]. Therefore, more efficient anti-mycobacterial
67 agents are needed.

68 Previously, we evaluated analogues of natural Cyclophostin and Cyclipostins (**CyCs**) for
69 their activity against *M. tuberculosis* and demonstrated that they efficiently inhibited *M.*
70 *tuberculosis* growing either extracellularly or within macrophages [6]. Of major importance,
71 no cytotoxicity towards the host mammalian cells has been observed for these **CyC** compounds
72 at concentrations up to 100 µM (**Table 2**) [6]. Using competitive labelling/enrichment assays
73 and mass spectrometry, 23 putative **CyC** targets were identified, all belonging to the

74 serine/cysteine hydrolase family proteins known to play a role in the lifecycle and/or
75 pathogenesis of *M. tuberculosis* [7].
76 Herein, we examined the selectivity and activity of a set of 26 **CyC** analogs (**Figure S1**)
77 against a variety of Gram-positive and Gram-negative bacteria. We also included a large panel
78 of mycobacterial clinical isolates. Our results strongly support the potent and specific activity
79 of **CyCs** against mycobacteria. These molecules constitute an unexploited chemical class of
80 active compounds with promising translational development possibilities for the treatment of
81 mycobacterial infections.
82

83 **2. Materials and Methods**

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

85 The 26 **CyC** analogs were previously synthesized and obtained at 98% purity as reported
86 in [8-10]. Stock solutions (4 mg/mL) of each **CyC** were prepared in dimethyl sulfoxide
87 (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-
94 CF isolates). *M. smegmatis* mc^{2}155 strain was routinely grown in Middlebrook 7H9 broth (BD
95 Difco) supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma-Aldrich) (7H9-S). *M.*
96 *marinum* ATCC BAA-535/M, *M. bovis* BCG Pasteur, *M. abscessus* CIP104536^T with either a
97 smooth (S) or rough (R) morphotype, and *M. tuberculosis* mc^{2}6230 (H37Rv *ΔRD1 ΔpanCD*
98 [12]) strains were grown in this 7H9-S medium supplemented with 10% oleic acid, albumin,

99 dextrose, catalase (OADC enrichment; BD Difco) (7H9-S^{OADC}). In the case of *M. tuberculosis*
100 mc^{2}6230, 24 µg/mL D-panthothenate (Sigma-Aldrich) was also added in the 7H9-S^{OADC}
101 medium. All cultures were kept at 37°C without shaking, except *M. marinum* which was grown
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* mc^{2}6230) on Middlebrook 7H10 agar (BD Difco)
112 supplemented with 10% OADC and 24 µg/mL D-panthothenate (*M. tuberculosis* mc^{2}6230).
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 µM 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
119 culture at 5×10^5 cells/mL. Incubation time varied from 1 day to 2 weeks depending on the
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 µg/mL D-panthothenate when needed). Then 100 µL of
131 the above inoculum (*i.e.*, 5×10^5 cells per well) was added to each well containing 100 µL 7H9-
132 S^{OADC} medium, serial two-fold dilutions of the selected **CyC** analog or controls to a final
133 volume of 200 µL. Growth controls containing no inhibitor (*i.e.*, bacteria only = *B*), inhibition
134 controls containing 50 µg/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*)
137 or 10-14 days (*M. marinum*, *M. bovis* BCG, *M. tuberculosis* $mc^2$6230). Then, 20 µL of a 0.025%
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
147 defined as the MIC_{90}. Isoniazid (INH), amikacin (AMK), imipenem (IPM) and cefoxitin (FOX)
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* mc^{2}6230)
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
166 (0.6-2.2 µg/mL) MIC$_{90}$. Moreover, the obtained MICs against *M. tuberculosis* mc^{2}6230 were
167 consistent with those recently reported against *M. tuberculosis* H37Rv extracellular growth [6].
168 Among the 10 **CyCs** tested, only **CyC$_{18(\beta)}$** and **CyC$_{17}$** inhibited the growth of all mycobacteria
169 investigated with MICs ranging from 0.18 to 11.2 µg/mL (**Table 2**). Of importance, **CyC$_{17}$** 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].

173

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, **CyC_{17}** was only active against
178 *M. abscessus* and *M. chelonae* isolates (**Table 3**). Of interest, MIC_{50} values for *M. abscessus*
179 and *M. chelonae* (10 and 40 µg/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**).

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

190

191 **4. Conclusion**

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

198

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Competing interest

None.

Ethical approval

Not required.

Supplementary data

Supplementary data associated with this article can be found in the online version.

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277

278

279 **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		
M. bovis BCG Pasteur	Reference strain	
M. tuberculosis $mc^2$6230	Reference strain	[12]
M. marinum M	Reference strain	
M. smegmatis $mc^2$155	Reference strain	
M. abscessus 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]

281

282

Table 2.

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

Compounds	MIC$_{90}$ (µg/mL) [a]						CC$_{50}$ [b] (µg/mL)
	M. smegmatis mc^{2}155	*M. abscessus* CIP 104536^T		*M. marinum*	*M. bovis* BCG	*M. tuberculosis* mc^{2}6230	
		Smooth	Rough				
CyC$_{7(\alpha)}$				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] MIC$_{90}$ 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] CC$_{50}$: compound concentration leading to 50% cell toxicity, determined on murine (raw264.7) macrophages – data from [6]. INH: isoniazid; AMK: amikacin; IPM: imipenem; FOX: cefoxitin.

Table 3.

MICs (in µg/mL) of **CyC$_{17}$** on a panel of *M. abscessus* and *M. chelonae* clinical strains isolated from cystic fibrosis and non-cystic fibrosis patients [a]

Clinical isolates (number of strains)	N° of strains with indicated MIC							
	< 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	

[a] MICs are expressed as mean values of triplicate. Respective MIC$_{50}$ / MIC$_{90}$ values of standard antimicrobial agents against the *M. chelonae* – *M. abscessus* clinical isolates: imipenem 16 / 32 µg/mL and cefoxitin 32 / 64 µg/mL [19].