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

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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 (CyCs) 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, CyC₁₇, 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*.

1. Introduction

The *Mycobacterium* genus comprises more than 200 species classified mainly on their pathogenicity and growth rates. Mycobacteria can be separated into rapidly-growing 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) comprising strict and opportunistic pathogens, such as the *M. tuberculosis* complex and the *M. avium* complex, respectively [1]. Treatment of mycobacterial infections remains challenging, 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 permeability to many antibiotics, thus limiting the therapeutic options. In addition, the treatment duration is long, ranging from 6 to 9 months for tuberculosis and for up to 2 years for several mycobacterial lung infections due to atypical mycobacteria, such as *M. kansasii* or *M. abscessus*. The emergence of multi-drug resistant mycobacteria such as *M. abscessus*, or *M. 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 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 chemotherapeutic regimens are still lacking [5]. Therefore, more efficient anti-mycobacterial 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.

2. Materials and Methods

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.

2.2. Bacterial strains and growth condition.

Six Gram-negative, 5 Gram-positive bacteria, 29 RGM (mainly clinical isolates) and 3 SGM were included in this study (**Table 1**). The clinical isolates were collected during the epidemiological study performed in 2004 [11] and include representatives of the *M. chelonae-abscessus* clade (10 *M. abscessus*, 4 *M. massiliense*, 2 *M. bolletii*, and 10 *M. chelonae* strains) 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 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 smooth (S) or rough (R) morphotype, and *M. tuberculosis* mc²6230 (H37Rv Δ RD1 Δ panCD [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* mc²6230, 24 µg/mL D-panthothenate (Sigma-Aldrich) was also added in the 7H9-S^{OADC} medium. All cultures were kept at 37°C without shaking, except *M. marinum* which was grown at 32°C. The 5 Gram-positive strains (*i.e.*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Enterococcus faecium*) and the 6 Gram-negative strains (*i.e.*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Serratia marcescens*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex) were grown at 37°C in LB Broth Base medium (ThermoFisher Scientific).

2.3. Drug susceptibility testing on solid medium.

This was performed in 24-well suspension culture plates (Greiner bio-one) as described in [13]. *E. coli* and *P. aeruginosa* were grown on LB agar medium at 37°C. All mycobacteria were grown at either 32°C (*M. marinum*) or 37°C (*M. smegmatis*, *M. bovis* BCG, *M. abscessus* S and R variants, and *M. tuberculosis* mc²6230) on Middlebrook 7H10 agar (BD Difco) supplemented with 10% OADC and 24 µg/mL D-panthothenate (*M. tuberculosis* mc²6230). The wells were filled with 1 mL of the appropriate medium containing each of the **CyC** analogs at a single 30 µM final concentration. Each screening plate contained negative (DMSO) and positive (50 µM antibiotics) controls, as well as one well for sterility control (*i.e.*, medium alone). For the 100% inhibition control we used 50 µM kanamycin (Sigma Aldrich) for *M. marinum*, *M. abscessus*, *M. smegmatis*, *M. bovis* BCG, *M. tuberculosis* and *E. coli*; and 50 µM 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 strain tested. The **CyC** compounds leading to a minimum of 50% growth inhibition were selected for subsequent minimal inhibitory concentrations (MIC) determination using the REMA assay.

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

Susceptibility testing was performed using the Middlebrook 7H9 broth microdilution method. All assays for each strain were carried out at least in triplicate. MICs of the **CyCs**, selected from solid medium screening against the various bacterial strains, were determined in 96-well flat-bottom Nunclon Delta Surface microplates with lid (ThermoFisher Scientific, ref. 167008) using the resazurin microtiter assay (REMA [14, 15]). Briefly, log-phase bacteria were diluted to a cell density of 5×10^6 cells/mL in 7H9-S^{OADC} (7H9 broth + 10% OADC + 0.2% glycerol + 0.05% Tween 80, and 24 µg/mL D-panthothenate when needed). Then 100 µL of the above inoculum (*i.e.*, 5×10^5 cells per well) was added to each well containing 100 µL 7H9-S^{OADC} medium, serial two-fold dilutions of the selected **CyC** analog or controls to a final volume of 200 µL. Growth controls containing no inhibitor (*i.e.*, bacteria only = B), inhibition controls containing 50 µg/mL kanamycin and sterility controls (*i.e.*, medium only = M) without inoculation were also included. Plates were incubated at 37°C (32°C for *M. marinum*) in a 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% (*w/v*) resazurin solution was added to each well, and the plates were incubated at 37°C for color change from blue to pink or violet and for a reading of fluorescence units (FU). Fluorescence corresponding to the resazurin reduction to its metabolite resorufin was quantified using a Tecan Spark 10M multimode microplate reader (Tecan Group Ltd, France) with excitation at 530 nm and emission at 590 nm. For fluorometric MIC determinations, a background subtraction was performed on all wells with a mean of M wells. Relative fluorescence units were defined as: RFU% = (test well FU/mean FU of B wells) × 100. MIC values were determined by fitting the RFU% sigmoidal dose-response curves [15] in Kaleidagraph 4.2 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) were used as reference drugs.

3. Results and Discussion

3.1. Screen of CyCs susceptibility testing on solid medium

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

3.2. MIC determination

The potency of the selected CyC_{7(α,β)}, CyC_{8(α,β)}, CyC_{9(β)}, CyC_{10(α)}, CyC₁₁, CyC₁₇ and CyC_{17(α,β)} was next confirmed by determining their MICs towards each respective mycobacterial strain, using the REMA assay [14, 15] (**Table 2**). Nearly all selected CyCs were active against *M. marinum* and *M. bovis* BCG growth with moderate (4.2-25.9 µg/mL) to good (0.6-2.2 µg/mL) MIC₉₀. Moreover, the obtained MICs against *M. tuberculosis* mc²6230 were consistent with those recently reported against *M. tuberculosis* H37Rv extracellular growth [6]. Among the 10 CyCs tested, only CyC_{18(β)} and CyC₁₇ inhibited the growth of all mycobacteria investigated with MICs ranging from 0.18 to 11.2 µg/mL (**Table 2**). Of importance, CyC₁₇ was also highly active against both R and S variants of *M. abscessus* with a lower MIC (0.18 µg/mL and 6.4 µg/mL against *M. abscessus* R and S, respectively) than AMK, IMP and FOX used as reference drugs (**Table 2**) as well as most conventional antibiotics used in clinical settings [17].

3.3. Potency and selectivity of the best **CyC₁₇** inhibitor against clinical isolates.

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

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, **CyC₁₇** was found to be active against the multi-resistant species of the *M. abscessus* complex. Further work to decipher the physiological target(s) of **CyC₁₇** and to elucidate its mode of action in *M. abscessus* is currently in progress.

199

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211 **Competing interest**

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215 Not required.

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217 **Supplementary data**

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

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279 **Table 1.**

280 Bacterial strains used in the study.

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

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

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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)
	<i>M. smegmatis</i> mc ² 155	<i>M. abscessus</i> CIP 104536 ^T		<i>M. marinum</i>	<i>M. bovis</i> BCG	<i>M. tuberculosis</i> mc ² 6230	
		Smooth	Rough				
CyC_{7(α)}				11.8	25.9		>40
CyC_{7(β)}				2.2	17.7	13.9	>40
CyC_{8(α)}				0.63	8.5	18.6	>40
CyC_{8(β)}				4.2	9.3		>10
CyC_{9(β)}				7.8	26.2		>45
CyC_{10(α)}				11.4			>50
CyC₁₁				9.0			>45
CyC₁₇	0.81	6.4	0.18	0.74	0.58	1.2	>45
CyC_{18(α)}				7.8	9.6	19.4	>45
CyC_{18(β)}	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.

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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

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Clinical isolates (number of strains)	N° of strains with indicated MIC						MIC ₅₀	MIC ₉₀
	< 2	5	10	20	40	80		
Mycobacteria								
All (26)	2	2	8	5	4	5	10	80
<i>M. abscessus</i> (10)	1	2	3	3	1		10	40
<i>M. massiliense</i> (4)			2		2		10	10
<i>M. bolletii</i> (2)	1		1				<2	10
<i>M. chelonae</i> (10)			2	2	1	5	40	80
Gram-negative bacteria								
<i>Escherichia coli</i>							>820	
<i>Pseudomonas aeruginosa</i>							>820	
<i>Serratia marcescens</i>							>820	
<i>Stenotrophomonas maltophilia</i>							>820	
<i>Burkholderia cepacia complex</i>							>820	
Gram-positive bacteria								
<i>Staphylococcus aureus</i>							>820	
<i>Staphylococcus epidermidis</i>							>820	
<i>Streptococcus pneumoniae</i>							>820	
<i>Enterobacter aerogenes</i>							>820	
<i>Enterococcus faecalis</i>							>820	
<i>Enterococcus faecium</i>							>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 µg/mL
 302 and ceftazidime 32 / 64 µg/mL [19].

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