



Preliminary evaluation of the positively and negatively charge effects of tetra-substituted porphyrins on photoinactivation of rapidly growing mycobacteria

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ARTICLE INFO

Keywords:

Porphyrins
Cationic porphyrins
Mycobacteria
Photoinactivation

ABSTRACT

This manuscript reports, at the first time, the photoinactivation evaluation of tetra-cationic and anionic porphyrins as photosensitizers (PS) for the photodynamic inactivation (PDI) of rapidly growing mycobacteria strains. Two different charged porphyrin groups were obtained commercially. PDI experiments in the strains *Mycobacterium massiliense* e *Mycobacterium fortuitum* conducted with adequate concentration (without aggregation) of photosensitizer under white light at a fluence rate of 50 mW/cm² over 90 min showed that the most effective PS caused a 100 times reduction in the concentration of viable mycobacteria. The present results show that porphyrin with positively charge are more efficient PS than anionic porphyrin (negatively charged) against *M. massiliense* e *M. fortuitum*. It is also clear that the effectiveness of the molecule as PS for PDI studies with mycobacteria is strongly related with the porphyrin peripheral charge, and consequently their solubility in physiological media. Cationic PSs might be promising anti-mycobacteria PDI agents with potential applications in medical clinical cases and bioremediation.

1. Introduction

Non-tuberculous mycobacteria (NTM) are microorganisms responsible for diseases known as mycobacteriosis and comprise species other than the *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*. This group has been gaining attention due to the increase of reported cases and the improvement of isolation techniques and identification of these pathogens [1,2], comprising today a group with more than 190 species, of which, approximately 40 species are considered pathogenic [3,4].

Mycobacteriosis can be characterized by both isolated and dispersed infection and represent an increasing number of hospital cases in immunocompromised patients, either with acquired immunodeficiency virus, in chemotherapeutic treatment or recently transplanted [5,6]. However, cases involving immunocompetent patients are cited, and in general, they are associated with invasive procedures or previous site injury [7–9].

Rapidly growing mycobacteria (RGM) represent an important group among NTM, being characterized as non-pigmented and with great growth capacity in hostile environments [10]. They are considered

ubiquitous and therefore are often isolated from sewage treatment plants, soil, swimming pools and water sources [11–13]. However, its clinical importance has been highlighted by its great capacity for bio-film formation [9,14,15]. This is strongly related to high resistance and the large number of infections associated with surgical procedures and in patients with previous lung disease [16–20].

RGM are commonly isolated in clinical laboratories and, among NTM, are the most important pathogens related to skin and soft tissue infections [21]. *Mycobacterium fortuitum*, *Mycobacterium chelonae* and *Mycobacterium abscessus* are the main RGM reported in cases of post-surgical or post-traumatic infections, but new species such as *Mycobacterium goodii* and *Mycobacterium massiliense* have recently been reported by Faria and co-workers [19]. The treatment of diseases caused by NTM is complex, since it involves a lengthy process of identification and determination of sensitivity in vitro to antibiotics. In addition, they are naturally resistant or have low sensitivity to tuberculostatic drugs. These factors and a low rate of clinical suspicion often result in ineffective treatment of NTM infections [3,4,6].

In this context, photodynamic inactivation (PDI), originally tested for the treatment of tumor cells, show up as an alternative in the control

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<https://doi.org/10.1016/j.tube.2019.06.001>

Received 6 March 2019; Received in revised form 25 May 2019; Accepted 5 June 2019

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and inhibition of RGM [22]. Their proposal is based on the arrangement between a photosensitizer (PS) molecule which, through a given wavelength is able to interact with molecular oxygen and generate reactive oxygen species (ROS), these have the ability to photo-oxidize biological substances and may lead to cell lysis.

Several compounds are studied as a source of ROS in PDI [23–26], but the use of porphyrins is notable, either because of the great applicability or the strong ROS production [27–29]. These compounds are tetrapyrrole macrocycles capable of absorbing at wavelengths in the visible range, obtaining their absorption peak at 400 nm.

The use of PDI in microbiological control has gained clinical importance due to the increased resistance demonstrated by the pathogens [27,30–32]. Photo-oxidation acts in a non-specific manner, preventing the development of microbial resistance mechanisms [33]. Its use demonstrates significant effects on the lysis of membrane and genetic material by the activation of the PS demonstrating its importance in the control of mycobacteriosis, since these pathologies are related to strains with great resistance to the antimicrobials recommended in the clinic [18,34,35].

Since the antimycobacterial therapy to combat mycobacteria is becoming increasingly complex together with the evolution and improvement of resistance mechanisms of RGM, this study evaluated for the first time the antimycobacterial activity of commercially available *meso*-tetra-methylated porphyrin (TMePyP⁺) and *meso*-tetra-sulfonated porphyrin (TPPS⁻) against of rapidly growing mycobacteria strains.

2. Materials and methods

2.1. Photosensitizers (PS)

Cationic *meso*-tetra(*N*-methyl-4-pyridyl) porphyrin tetrachloride salt (TMePyP⁺) and anionic *meso*-tetra(4-sulfonatophenyl) porphyrin tetrasodium salt (TPPS⁻) water-soluble porphyrins (Fig. 1) were purchased from Frontier Scientific® (Logan, Utah, USA).

2.2. Photosensitizer stock solutions

Stock solutions of photosensitizers to be used in photoinactivation assays were prepared directly in PBS pH 7.2 buffer solution, at a concentration of 500 mM.

2.3. Light source

All the irradiation experiments were performed under white-light

LED irradiation (370–800 nm) at 50 mW/cm² and light source 10.9 J/cm². All experiments were performed in triplicate and independently.

2.4. Microorganisms

Standard strains of *Mycobacterium fortuitum* (ATCC 6841) and *Mycobacterium massiliense* (ATCC 48898) were used for this study. These strains were maintained at –80 °C and were reactivated in Löwenstein-Jensen medium for use (HiMedia Laboratories Pvt. Ltd, India).

2.5. PDI experimental setup

The determination of the irradiation influence on the mycobacteria through the PS activity was performed by the variation of the lighting time irradiation (15, 45 and 90 min). One plate was prepared for each illumination time, which were irradiated with visible light at the incubation times of 0, 24 and 48 h and held for 72 h at 37 °C. The plates were closed according to biosafety standards and the required distance between the plate and the white-light source was 8–10 cm.

Two controls were included in each irradiation experiment: a light control (LC) submitted to the same irradiation conditions as the samples but without PS, and a dark control (DC) containing the PS in the same concentrations but kept in the dark.

2.6. Determination of minimum inhibitory concentration (MIC)

The anti-mycobacterial activity of PS was evaluated by the broth microdilution method, following the guidelines of CLSI M24-A2.26 [36]. The PS concentration was evaluated in serial dilutions of factor 2 in Mueller Hinton broth (HiMedia Laboratories Pvt. Ltd, India). The inoculum was standardized according to the 0.5 MacFarland density scale and 100 µL were transferred to each well of the microplate. Minimum inhibitory concentration (MIC) was determined using 2,3,5-triphenyltetrazolium chloride (TTC) (Vetec®, Rio de Janeiro, RJ, Brazil).

2.7. Dose response curve

The evaluation of PS photo-activity during growth time of the RGM strains was performed through the dose response curve. Aliquots of 10 µL of each well corresponding to the concentrations of 2xMIC, MIC, ½MIC and ¼MIC, respectively, and were plated for surface plating in Mueller Hinton Agar (HiMedia Laboratories Pvt Ltd., India). The procedure was performed at times 0, 24, 48, 72, 96 and 120 h of bacterial growth and under white-light illumination times (15, 45 and 90 min)

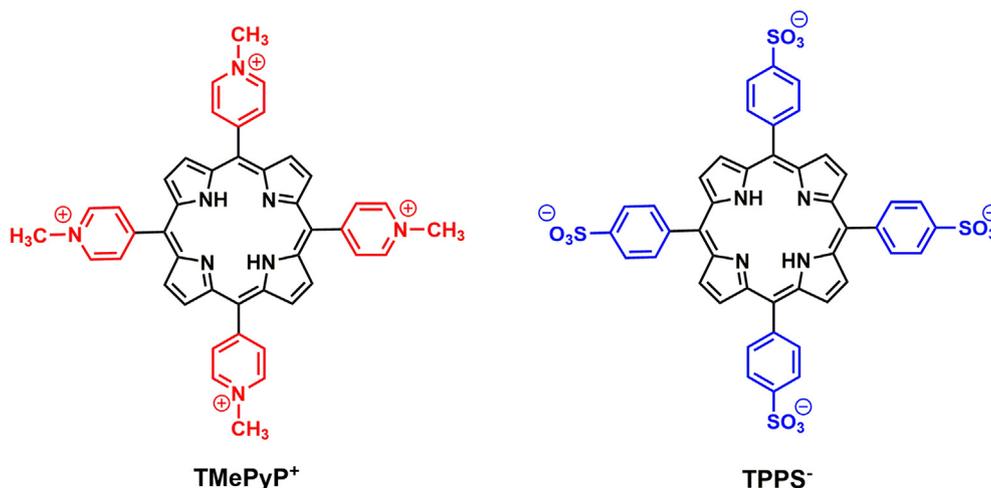


Fig. 1. Representative structure of water-soluble porphyrins used in this work.

for plaque at 24 h intervals, as well as described in the MIC determination. The plates were incubated at 37 °C for 72 h and, after this time, the viable microorganisms were counted.

2.8. Scavengers of reactive oxygen species experiments

The experiments were carried out by previously determining the MIC values of four compounds described in the literature as ROS scavengers, among them are: dimethyl sulfoxide (DMSO) and Mannitol (hydroxyl radical species), Ascorbic Acid (singlet oxygen species) and Potassium Iodide (superoxide species). The evaluation of the ROS involved in the anti-mycobacterial activity of cationic porphyrin was evaluated by the broth microdilution method, following the guidelines of CLSI M24-A2. The tetra-sulfonated porphyrin (anionic compound) was not tested because of its low inactivation activity in the previous experiments.

Dilution of cationic porphyrin followed the same criteria of MIC determination (ranging from 100 µM to 0.0488 µM range). The inoculum was standardized according to the MacFarland 0.5 density scale, next to it the ROS sequestrant was added at the desired concentration and 100 µL were transferred to each well of the microplate. The plates were irradiated for 90 min (white-light LED irradiation at 50 mW/cm²) for 3 times at 24 h intervals. Plates were prepared for each of the hijackers tested (Ascorbic Acid, Potassium Iodide, DMSO and Mannitol) in addition to control only with cationic porphyrin and control without white-light dosage. The plates were kept for 72 h at 37 °C in a growing greenhouse and remained closed according to biosafety standards, the distance required between the plate and the light source being 8–10 cm. Minimum inhibitory concentration (MIC) was determined using 2,3,5-triphenyltetrazolium chloride (TTC) (Vetec[®], Rio de Janeiro, RJ, Brazil).

2.9. Statistical analysis

All PDI tests were performed in triplicate and the results were expressed as the mean ± standard deviation. The dose response curve was performed using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA) and statistical differences were considered when $p < 0.05$.

3. Results

The use of photodynamic applications in the control of neoplastic cells has been gaining ground as an antimicrobial alternative, its use against infections caused by viruses, fungi and bacteria has shown an important therapeutic possibility in the fight against pathogens [23,37]. In addition, the use of porphyrins and derivatives, such as PS, in the therapy reveals good activity due to the extensive production of ROS and medium lipophilicity with consequent interaction with the bacterial membrane [38].

3.1. Determination of minimum inhibitory concentration (MIC) of PS

The potential for inhibition of PS against non-irradiated RGM strains (dark conditions) did not demonstrate high photo-oxidation capacity, where cationic **TMePyP**⁺ was able to inhibit both strains at 50 µM, and anionic **TPPS**⁻ used twice the concentration (100 µM) to inhibit bacterial growth. However, with exposure to white-light irradiation at the choice times described (15, 45 and 90 min) there was significant inhibition of bacterial growth (Table 1). Meso-tetra(*N*-methylated)porphyrin (**TMePyP**⁺) had lower inhibition concentrations, which represented a decrease greater than 100-fold when compared to MIC values without illumination conditions. In addition, the expressed values were linear, demonstrating a response time-dependent radiation (Figs. 2 and 3).

Anionic meso-tetra(sulfonated)porphyrin (**TPPS**⁻) showed half-dose

Table 1

MIC values for PS with and without white-light irradiation at different exposure times to the light source (white LED; 50 mW/cm²) compared to RGM strains.

Microorganism	Cationic TMePyP ⁺ (µM)			Anionic TPPS ⁻ (µM)		
	Dark conditions			Dark conditions		
	15 min	45 min	90 min	15 min	45 min	90 min
Mycobacterium fortuitum	50.0	50.0	50.0	100	100	100
Mycobacterium massiliense	50.0	50.0	50.0	100	100	100
Microorganism	Cationic TMePyP ⁺ (µM)			Anionic TPPS ⁻ (µM)		
	Light conditions			Light conditions		
	15 min	45 min	90 min	15 min	45 min	90 min
Mycobacterium fortuitum	1.562	0.781	0.391	50.0	50.0	50.0
Mycobacterium massiliense	1.562	0.781	0.391	50.0	50.0	50.0

MIC value reduction when applied the same irradiation conditions, the value of 50 µM did not decrease even at the highest periods of light exposure. The values shown in Table 1 represent the data of both strains tested.

3.2. Dose response curve

In the dose response curve, the bactericidal action of **TMePyP**⁺ was observed, which was able to inhibit the growth of the RGM strains tested at concentrations corresponding to 2xMIC and MIC at all proposed lighting irradiation times. There was a difference between the strains and the time in which this occurred, where *M. massiliense* had its inhibited growth in 24 h of experiment, whereas *M. fortuitum* needs two periods of whit-light illumination to inhibit its growth in the mentioned PS concentrations (Table 2).

3.3. Scavengers of reactive oxygen species

In order to investigate which reactive oxygen species are participating in the photo-inactivation process, experiments were carried out with the reactive oxygen species scavengers. Table 3 shows MIC values in the absence and presence of the ROS scavengers.

In general, we observed that in the presence of radical species scavengers (hydroxyl - mannitol and superoxide - KI), the values found for MIC were close to those presented for the mycobacteria photo-inactivation, in the absence of the scavenger species. As for the presence of ascorbic acid (oxygen singlet quencher) we can notice a significant increase of the MIC values in both microorganisms, in relation to the values observed in the absence of ROS scavengers. These data show that probably the species that is acting in greater quantity in the photo-oxidation process of the mycobacteria is the species of singlet oxygen (¹O₂), since it is reported that these tetra-cationic porphyrins have moderate values for the generation of this type of reactive oxygen species (for **TMePyP**⁺ in PBS; Φ_Δ = 0.67) [39].

4. Discussion

Much of the expected results of PDI are can be attributed to the ROS-generating potential, especially singlet oxygen species (¹O₂). In addition, an alternative mechanism is attributed to the oxidizing potential of PS itself. However, both processes lead to the oxidation of biological materials, including the bacterial wall and cell membrane [40]. The resistance presented by microorganisms against drugs recommended for the treatment of infections represents a major public health problem worldwide [41,42]. Therefore, the use of PDI against

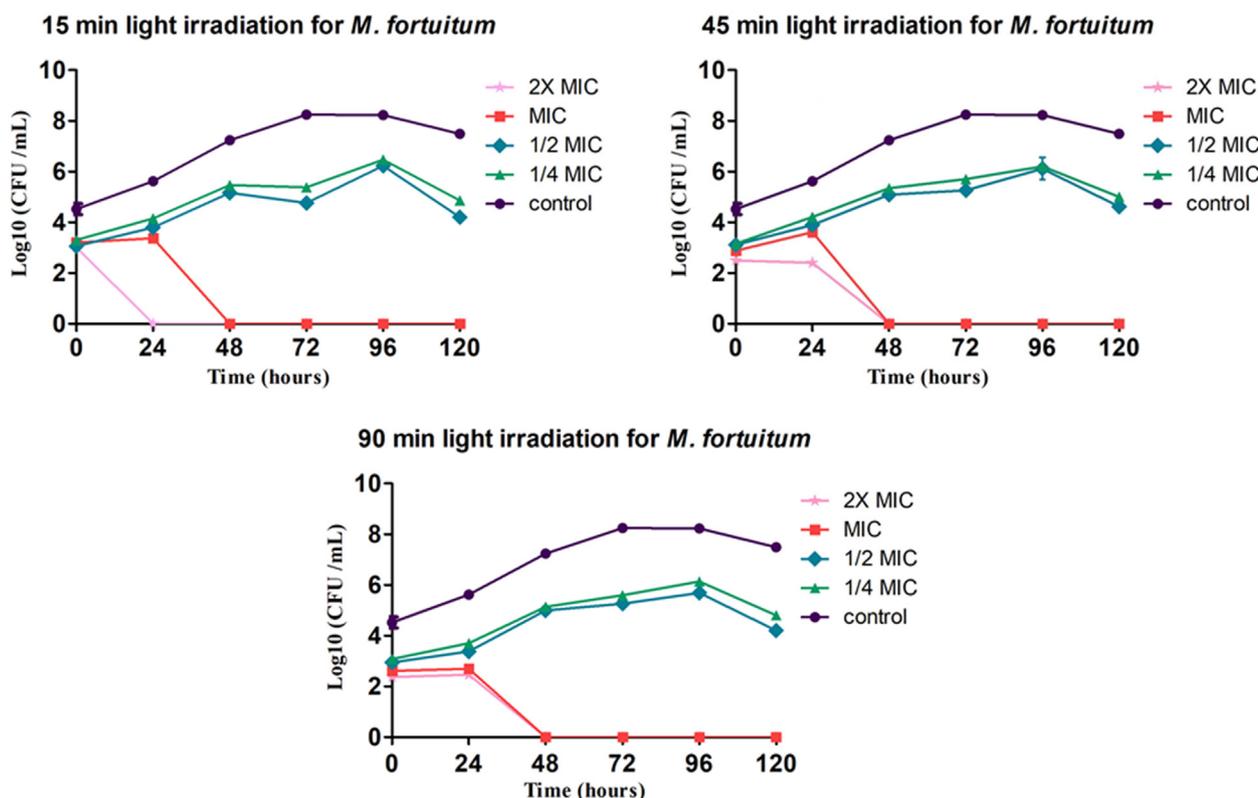


Fig. 2. Survival of *Mycobacterium fortuitum* during PDI experiments of cationic porphyrins TMePyP⁺ with white-light irradiation at a fluence rate of 50 mW/cm² at 90 min. Control: mycobacteria + light irradiation, without porphyrin. Values correspond to the average of two independent experiments. Error bars represent standard deviation. Lines were used to connect the data points.

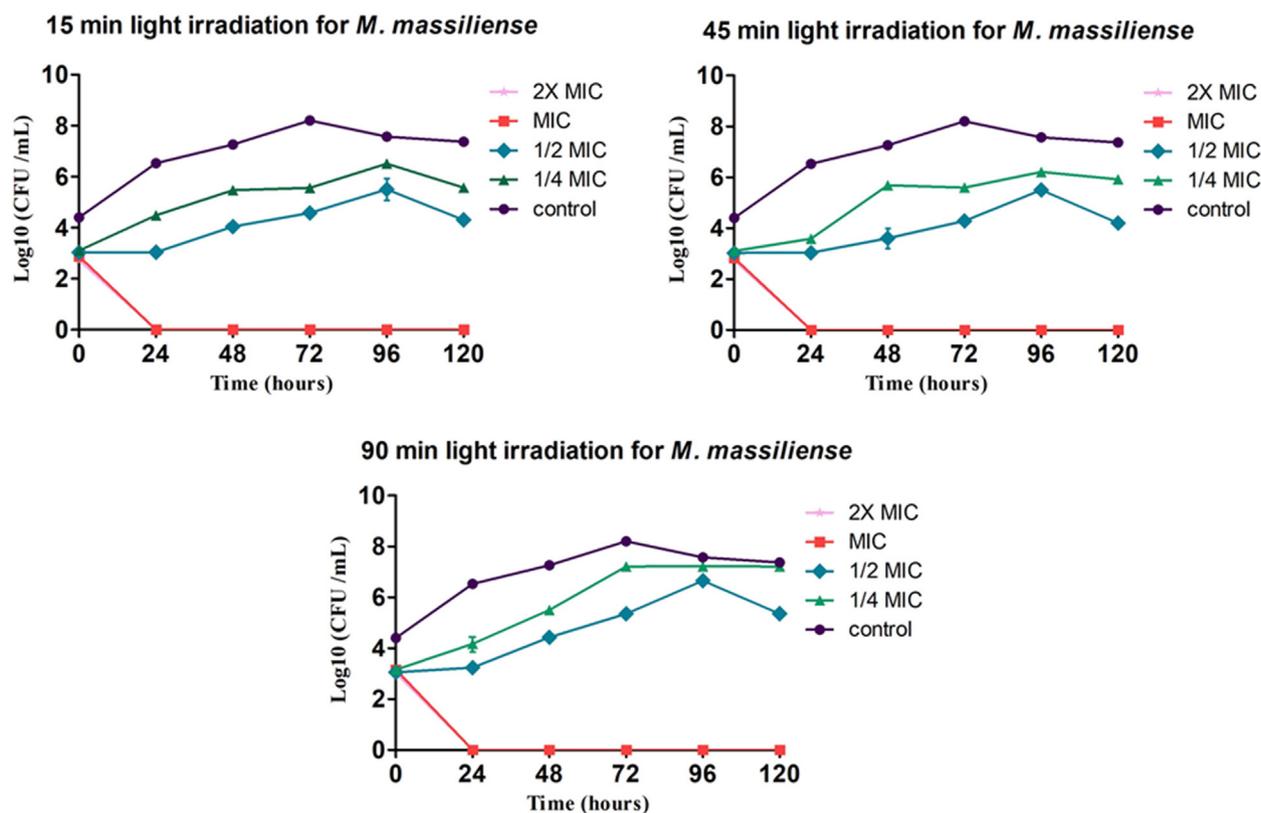


Fig. 3. Survival of *Mycobacterium massiliense* during PDI experiments of cationic porphyrins TMePyP⁺ with white-light irradiation at a fluence rate of 50 mW/cm² at 90 min. Control: mycobacteria + light irradiation, without porphyrin. Values correspond to the average of two independent experiments. Error bars represent standard deviation. Lines were used to connect the data points.

Table 2

Concentration dependence of MIC values for cationic porphyrin with white-light irradiation at different exposure times to the light source (white LED; 50 mW/cm²) compared to RGM strains.

Microorganism	2xMIC (μM)			MIC (μM)		
	15 min	45 min	90 min	15 min	45 min	90 min
<i>Mycobacterium fortuitum</i>	3.125	1.562	0.781	1.562	0.781	0.391
<i>Mycobacterium massiliense</i>	3.125	1.562	0.781	1.562	0.781	0.391
Microorganism	½MIC (μM)			¼MIC (μM)		
	15 min	45 min	90 min	15 min	45 min	90 min
<i>Mycobacterium fortuitum</i>	0.781	0.391	0.195	0.391	0.195	0.098
<i>Mycobacterium massiliense</i>	0.781	0.391	0.195	0.391	0.195	0.098

resistant organisms is an alternative in the combat and control of pathogens classified as having multiple resistance. Alves and collaborators [31] evaluated the efficacy of this *in vivo* therapy against fluconazole resistant *Candida albicans* showing a significant reduction in the viability of the tested strains. In addition, histological analysis revealed that PDI does not cause side effects in tissues. Along these lines, Tasli and co-workers [43] demonstrate the activity of porphyrin cationic derivatives against methicillin resistant *Staphylococcus aureus* (MRSA) through PDI, with a reduction of up to 99% in bacterial cell viability.

Much of the resistance found in the control of mycobacteria is due to its thick and complex cell wall, which is a characteristic extended to all genres. Its composition is mainly due to four structural components, peptidoglycan associated with arabinoglycan giving rigidity. Added to this is the presence of mycolic acids and glycolipids, which give the genus the ability to withstand nutrient and water scarcity and exposure to xenobiotics [44].

The difficulty of contacting antimicrobials to the plasma membrane and to the mycobacterial cytoplasm reduces their activity and consequently many of the drugs do not demonstrate efficiency for the treatment of mycobacteriosis. Several cellular structures are targeted by PDI field, highlighting the activity against the biological membrane and nucleic acids [26,45,46]. However, it is not possible to induce mechanisms of resistance, since it acts in a non-specific manner, breaking the wall and cell membrane, with consequent extravasation and bacterial death [30]. In this sense, photodynamic processes become an alternative, since in a non-specific way, it acts on the main physical barrier of defense of the genus, conferring success in the treatment of infections caused by mycobacteria [35,47].

Infections involving *M. fortuitum*, *M. massiliense* or *Mycobacterium marinum* are cited for their high potential associative to the number of skin-related infectious processes [48–50]. Cases reporting the use of PDI versus cutaneous infections caused by NTM are punctual and rarely cited, but described as a powerful treatment alternative [22]. The same author reported significant improvement after two sections of light exposure using ALA-PDT as PS, with a reduction of up to 78.9% in the

Table 3

MIC values for cationic porphyrin (TMePyP⁺) without and with ROS scavengers, at white-light irradiation conditions (90 min; white LED; 50 mW/cm²) compared to RGM strains.

Microorganism	MIC values (μM)			
	without scavengers		with ROS scavengers	
	Absence	Ascorbic Acid	Potassium iodide	Mannitol
<i>Mycobacterium fortuitum</i>	0.391	25.0	6.250	0.780
<i>Mycobacterium massiliense</i>	0.391	25.0	6.250	1.562

M. fortuitum count in the patient.

4.1. Charge-effect of cationic and anionic porphyrin

By analyzing the results obtained in the susceptibility test of the RGM strains against TMePyP⁺ and TPPS⁻, a higher antimycobacterial activity of cationic derivative TMePyP⁺, with MIC value, was observed for the two mycobacterial strains, under the two conditions tested, lower than those obtained for the TPPS⁻ analogue. Still on incubation conditions, TMePyP⁺ plates submitted to white-light illumination varied with the increase of the time of luminosity, in addition to suffering a reduction of MIC over 100 times with the increase of the time of exposure to light, while the TPPS⁻ plates were not influenced by different lighting irradiation times, but had their MIC reduced by half when illuminated.

It is believed that the superior antimycobacterial activity of positively charged TMePyP⁺ against RGM strains occurs due to a possible selection of bacterial membrane load, as mentioned by Feese and Ghilandi [51], that through fluorescence spectroscopy correlated the antimicrobial activity of the compounds with the degree of association with the microorganism. It is known that anionic compounds, including negatively charged TPPS⁻, do not demonstrate a great potential for association with the membrane of mycobacteria and together with this, little or no ability to bacterial lysis, unlike cationic porphyrins, such as TMePyP⁺, which tend to (Eqs.), as well as the presence of ROS (Table 1). Still in this context, in a study with *Mycobacterium smegmatis*, TMePyP⁺ despite not having the highest degree of association with the membranes, compared to other cationic porphyrins, obtained the highest activity in the treatment of this strain, even in low periods of white-light irradiation. Therefore, although the positive partial load is related to the activity and degree of adhesion to the mycobacterial membrane, this should not be the only mechanism that contributes to treatment efficacy [46,51].

From the susceptibility assay, the behavior of mycobacteria against PS was evaluated. Due to the higher antimycobacterial action of TMePyP⁺, it was selected for the dose-response curve assay. In this study the bactericidal character of the PS was observed, from 24 h for *M. massiliense* and from 48 h for *M. fortuitum*. In addition, the curves demonstrate photoinactivation potential of viable bacterial cells dependent on time and dose of illumination applied. It is observed that in the subinhibitory concentrations (½ and ¼ MIC values) photoinactivation significantly reduces bacterial growth. This can be attributed to the limited concentration of ROS produced during the process, which are able to photo-oxidize a restricted number of bacterial cells, thus explaining the decrease in counting relative to the positive control. This result is similar for both strains and at all times and concentrations tested, but more evident for *M. fortuitum*.

Moreover, two times MIC did not show great changes in the results for *M. massiliense* presenting the curve similar to the MIC concentrations. For *M. fortuitum* at longer lighting times, the curves corresponding to MIC and 2xMIC were equivalent, demonstrating the need for repeated doses of light for the formation of ROS and consequently bacterial death. However, in the shortest time (15 min) For *M. fortuitum* bacterial death was observed in only one light exposure (24 h) and

could be related to the highest concentration used in the experiment (3.125 μM).

Similarly, Sung and collaborators [47] investigated the anti-tuberculosis activity by PDI in clinical strains of multidrug-resistant *M. tuberculosis* and observed that the therapy only obtained antimycobacterial activity through prolonged and repeated lighting times. Therefore, considering that the mycobacterium genus has a milder growth rate than most pathogenic mesophiles, it is necessary to increase the dose of lighting or increase the irradiation intervals to follow the growth time of these microorganisms and consequently to obtain efficacy in the treatment.

5. Conclusions

This work demonstrates that tetra-cationic porphyrins can be considered as promising PS for the inactivation mycobacteria although the performance could be highly variable between molecules with different N-alkyl groups. The higher singlet oxygen production (comparing cationic to anionic commercially porphyrin), more photostable, lower aggregation in aqueous solution and a more effective irradiate effects with the mycobacteria are the most likely factors underlying the better performance of tetra-cationic(N-methylated)porphyrin against *M. massiliense* and *M. fortuitum*. The current treatment proposed in MCR skin infections is expensive, costly and leads to many adverse effects due to the systemic use of antimicrobials. The photo-inactivation process presents as a promising alternative in this case, especially in topical applications in the mycobacterioses, since it acts directly on the microorganisms. This targeting, mediated by ROS, results in a reduction in the treatment time of the patient, as well as acting in low doses on the membrane and lead more easily to lysis and later cell death. Its use can also be applied to deep infections and poorly perfused tissues and is limited to adjuvant therapy in antimicrobial treatment. The results of this preliminary work confirm the potential of cationic porphyrin derivatives for antimicrobial PDI in broad-field applications such as clinical medicine and bioremediation, related to human health.

Ethical approval

None.

Conflicts of interest

None.

Acknowledgments

This study was financed by CNPq, CAPES and FAPERGS. Bernardo A. Iglesias thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil; Universal Grants 409150/2018-5) and Marli Matiko Anraku de Campos thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil; Grants 404541/2016-0).

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