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ARTICLE

In vitro and ex vivo activity of the fluoroquinolone DC-159a against mycobacteria

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Abstract

Antimicrobial resistance is a global health problem. In 2021, it was estimated almost half a million of multidrug-resistant tuberculosis (MDR-TB) cases. Besides, non-tuberculous mycobacteria (NTM) are highly resistant to several drugs and the emergence of fluoroquinolone (FQ) resistant *M. tuberculosis* (*Mtb*) is also a global concern making treatments difficult and with variable outcome. The aim of this study was to evaluate the activity of the FQ, DC-159a, against *Mtb* and NTM and to explore the cross-resistance with the currently used FQs.

A total of 12 pre-extensively drug-resistant (XDR) *Mtb*, 2 XDR, 36 fully drug susceptible strains and 41 NTM isolates were included to estimate the in vitro activity of DC-159a, moxifloxacin (MOX) and levofloxacin (LX), using minimal inhibitory and bactericidal concentration (MIC and MBC). The activity inside the human macrophages and pulmonary epithelial cells were also determined.

DC-159a was active in vitro and ex vivo against mycobacteria. Besides, it was more active than MOX/LX. Moreover, no cross-resistance was evidenced between DC-159a and LX/MOX as DC-159a could inhibit *Mtb* and MAC strains that were already resistant to LX/MOX.

DC-159a could be a possible candidate in new therapeutic regimens for MDR/ XDR-TB and mycobacterioses cases.

Introduction

Antimicrobial resistance (AMR) is a public health concern worldwide and it could become the leading cause of death [1]. In 2019, it was estimated that approximately 500,000 multidrug-resistant tuberculosis (MDR-TB) cases, caused by *Mycobacterium tuberculosis* (*Mtb*) were resistant to isoniazid and rifampicin (RIF) [2].

World Health Organization defined the extensively drug-resistant TB (XDR-TB) [3, 4] in 2006 and it was re-defined in

2020. Pre-XDR-TB is the MDR/RR-TB (RIF resistant-TB) also resistant to any fluoroquinolone (FQ) such as levofloxacin (LX) and moxifloxacin (MOX) which are the FQs recommended for MDR-TB treatment. XDR-TB are those MDR-TB that are resistant to any FQ, bedaquiline and/or linezolid [2]. The emergence of FQ resistance (FQ-R) makes currently approved FQs inadequate to treat pre-XDR-TB/ XDR-TB [5]. Bactericidal activity differs greatly among FQs and cross-resistance among them has been demonstrated [6].

FQ-R occurs mainly due to mutations in *gyrA* and *gyrB* genes that code for the enzyme DNA gyrase [7–9]. Point mutations in the quinolone-resistant determining region of *gyrA* gene, mainly mutations in codons 90 and 94, are the most important molecular mechanism described for FQ-R in clinical isolates [6].

Non-tuberculous mycobacteria (NTM) group all mycobacteria but *Mtb* and *M. leprae*.

NTM can be found in the environment and more than 180 species with different capability to cause disease generally named mycobacterioses have been identified. NTM can cause almost 30% of extra-pulmonary disease [10–15] and they are emerging pathogens associated with HIV co-infection, affecting not only immunocompromised but also immunocompetent patients [16]. Members of the *M. avium*

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Fig. 1 Structures of FQs. this figure shows the structures of the three fluoroquinolones included in this study, DC-159a, LX: levofloxacin (LX) and moxifloxacin (MOX)
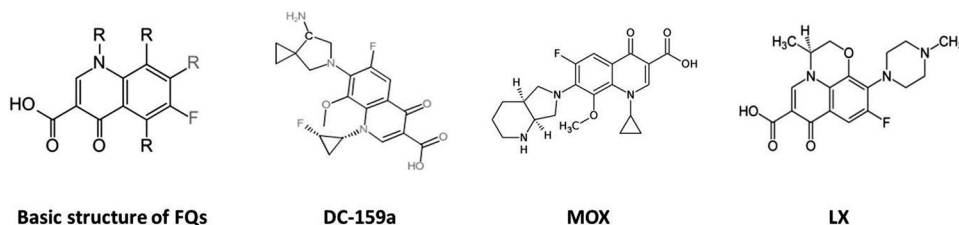
complex (MAC) are the main etiological agents of mycobacterioses [17–20] and *M. avium sp. hominissuis* (MAH) and *M. intracellulare* (MAI) are the leading members causing disseminated or pulmonary disease in humans [21–24]. The worldwide incidence and prevalence of mycobacterioses remain unknown [16]. Nevertheless, the global burden of these diseases is increasing worldwide [25, 26]. In Argentina, around 6% of pulmonary disease caused by mycobacteria had a NTM as etiological agent [19]. NTM treatment is difficult, not well standardized and with variable outcomes therefore it is necessary to find alternative drugs with bactericidal effect [27, 28].

DC-159a is a broad-spectrum 8-methoxy FQ with a potent antimicrobial activity against *Streptococcus* spp., *Staphylococcus* spp. *and Salmonella* spp. as well as against *Mtb*, *M. leprae* and NTM [29–32]. It is active against *Mtb* carrying mutations in *gyrA* gene, and therefore DC-159a may be useful in Pre-XDR/XDR-TB [6, 33–36]. Moreover, the structural characteristic of DC-159a suggests a reduced likelihood of adverse effects [31]. Therefore, DC-159a could be a promising therapeutic tool for the treatment of TB and mycobacterioses [30, 31].

Mycobacteria are intracellular pathogens that cause mainly pulmonary diseases. Once inhaled, they primarily infect alveolar macrophages (Mϕ) but also infect and adhere to lung epithelial cells. Upon infection, cells can kill intracellular mycobacteria or become a niche for bacterial growth. Infected cells produce pro-inflammatory cytokines, growth factors, and chemokines that recruit and activate fresh phagocytes to the site of infection controlling pathogen growth [37–39]. Given that Mϕ and pulmonary epithelial cells initiate host immune response, it becomes relevant to study the effect of anti-mycobacterial drugs inside infected cells. Therefore, the aim of this study was to evaluate the in vitro and ex vivo activity of DC-159a against *Mtb* and NTM strains and to explore the degree of cross-resistance with the currently used FQs.

Materials and methods

Drugs

MOX, LX, and DC-159a were included. DC-159a was provided by Daiichi Sankyo Co., Ltd. (Tokyo, Japan).

MOX and LX were purchased from Sigma Aldrich (Merck, Buenos Aires, Argentina). The chemical structures of these FQs are shown in Fig. 1.

Mycobacterial isolates

A total of 41 NTM isolates (21 MAH, 20 MAI) and 50 *Mtb* strains (36 susceptible TB (S-TB)), 12 pre-XDR: MDR plus LX-R (n: 2), MDR plus LX-R and MOX-R (n: 6), MDR plus LX-R and ofloxacin-R (n: 2), MDR plus linezolid-R (n: 2), 2 XDR (MDR plus LX-R and linezolid-R), were included in the study.

These strains were isolated in the Reference Laboratory of TB Control Program from patients living in the Northern region of Buenos Aires Province that received medical attention at Dr. Cetrangolo Hospital between 2014 and 2018. *Mtb* H37Rv ATCC 27294, *M. bovis* BCG, and *M. avium avium* D4 (avian PPD strain) were included as reference strains.

Genotyping of mycobacterial isolates

Isolates were genotyped to verify the diversity among them. Spoligotyping was used for genotyping *Mtb* as described van Embden et al. [40, 41].

Related *Mtb* isolates were grouped in genetic clades (spoligotyping families, SPOF) according to MIRU-VNTRplus database (https://www.miru-vntrplus.org/).

Genotyping of MAC was performed through MIRU-VNTR of eight loci (292, X3, 25, 47, 3, 7, 10, 32), according to the previously described protocol [21, 42].

The genotype pattern (INMV) was determined using the MAC-INMV database (http://mac-inmv.tours.inra.fr/index.php?p=nomenclature).

In vitro activity of DC-159a

Minimal inhibitory concentration (MIC)

In vitro mycobacterial susceptibility to DC-159a as well as cross-resistance with MOX and LX was estimated through MIC determination by the microplate colorimetric method using resazurin as redox indicator [6].

a_1) *Preparation of drugs*: stock solutions ($10\,000\,\mu g\,ml^{-1}$) were prepared for each drug. These solutions were aliquoted and frozen until use, no longer than 3 months. For *Mtb* and MAC strains, the drugs were tested in wide range of $32.00–0.03\,\mu g\,ml^{-1}$.

a_2) *Inoculums preparation*: mycobacterial suspension with turbidity comparable to 1Mc Farland solution ($10^6–10^8\,CFU\,ml^{-1}$, colony forming units per milliliter) was prepared (suspension Y). A 1/25 dilution of suspension Y in Middlebrook 7H9 (M7H9) supplemented with OADC (BD; Argentina) (suspension Z) was used to load the plates.

a_3) *Preparation of the plates*: flat-bottom 96 well plates were prepared as previously described [43, 44], Supplementary Fig. 1. Briefly, each well from rows A and H were filled with $200\,\mu l$ of sterile water to avoid desiccation during incubation. In column 1, well B (sterile control) was filled with $100\,\mu l$ of M7H9/OADC and wells C to G (growth controls) were filled with $100\,\mu l$ of M7H9/OADC plus $100\,\mu l$ of suspension Z. In other columns, $100\,\mu l$ of M7H9/ OADC were added. Then, $100\,\mu l$ of the drug solution was added and two-fold serial dilutions were performed. The wells were inoculated with $100\,\mu l$ of suspension Z.

Inoculated plates covered with lids, sealed and light protected were incubated at $37\,^\circ C$. At day 5, $30.0\,\mu l$ of resazurin was added to one growth control well and the plate incubated for 24 h. If well color did not change, this process was repeated in another growth control well until bacterial growth was detected. Finally, all wells were filled with resazurin and incubated for further 24 h for the final reading. Experiments were carried out in triplicate for each strain.

MIC was defined as the lowest drug concentration that completely inhibited all visible mycobacterial growth and it was evidenced by the absence of color change of resazurin in the respective well [21, 43–45]. Besides, MIC_{50} and MIC_{90} were defined as the MIC that inhibited 50% and 90% of the strains.

a_4) *Comparison the in vitro activity of DC-159a with other FQs*.

MIC of DC-159a results were compared with those obtained with MOX and LX in order to determine the activity of these drugs and the cross-resistance among them.

Minimal bactericidal concentration (MBC)

During MIC determination, NTM and *Mtb* strains were loaded twice in the same plate, and one of the duplicates was not stained with resazurin. Instead, suspension from drug-containing wells and free of resazurin that did not show any visible growth at the end of total incubation period, were taken out of the plates, inoculated onto fresh M7H11/OADC agar plates and incubated for other 21–28 days. After that, the $CFU\,ml^{-1}$ was counted, related to the original bacterial suspension Y, and recorded. These experiments were carried out in triplicate for each strain.

MBC was defined as the lowest drug concentration that irreversibly inhibited the development of the original bacterial population. It means the drug concentration that kills the microorganisms.

In practice, MBC was the lowest concentration of DC-159a that, once inoculated onto the solid culture medium, demonstrated no bacterial development at the end of the incubation period.

Ex vivo activity of DC-159a

Intracellular MIC and MBC of DC-159a

For MIC and MBC intracellular determination, two ex vivo systems were used: a_1) peripheral blood mononuclear cells (PBMC)-derived macrophages (Mϕ) from healthy donors (HDs), a_2) the human cell line of the alveolar epithelium A-549 (ATCC® CCL-185™), type II pneumocytes from basal alveolar adenocarcinoma.

The ex vivo activity of DC-159a was estimated through intracellular MIC and MBC determination. Experiments were carried out in triplicate (supplementary Fig. 2), using the reference strains, *M. bovis* BCG as a member of *Mtb* complex and *M. avium* D4 as a member of MAC.

a_1) *PBMC-derived Mϕ*: blood samples from BCG-vaccinated HDs were obtained from the Hemotherapy Center, Garrahan Hospital, upon written informed consent. PBMC were isolated from heparinized blood by Ficoll-Triyosom and monocytes were obtained from PBMC by plastic adherence. PBMC suspended in RPMI 1640 (HyClone; Thermo Scientific) were plated at 2×10^6 cells/ well in flat 96-well plates for 2 h at $37\,^\circ C$. After removing the non-adherent cells, monocytes (2×10^5 cells/well) were washed and cultured in complete RPMI medium [RPMI containing $100\,mg\,ml^{-1}$ streptomycin, 100 U/ml penicillin and 10% heat-inactivated fetal calf serum (FCS, Invitrogen, Gibco)] for 6 days at $37\,^\circ C$ in a humidified 5% CO_2 atmosphere until their differentiation to Mϕ.

a_2) *A-549 cells:* A-549 cells were grown in 75-ml flasks (Corning® CellBIND® Surface Sterile, Polystyrene), using complete DMEM (DMEM plus 10% FBS, 2 mM Glutamine, $100\,UI\,ml^{-1}$ penicillin, $50\,\mu g\,ml^{-1}$ streptomycin) at $37\,^\circ C$, 5% CO_2. Once 80% of confluence was achieved, A-549 were detached from flasks using TrypLE™ Express (Gibco, Thermofisher). Trypan blue 0.4% (Sigma) was used to assess cell viability. Finally, A-549 were seeded into 24 well plates (2×10^5 cells ml^{-1}) and incubated at $37\,^\circ C$, 5% CO_2 until confluence.

Mycobacterial infection: Mϕ and A-549 were infected with D4 and BCG. Mycobacteria were harvested at exponential growth phase and suspended at $10^6\,CFU\,ml^{-1}$ (OD_{600nm}: 0.1) in RPMI (Mϕ) or DMEM (A-549). Upon removing culture medium, Mϕ and A-549 were incubated

with 1 ml of mycobacterial suspension at different MOIs (10:1, 5:1, 2:1), 3 h at 37 °C, 5% CO_2 and then washed with 200 µl 1X PBS (Gibco) to rule out non- phagocytosed bacteria. Thereafter, 1.5 ml of the corresponding culture medium alone or containing DC-159a (0.03–4.00 µg ml^{-1}) was added to each well and infected cells were cultured for further 7 days at 37 °C, 5% CO_2. To verify the inoculated bacteria, an aliquot of the initial bacterial inoculum was grown in M7H11/OADC, and the CFU ml^{-1} count was determined. Cells cultured without drug were considered as negative controls.

Determination of intracellular replication of mycobacteria: after 7 days of infection and exposure to the drug, CFU count was performed to determine the intracellular MIC and MBC. For this purpose, cell monolayers were washed with PBS 1X and treated with 0.2% Triton X-100 (Sigma) followed by neutralization buffer (BSA 20%). The lysates were undiluted or diluted (1/10, 1/100, 1/1000) in M7H9/OADC and placed on M7H11/OADC plates and incubated at 37 °C for 21–28 days. Wells with infected cells that were untreated served as the growth control.

Intracellular MIC was considered the drug concentration that prevented mycobacterial development and caused, in general, a 10-fold decrease in the CFU ml^{-1} initially inoculated. The drug concentration that caused a decrease of at least 100 times the number of bacilli initially inoculated was considered the intracellular MBC [46].

Cell viability: MTT cell proliferation assay was performed to evaluate the cytotoxic effect of DC-159a and whether it affects Mϕ and A-549 proliferation. MTT is a tetrazolium salt and its ring is cleaved in active mitochondria. The reduction reaction obtaining formazan occurs only in living cells being the signal generated dependent on the degree of cell activation [47].

Mϕs and A-549 containing plates were prepared as indicated previously (5 a1, a2). Some wells were exposed to DC-159a (4.00–0.13 µg ml^{-1}) and others were infected with BCG or D4 as described above.

After the incubation period, 10 µl or 40 µl of MTT (5 mg ml^{-1}) was added to 96 well or 24 well plates, respectively. Plates were incubated at 37 °C, 4 h. Then, to solubilize the formazan, the culture media was removed and 100 µl of DMSO was added. Plates were re-incubated at 37 °C, 15 min. The absorbance was measure at 570 nm on a spectrophotometer [47].

Statistical methods

Data were collected in Microsoft Excel 7.0 and exported to the Graph Pad Prism 8.0 software. The statistical was performed for each drug, the Fischer's exact or w^2 tests were used to evaluate the differences among DC-159a with MOX and LX [48].

Results

Genotyping of mycobacterial isolates

MAC isolates

MIRU-VNTR assay showed a high diversity of MAC strains with 12 INMV patterns.

The most represented were INMV 92 and 121, followed by INMV 97, 103, 50, and 40. The less represented genotypes were INMV 144, 145, 146, 147 and 148 (Table 1).

Mtb isolates

Spoligotyping showed high diversity among *Mtb* isolates, represented by 7 SPOF, being the most prevalent the Tuscany (T1, T2), Haarlem (H2, H3) and LAM (LAM 5, LAM 9, and LAM 3) (Table 1).

In vitro activity of DC-159a

Minimal inhibitory concentration (MIC)

In the first experiments, 41 MAC, 36 S-TB, 12 pre-XDR and 2 XDR *Mtb* strains were inhibited by less than 1.00 µg ml^{-1} of DC-159a. Therefore, the serial two-fold dilution range from 1.00–0.03 µg ml^{-1} was chosen to

Table 1 Genotypes of *Mtb* and MAC isolates

Mycobacterial strains	Genotype		n (%)
MAC (no.: 41)	INMV	92	8 (19.5)
		121	8 (19.5)
		97	4 (9.8)
		103	4 (9.8)
		50	4 (9.8)
		40	4 (9.8)
		144	2 (4.9)
		145	2 (4.9)
		146	2 (4.9)
		147	2 (4.9)
		148	1 (2.4)
Mtb (no.: 14)	SPOF	T1	3 (21.4)
		H2	3 (21.4)
		H3	2 (14.3)
		LAM 5	2 (14.3)
		LAM 9	2 (14.3)
		LAM 3	1 (7.1)
		T2	1 (7.1)

MAC M. avium complex, Mtb M. tuberculosis, n number, *INMV* MIRU pattern, *SPOF* spoligotyping family, *T* Tuscany, *LAM* Latin American Mediterranean, *H* Haarlem

Table 2 MIC results for MAC and *Mtb* strains

Isolates	DC-159a[d]		LX[e]		MOX	
	MIC_50 (μg ml^{-1})	MIC_90 (μg ml^{-1})	MIC_50 (μg ml^{-1})	MIC_90 (μg ml^{-1})	MIC_50 (μg ml^{-1})	MIC_90 (μg ml^{-1})
MAH[a]	0.06	0.13	0.50	8.00	0.25	8.00
MAI[b]	0.06	0.25	0.25	8.00	0.13	2.00
pre-XDR-TB/XDR-TB[c]	0.03	0.06	2.00	≥2.00	2.00	≥2.00

[a]p (DC-159a vs LX): 0.0010; p (DC-159a vs MOX): 0.0078; p (MOX vs LX): 0.7344

[b]p (DC-159a vs LX): 0.0430; p (DC-159a vs MOX): 0.2500; p (MOX vs LX): 0.1563

[c]p (DC-159a vs LX): 0.0355; p (DC-159a vs MOX): 0.00154; p (MOX vs LX): 0.6897

[d]p (MAHa vs MAI): 0.4343

[e]p (MAHa vs MAI): 0.3791

determine the MIC in the next experiments. MIC of DC-159a obtained for H37Rv and BCG (0.03 μg ml^{-1}) was lower than that obtained for D4 (0.13 μg ml^{-1}).

Table 2 shows MIC_50 and MIC_90 results for DC-159a, LX, and MOX.

In general, MAC and *Mtb* were more susceptible to DC-159a than to MOX and LX. No difference was found between MOX and LX ($p > 0.05$) within the included isolates (Table 2).

MIC_50 for DC-159a against MAH was 0.06 μg ml^{-1}, 4-to 8-fold lower than those obtained with MOX and LX, respectively (MIC_50 for MOX: 0.25 μg ml^{-1}; MIC_50 for LX: 0.50 μg ml^{-1}). For MAI isolates, MIC_50 for DC-159a was 0.06 μg ml^{-1}, 2- to 4-fold lower than MIC_50 for MOX and LX respectively (MIC_50 for MOX: 0.13 μg ml^{-1}; MIC_50 for LX: 0.25 μg ml^{-1}).

Besides, 15 MAC isolates (6 MAI, 9 MAH) when tested against LX showed the MIC susceptibility range of 8.0–1.0 μg ml^{-1} and were inhibited by a range of MIC between 0.03 to 0.06 μg ml^{-1} for DC-159a; 11 MAC isolates (9 MAH, 2 MAI) showed MIC range of 8.0 and 1.0 μg ml^{-1} for MOX and were susceptible to DC-159a (MIC: 0.03–0.06 μg ml^{-1}). These findings confirm no cross-resistance between DC-159a and MOX/LX for the tested strains.

Regarding the pre-XDR/XDR isolates, MIC_50 for DC-159a was 0.03 μg ml^{-1}, 4- to 8-fold lower than with MOX and LX, respectively (MIC_50 for MOX: 0.13 μg ml^{-1}; MIC_50 for LX: 0.25 μg ml^{-1}).

Those pre-XDR isolates were only resistant to LX, MOX or both FQs, but showed MIC for DC-159a of 0.03 μg ml^{-1} (n: 6) and 0.06 μg ml^{-1} (n: 4) showing no cross-resistance between DC-159a and MOX/LX.

Minimal bactericidal concentration (MBC)

BCG, D4 and H37Rv strains, showed the same MIC for DC-159a as the MBC. For BCG and H37Rv strains the

Table 3 Relationship between the inhibitory and bactericidal effects of DC-159a

MIC/MBC DC-159a (μg ml^{-1})	MAH (n: 22)	MAI (n:19)	*Mtb* (n:14)	Reference strains		
				Mtb H37Rv	*M. bovis BCG*	*MAA D4*
1.00/1.00	-	1	-			
0.50/0.50	-	-	-			
0.25/0.25	-	1	-			
0.13/0.13	2	6	-			X
0.13/0.25	-	2	-			
0.06/0.06	9	6	5			
0.06/0.13	2	1	2			
0.03/0.03	4	-	4	X	X	
0.03/0.06	5	-	3			

MIC minimal inhibitory concentration, *MBC* minimal bactericidal concentration, *MAH M. avium hominissuis*, *MAI M. avium intracellulare*, *Mtb M. tuberculosis*, *MAA M. avium avium*, n: number

MIC/MBC for DC-159a was 0.03 μg ml^{-1}, for D4 the MIC/MBC for DC-159a was 0.13 μg ml^{-1}. Table 3 shows the MBC values for MAC and *Mtb*, and 70.7% (29/41) of MAC and 9/14 of *Mtb* isolates had the same MBC/MIC.

Genotyping patterns and MIC values

Table 4 shows the distribution of the INMV genotypes of MAC and SPOF of *Mtb* strains inhibited by different DC-159a concentration.

Genetic diversity among MAC was observed and almost all of the strains were inhibited by the same values of MIC for DC-159a regardless the genetic pattern of the strain. Only the INMVs 40 and 92 were inhibited by higher MIC values.

INMV 40 was represented only by MAI, while INMV 92 with MIC for DC-159a 0.25 μg ml^{-1} was represented by MAI and MIC for DC-159a 0.03 μg ml^{-1} by MAH.

Table 4 Distribution of the INMV and SPOF genotypes according to the DC-159a concentration

MIC_ DC-159a (μg ml^{-1})	MAC_INMV	*Mtb*_SPOF
<0.03	40, 92, 103, 146	LAM 3, LAM 5, LAM 9, T1, T2
0.06	40, 50, 121, 148	H2, H3
0.125	145, 147	-
0.250	92	-
0.50	-	-
1.0	97	-

SPOF spoligotyping family, *INMV* genetic pattern of MAC, *MIC_ DC-159a* minimal inhibitory concentration to DC-159a, *Mtb M. tuberculosis*, *LAM* Latin-American Mediterranean family, *T* Tuscany, *H* Haarlem

Regarding the *Mtb* almost all SPOF were inhibited by MIC to DC-159a 0.03 μg ml^{-1}. MIC to DC-159a 0.06 μg ml^{-1} was represented only by H2 and H3.

Ex vivo activity of DC-159a

Intracellular MIC and MBC

To evaluate the DC-159a ability to reduce intracellular mycobacterial growth, Mϕ and A-549 cells were infected with D4 and BCG and treated with different DC-159a concentration. Lysates derived from infected cells were then employed to evaluate CFU ml^{-1} and calculate MIC and MBC.

We used BCG as a representative of the *Mtb* complex rather than H37Rv, for biosafety issues as it showed the same MIC/MBC for DC-159a.

MOIs 10:1; 5:1 and 2:1 were assayed to determine the most suitable one for intracellular MIC and MBC assays. A MOI of 10:1 was selected for MAC and 2:1 for *Mtb*.

Tables 5 and 6 show the DC-159a inhibition rate obtained for each type of cell, for D4 and BCG. The intracellular MIC for DC-159a of D4 was 0.50 μg ml^{-1} while the MBC was 2.00 μg ml^{-1} in Mϕ. In A-549 the MIC for DC-159a of D4 was 0.13 μg ml^{-1} and the MBC was 1.00 μg ml^{-1}. Regarding the BCG, the MIC for DC-159a was 0.25 μg ml^{-1} and the MBC was 1.00 μg ml^{-1} within Mϕ, while in A-549 cells the MIC was 0.06 μg ml^{-1} and the MBC was 1.00 μg ml^{-1}.

In the ex vivo system, D4 showed a 33.3% of inhibition and a decrease of 1.5 CFU when exposed only to 0.03 μg ml^{-1} of DC-159a while when it was exposed to 0.50 μg ml^{-1}, the inhibition increased up to 96.7% with a 24.0 CFU reduction. Regarding BCG, 85.0% of inhibition and 8.0 decreases in CFU was observed with 0.03 μg ml^{-1}, while when exposed to 0.50 μg ml^{-1} the inhibition was 98.5% and 30.5 CFU decrease.

Table 5 Ex vivo activity of DC-159a expressed as the percentage of inhibition growth and decrease in CFU of the reference strains D4 and BCG in a PBMC_ Mϕ system

PBMC_Mϕ plus DC-159a (μg ml^{-1})	Strains			
	MAA D4		*M. bovis* BCG	
	% inhibition	CFU (times) ↓	(% inhibition)	CFU (times) ↓
0.03	18.2	1.22	25.2	1.33
0.06	34.3	1.52	54.3	2.2
0.13	58.6	2.41	70.4	3.4
0.25	75.7	4.1	90.5	10.5
0.5	90.0	10.0	95.6	22
1.0	94,5	18	99.2	125
2.0	99.1	111	99.4	167
4.0	99.6	250	99.8	500

CFU colony forming units, intracellular MIC_DC-159a: 0.5 μg ml^{-1} (D4); intracellular MIC_DC159a: 0.25 μg ml^{-1} (BCG); intracellular MBC_DC-159a: 2.0 μg ml^{-1} (D4); intracellular MBC_DC-159a: 1.0 μg ml^{-1} (BCG)

Table 6 Ex vivo activity of DC-159a expressed as the percentage of inhibition growth and the decrease in CFU of D4 and BCG in A-549 cells

A-549 plus DC-159a (μg ml^{-1})	Strains			
	MAA D4		*M. bovis* BCG	
	% inhibition	CFU (times) ↓	% inhibition	CFU (times) ↓
0.03	33.3	1.5	85	8.0
0.06	86	2.0	92	12.5
0.13	95	24.0	96.5	22.6
0.50	96.7	30.0	98.5	30.5
1.00	99.2	120.0	99.2	120.0
2.00	98.3	60.0	100	-
4.00	100	-	100	-

CFU colony forming units, intracellular MIC_DC-159a: 0.13 μg ml^{-1} (D4); intracellular MIC_DC159a: 0.06 μg ml^{-1} (BCG); intracellular MBC_DC-159a: 1.0 μg ml^{-1} (D4); intracellular MBC_DC-159a: 1.0 μg ml^{-1} (BCG)

Each MIC and MBC determination was performed in triplicate, so results represented the average of them.

Cell viability: A-549 and Mϕs were incubated with different drug concentrations (4.00–2.00–1.00–0.50–0.25–0.13 μg ml^{-1}) and cell death due to DC-159a was evaluated by MTT cell proliferation assay. The developed color was directly proportional to the number of cells and the metabolic cellular activity. This activity was measured using a spectrophotometer. Figure 2 shows the absorbance (570 nm) according the DC-159a concentration. It was observed a very slightly decrease in A549/Mϕ cell viability within the range of concentration used (0.13–1.00 μg ml^{-1}) and a loss of viability at higher drug concentrations.

Fig. 2 Cell viability according with the DC-159a concentration. MTT cell proliferation assay to evaluate A-549 and Mφs death after the exposure to different DC-159a concentrations (4.00–2.00–1.00–0.50–0.25–0.13 µg ml⁻¹).The absorbance measured at 570 nm was directly proportional to the number of cells and the metabolic cellular activity. LX: levofloxacin (LX) and moxifloxacin (MOX). **a** Macrophages. **b** A-549 cells

(a) Macrophages

(b) A-549
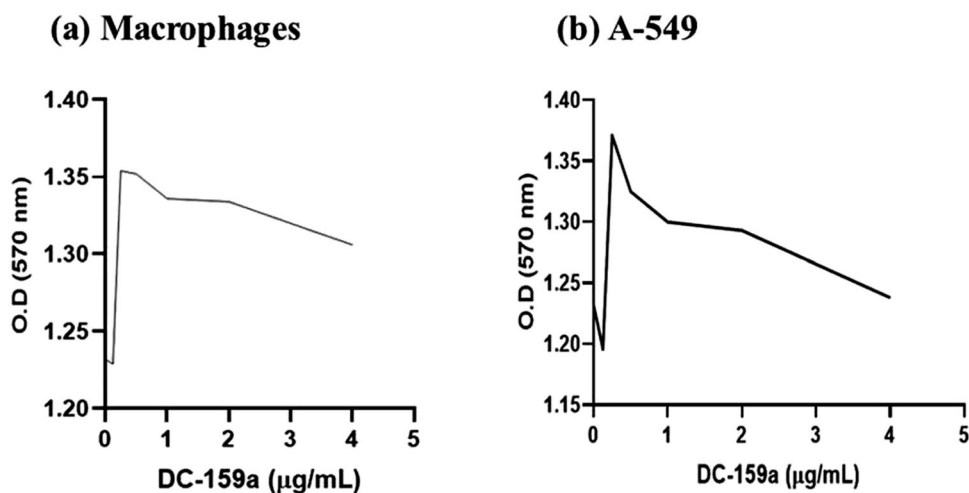
Discussion

The in vitro and ex vivo activity of DC-159a on NTM and on pre-XDR/XDR strains were evaluated, and the in vitro activity was compared to LX and MOX activity.

Genotyping allowed the identification of the genetic diversity among the isolates.

DC-159a was active in vitro and ex vivo against mycobacteria. In accordance with previous studies, DC-159a was more active against *Mtb* and MAC isolates than MOX/LX [30], yielding the lowest MIC$_{50}$/MIC$_{90}$ among the tested FQs. And also, in agreement with other authors who reported a major susceptibility level to DC-159a in *Mtb* compared to several NTM species, we found that, among the tested isolates, in vitro susceptibility to DC-159a was greater against *Mtb* than in MAC. Additionally, MAH isolates showed a higher MIC$_{90}$ than MAI isolates. This finding is also in agreement with previous studies results. All the species were highly susceptible to the drug [30]. Importantly, the MIC average of DC-159a against MAC was 2- to 8-fold lower than those obtained with MOX/LX. These findings support the previous findings about MIC$_{90}$ of DC-159a against the *M. avium-M. intracellulare* complex of being 2- to 16-fold lower than those obtained with other FQs [30].

A narrow range of MIC$_{50}$ and MIC$_{90}$ to DC-159a was observed for all the species tested. The lack of dispersion of these results showed a very compact response to the drug and it could be useful to estimate its possible use in future therapeutic schemes. In this study, no cross-resistance was evidenced among DC-159a and LX/MOX as DC-159a could inhibit *Mtb* and MAC strains that were already resistant to LX/MOX. This finding is important and is in line with other authors who reported that DC-159a remains active against FQ-R *Mtb* with the most common *gyrA* mutations. However, the exact mechanism of DC-159a resistance in *Mtb* is under investigation [30, 33–36]. As far

as the authors know, there is no previous report about detection of neither *Mtb* nor MAC clinical isolates resistant to DC-159a. Previous studies demonstrated that H37Rv mutants carrying the Gly88Cys *gyrA* gene mutation had an increased MIC for DC-159a compared to the wild type strain. A Gly88Cys mutation in *gyrA* is one of the key alterations by which *Mtb* mutants acquire DC-159a resistance in vitro. But this mutation is so far rarely detected in clinical practice [49, 50]. Anyway, the frequency of resistance development of *Mtb* to this new FQ remains to be determined, since this would be a very important data when a drug is entered into clinical trials.

Besides, other authors that assessed the in vivo efficacy of DC-159a to shorten the TB treatment duration in a murine TB model reported a superior in vivo efficacy of DC-159a compared to MOX. It might be attributed to its rapid uptake, high penetration and concentrations in the lungs. Moreover, they stated that DC-159a containing regimens were superior to MOX containing regimens given that DC-159a would shorten the TB treatment duration [51].

Regarding the DC-159a activity, the MIC and MBC were lower in the in vitro than in the ex vivo system. Probably, because in vitro the drug interacts directly with the bacteria while ex vivo it interacts also with the cells. A previous study performed with MOX and THP-1 cell line (Mφ), reported that MOX accumulates into the cells and remains active intracellularly, but significantly less active than under in vitro conditions [52, 53].

Albeit further studies should be carried out, DC-159a activities was higher in the A-549a system than in Mφ and it could be related to each cells capacity to uptake the drug.

The fact that D4 and BCG when exposed to 0.50 µg ml⁻¹ of DC-159a showed more than 95.0% of inhibition and more than 20.0 of CFU reduction in the ex vivo system, and that the intracellular MBC values found for BCG and D4 were 1.00–2.00 µg ml⁻¹ suggest that it could be an attainable serum drug concentration after a dose of 100 mg kg⁻¹

already demonstrated in in vivo models [51]. Furthermore, doses of $5\,mg\,kg^{-1}$ of DC-159a were tested in a monkey model, reaching maximum serum CC: $2.20\,\mu g\,ml^{-1}$ [30].

Moreover, it was reported that after the same administered dose, a serum concentration of DC-159a similar to that of MOX and ofloxacin was achieved [5, 32].

AMR is a major global health problem and several reports point out the need to find and develop new anti-TB drugs capable of combating drug-resistant TB. It could be desirable that these drugs were also useful to treat the diseases caused by NTM that are becoming more relevant worldwide because are extremely resistant to the conventionally used antimicrobials. DC-159a is one of the drugs that are been studying and taking under consideration with very promising results.

Currently, DC-159a is in preclinical phase against *Mtb*, during which important activity and drug safety information are been collected, because the primary goals of preclinical studies are to determine the safe dose for the first-in-human study and to evaluate the safety profile of the drug. Data so far, shows that, DC-159a has better pharmacokinetic and pharmacodynamics properties than the currently used FQs. The DC-159a structure could facilitate the entrance through the bacillary cell wall and improve the pharmacokinetic properties. According to these study results, DC-159a could be a possible candidate to be evaluated in the design of new therapeutic regimes for MDR, pre-XDR-TB/XDR-TB and for mycobacterioses caused by MAC [54–56].

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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