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Evaluation of resistance development and viability recovery by a non-enveloped virus after repeated cycles of aPDT

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ABSTRACT

Nowadays, the emergence of drug resistant microorganisms is a public health concern. The antimicrobial photodynamic therapy (aPDT) has an efficient action against a wide range of microorganisms and can be viewed as an alternative approach for treating microbial infections. The aim of this study was to determine if a model target virus (T4-like bacteriophage), in the presence of the tricationic porphyrin 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py$^+$-Me-PF), can develop resistance to aPDT and recover its viability after photodynamic treatments. To assess the development of aPDT resistance after repeated treatments, a suspension of T4-like bacteriophage was irradiated with white light (40 W m^{-2}) for 120 min in the presence of 5.0 μM of Tri-Py$^+$-Me-PF (99.99% of inactivation) and new phage suspensions were produced from the surviving phages, after each cycle of light exposure. The procedure was repeated ten times. To evaluate the recovery of viral viability after photoinactivation, a suspension of T4-like bacteriophage was irradiated with white light for 120 min in the presence of 5.0 μM of Tri-Py$^+$-Me-PF on five consecutive days. In each day, an aliquot of the irradiated suspension was plated and the number of lysis plaques was counted after 24, 48, 72, 96 and 120 h of dark incubation at 37 °C. The profile of bacteriophage photoinactivation did not change after ten consecutive cycles and no recovery of viability was detected after five accumulated cycles of photodynamic treatment. The results suggest that aPDT represents a valuable and promising alternative therapy to treat viral infections, overcoming the problem of microbial resistance.

1. Introduction

The introduction of antimicrobial drugs in clinic, in the 1930s, coupled with the use of safe and effective vaccines, suggested that infectious diseases could be controlled and, eventually, eradicated. Although antimicrobials have saved countless lives and transformed the practice of medicine, the initial widespread optimism has proven to be premature and infections still remain the leading cause of mortality worldwide. Pathogenic microorganisms are widely spread in nature, there are numerous infection sources, and the unfit and prolonged antibiotic and antiviral treatments have led to greater microbial resistance to these substances.

Antimicrobial-resistant pathogens pose an enormous threat to the treatment of a wide range of serious infections. Nosocomial and community-acquired agents have developed resistance to a wide range of antimicrobials and have proven to be highly successful in their ability to develop resistance mechanisms, often transferable, against virtually all commonly used antimicrobial drugs (Kimberlin and Whitley, 1996; Pillay, 1998; Taylor et al., 2002; Hamblin and Hasan, 2004; Maisch, 2007; Wainwright, 2009).

Although there are now many different antimicrobial drugs on the market, the vast majority of antimicrobials are antibacterials and, consequently, resistance to antimicrobial drugs is now well documented in bacteria (Nishijima et al., 1993; Cookson, 1998; Kömerik et al., 2000; Fridkin, 2001; Sievert et al., 2002; Konopka and Goslinski, 2007; Maisch, 2007). Human and animal viruses can also develop resistance to antiviral drugs. This may not seem an issue of major concern due to the lack of antivirals available, but it should be kept in mind that the use of antiviral drugs is increasing and that viruses, being genetically flexible, may mutate quickly and, consequently, the emergence of antiviral drug resistance will come as no surprise and will be an everlasting issue. Moreover, the growing availability of antiviral drugs and the larger access to combination therapy can lead to the transmission of resistant viruses. Transmission of viruses with major resistance mutations may indeed result in partial virological response or even therapy failure (Tamalet et al., 2000; Duwe et al., 2001).

The emergence of antiviral drug resistance requires the research of new alternative methods unlikely to cause resistance. Photodynamic inactivation of microorganisms represents a potential alter-

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native to meet that need. The main advantages of aPDT are the non-target specificity and the potential absence of resistance mechanisms due to the mode of action and type of biochemical targets (multi-target process) (Jori et al., 2006; Winckler, 2007). Both oxidative mechanisms responsible for the photoinactivation of microorganisms, i.e., type I and type II pathways (Wainwright, 1998; Calin and Parasca, 2009), lead to highly toxic reactive oxygen species (ROS), such as $^1O_2$ and free radicals (superoxide and hydroxyl radical), that are able to irreversibly alter microorganisms' vital constituents, resulting in oxidative lethal damage (DeRosa, 2002; Ergaieg et al., 2008).

The main targets of the viral photodynamic activity are external structures, such as capsid proteins and envelope lipids, and nucleic acids (Vzorov et al., 2002; Egyeki et al., 2003; Wainwright and Crossley, 2004; Zupán et al., 2004). The damages to the external microbial structures are sufficient to photoinactivate the target microorganism through leakage of microbial contents or inactivation of enzymes (Li et al., 1997; Mettath et al., 1999). As the main targets of aPDT are the external microbial structures, the photosensitizer (PS) does not need to penetrate in the microorganism, having little possibility to create or operate any kind of anti-drug mechanisms (Winckler, 2007).

Until now, the majority of research in this field was done essentially with bacterial strains. Photosensitization-resistant mutants have not been found for bacteria (Lauro et al., 2002; Oliveira et al., 2009; Pedigo et al., 2009). *Actinobacillus actinomycetemcomitans*, *Peptostreptococcus micros*, *Vibrio fischeri* and *Escherichia coli* did not develop resistance to aPDT after at least ten cycles of subtotal photodynamic inactivation (Lauro et al., 2002; Pedigo et al., 2009; Tavares et al., 2010). Besides that, both *V. fischeri* and *E. coli* were unable to recover their metabolic activity (Tavares et al., 2010). Moreover, all studies that have examined the inactivation of antibiotic resistant bacteria by aPDT, have found them to be as susceptible as their native counterparts (Wainwright, 1998; Embleton et al., 2004; Maisch et al., 2005; Trannoy et al., 2006; Maisch, 2007; Grinholc et al., 2008;). In spite of all the work with antibiotic bacteria, however, there are few studies of aPDT using antiviral resistant strains of viruses. The PS benzoporphyrin derivative was investigated regarding its ability to destroy AZT-resistant strains of HIV-1. The results showed that AZT-resistant strains appear to be as susceptible to photodynamic inactivation as AZT-sensitive strains of HIV (North et al., 1994). Although the recovery of viral viability of hepatitis C virus after aPDT has already been tested (Cheng et al., 2010), studies concerning the development of resistance after repeated exposure to photodynamic treatment have not been performed yet.

The aim of this study is to determine if a model target virus (T4-like bacteriophage), in the presence of the tricationic porphyrin 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py$^+$-Me-PF), a very promising PS, can develop resistance to aPDT after repeated photodynamic cycles and recover its viability after photodynamic treatments. The T4-like phage was chosen because this phage is used as model system for understanding general features of viral multiplication (Madigan and Martinko, 2006) besides having some important characteristics that make it useful as an indicator of enteric viruses, namely the same lifetime, similar structure, size and morphology.

2. Material and methods

2.1. Photosensitizer

The tricationic porphyrin Tri-Py$^+$-Me-PF (Fig. 1) used in this work was prepared in accordance to the literature (Carvalho et al., 2010). The tricationic porphyrin Tri-Py$^+$-Me-PF was selected as PS because this porphyrin, already described by our group, has shown promising results on the photoinactivation of several types of microorganisms (Costa et al., 2008; Oliveira et al., 2009; Carvalho et al., 2010; Tavares et al., 2010).

2.2. Phage selection and quantification

A sample of wastewater from a secondary-treated sewage plant near the city of Aveiro (Portugal) was used to select the somatic bacteriophages of *E. coli* C (ATCC 13706) (Costa et al., 2008). The quantification of bacteriophages was conducted, in duplicate, by the agar double layer technique (Adams, 1959) using the aforementioned strain of *E. coli* (Costa et al., 2008).

2.3. Photoinactivation assay

Phage photoinactivation by Tri-Py$^+$-Me-PF, at the concentration of 5.0 μM, was achieved by exposing the T4-like phage in laboratory conditions to white light (13 fluorescent lamps OSRAM 21 of 18 W each, 380–700 nm) with a fluence rate of 40 W m^{-2} (measured with a light meter LI-COR Model LI-250) during defined time intervals, with agitation (100 rpm). Dark and light controls were also included in the experiment and were carried out simultaneously. In the light control (LC), the phage suspension without PS was exposed to the same irradiation protocol. In the dark control (DC), the beaker containing the phage suspension and the PS at the studied concentration (5.0 μM) was covered with aluminium foil to protect it from light exposure. Sub-samples of 1 mL of test and control samples were aseptically taken at times 0, 60, 90, 180, and 270 min. The rate of phage inactivation was evaluated through the quantification of the number of phages as previously referred (Costa et al., 2008). Two independent experiments were carried out with two replicates each.

2.4. aPDT resistance assay

To check for the development of resistance to aPDT, new phage suspensions were produced after each cycle of exposure to photodynamic treatment. In order to obtain a modest phage inactivation, the phage suspension in the presence of Tri-Py$^+$-Me-PF (5.0 μM) was exposed to white light in cycles of 120 min (28.8 J cm^{-2}) in the same conditions of the aforementioned photoinactivation assay. This will allow to test if the phages affected by the PS, though not in such a drastic way as occurs when they are irradiated for a long period (270 min), are able to develop resistance to aPDT. After each cycle of 120 min, sub-samples of 1 mL were aseptically taken and plated by the double agar layer technique. Three phage plates were picked up with an inoculation loop and added to 1 mL of an

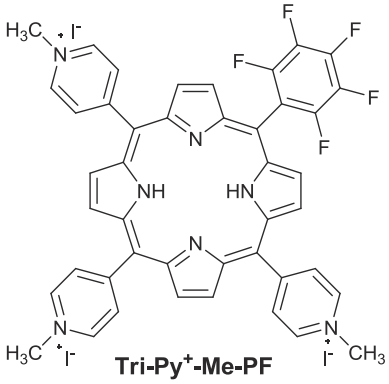

Fig. 1. Structure of the 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide used in this study.

E. coli exponential phase culture in TSB medium. After 4–5 h of incubation at 37 °C, the suspension was centrifuged at 10,000*g* during 10 min. The pellet was discharged and the supernatant was collected into a new tube, and kept as a new phage stock. An aliquot of 50 μL of the new phage stock was diluted 1:100 in PBS and submitted to a new cycle of photodynamic treatment, using the previously described irradiation protocol. Phage enumeration was also performed as previously described. Dark and light controls were included in all inactivation cycles. This procedure was repeated for ten consecutive cycles. The experiment was performed three times and the results were presented as the average means.

2.5. aPDT viability recovery assay

Phage photoinactivation in the presence of 5.0 μM Tri-Py$^+$-Me-PF was achieved by exposing the phage to white light (40 W m^{-2}) irradiation in the conditions described above for 120 min in order to obtain only a modest phage inactivation. This procedure allows us to check if the phages affected by the PS, though not in such a drastic way as occurs when they are irradiated during 270 min, are able to recover their viability. Dark and light controls were also prepared as described above and included in all inactivation cycles. Five sub-samples of 1 mL of the irradiated phage suspension in the presence of PS, LC and DC samples were aseptically taken at time 120 min and plated by the double agar layer technique. All petri plates were incubated at 37 °C, in the dark, and the number of lysis plaques was counted in each of the five petri plates, after 24, 48, 72, 96 and 120 h, in order to detect the delayed development of lysis plaques. The remaining phage suspensions, after the first cycle of irradiation, were kept at room temperature in dark conditions for 24 h. After this period, phage suspensions were submitted to a new cycle of irradiation (120 min, 40 W m^{-2}) and sub-samples taken and plated following the same procedure. This methodology was repeated five times intercalated by 24 h of dark incubation at room temperature.

2.6. Statistical analysis

SPSSWIN 15.0 was used for data analysis. The significance of differences in phage inactivation was assessed using one-way ANOVA. Only the data with normal distribution (assessed by Kolmogorov-Smirnov test) and with homogeneity of variances (assessed by Levene test) was used. A value of $p < 0.05$ was considered significant. All experiments were done at least in duplicate.

3. Results

3.1. Bacteriophage response to photoinactivation

After 270 min of white light irradiation, in the presence of 5.0 μM Tri-Py$^+$-Me-PF, the phage was almost completely inactivated (∼7 log) (Fig. 2). Consequently, in the aPDT resistance studies and recovery assays, a shorter irradiation time (120 min) was used, in order to obtain a modest phage inactivation (∼3 log). It was possible, with such procedure, to obtain a sizeable number of lysis plaques, which were used to study the possible development of resistance and phage recovery.

The results of light and dark controls show that phage viability was neither affected by irradiation itself nor by the direct effect of the PS in the dark (Fig. 2). This indicates that the reductions obtained on phage survival after irradiation of the treated samples are only due to the photosensitizing effect of the porphyrin.

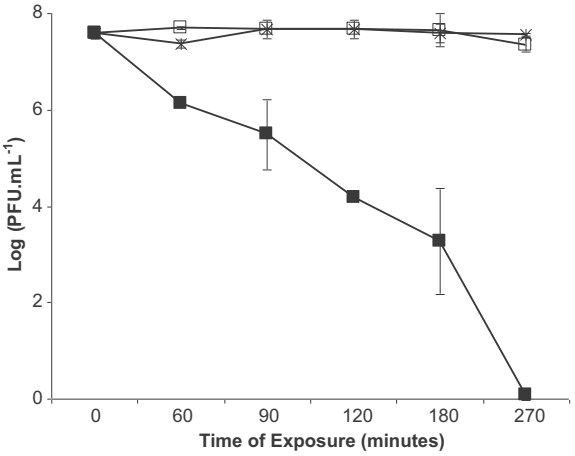

Fig. 2. Variation of the number of bacteriophages after irradiation with white light (40 W m^{-2}) in the presence of Tri-Py$^+$-Me-PF at 5.0 μM. (open square: LC, asterisk: DC, filled square: 5.0 μM). The values are expressed as the mean of two independent experiments; error bars represent the standard deviation (small bars are overlapped by the symbols).

3.2. aPDT resistance study

The rate of phage inactivation in ten consecutive treatments with Tri-Py$^+$-Me-PF at 5.0 μM was almost the same (Fig. 3). The number of surviving phages was approximately the same in all cycles of irradiation (∼5 log of surviving phages). That means no appreciable development of resistance in partially inactivated phages was observed. In fact, the efficiency of photoinactivation underwent no significant change in ten subsequent irradiation cycles (ANOVA, $p > 0.05$). In each phototreatment, the differences between replicates were not significant either (ANOVA, $p > 0.05$).

3.3. aPDT viability recovery study

After the first cycle of partial photodynamic inactivation, the phage numbers were reduced to ∼4 log and the number of lysis plaques did not vary significantly (ANOVA, $p > 0.05$) for each sample during the following 120 h (5 days) of incubation at 37 °C in the dark (Fig. 4a). After the second cycle of irradiation, the phage numbers were reduced to ∼2 log and, as for the first cycle, the number

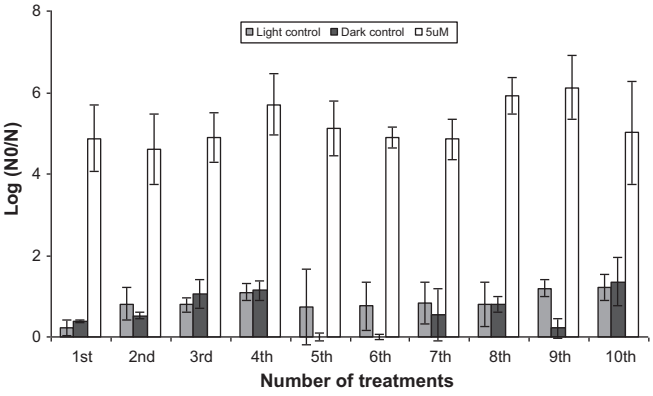

Fig. 3. Photodynamic inactivation efficiency during ten consecutive T4-like phage generations, in presence of 5.0 μM of Tri-Py$^+$-Me-PF after 120 min of irradiation with white light (40 W m^{-2}). The values are expressed as the mean of three independent experiments (three different phage plates); N_0 represents the number of initial phages determined by phage plaque counts before the irradiation and N represents the number of surviving phages determined by phage plaque counts after irradiation of each experiment; error bars represent the standard deviation.

of phage plaques did not increase after 120 h of incubation (ANOVA, $p > 0.05$) (Fig. 4a). After the third cycle of irradiation, the phages were inactivated to the detection limit and the formation of new lysis plaques was not observed during the following 120 h. The number of lysis plaques was similar in both controls during the 120 h of incubation at 37 °C, in the five cycles of irradiation (ANOVA, $p > 0.05$).

4. Discussion

In this study, it was demonstrated that: (1) the tricationic porphyrin Tri-Py$^+$-Me-PF has antiviral activity and effectively inactivates T4-like phages (reduction of ~7 logs) after irradiation with white light. The inactivated phages do not recover their viability after 120 h of dark incubation; (2) viruses that survived to short treatments, in the presence of 5.0 μM Tri-Py$^+$-Me-PF, do not develop resistance after ten repeating cycles of aPDT.

In general, the development of resistance to photosensitization by microorganisms should be considered as an unlikely event, because this is typically a multi-target process, which marks the difference between aPDT and the mechanism of action of most antimicrobial drugs (Maisch et al., 2004). Moreover, it is questionable if microorganisms can develop resistance to ROS, which mediate photodynamic inactivation. Up to now, there are only a few reports concerning a potential specific resistance mechanism against ROS. Although antioxidant enzymes, such as superoxide dismutase, catalase and peroxidase, give protection against some ROS, they do not protect against singlet oxygen (Wainwright and Crossley, 2004) that, according to the literature, is the main ROS through which the PS exert their photodynamic action (Nitzan et al., 1989; Müller-Breitkreutz et al., 1995; Hadjur et al., 1998; Maisch et al., 2005; Maclean et al., 2008). Moreover, these enzymes are also inactivated by singlet oxygen (Kim et al., 2001). The results of this study corroborate the literature (Lauro et al., 2002; Tavares et al., 2010) in what concerns to the absence of resistance mechanisms in bacteria exposed to photodynamic inactivation events. This finding is of particular interest mostly because the effect of consecutive phototreatments on the possible gain of viral resistance had not yet been addressed.

The results of the resistance assay show that, after ten consecutive phototreatments, the T4-like phage did not exhibit any evident signs of resistance to aPDT. The porphyrin Tri-Py$^+$-Me-PF can potentially lead to simultaneous binding to several components/subunits of the viral capsid and to the nucleic acid (DNA), avoiding the development of resistance to photosensitization. The virus would require multi-site mutations to become resistant, an event with significantly lower probability than single-site mutation which is often sufficient for conferring resistance to small molecule inhibitors.

When the phage was subjected to ten consecutive aPDT cycles, the fraction of non-inactivated viruses did not show strong variation between cycles. After the first exposure to white light, the phage plaques produced after irradiation of the viral suspension were used to produce new phage stocks in the presence of host bacterial cells. After ten consecutive sessions of 120 min with 5.0 μM Tri-Py$^+$-Me-PF, any reduction in the efficacy of the photosensitization of T4-like phage was observed. If phage resistance to aPDT would occur, significant reductions on phage photoinactivation efficiency would be detected between experiments. This would be interpreted as an indication of enhanced resistance of the phages of later generations in relation to the ones in the initial stocks. However, this was not observed, thus invalidating the hypothesis of the acquisition of resistance to aPDT by bacteriophages. Although there were some variations between treatments and even between the three replicas of each treatment, these differences were not significant (ANOVA, $p > 0.05$) and were possibly due to different concentrations of the distinct suspensions of phages produced before each cycle of photoinactivation.

The multi-target property of aPDT can also explain the irreversible effects on the bacteriophage viability by the tricationic porphyrin Tri-Py$^+$-Me-PF after 120 min of irradiation. The T4-like bacteriophage was irradiated for 120 min in order to obtain only a modest phage inactivation. This allowed us to test if the phages were affected by the PS, though not in such a drastic way, as happened when irradiated for long period of time (270 min) during which all phages are inactivated and are able to recover their viability. During 120 h of incubation after phototreatment, the phages, by being in the presence of their host, have all the necessary conditions to recover from the photodynamic treatment. If new phage plaques would appear in the petri plates during the 5 days of dark incubation it would mean that the bacteriophages, previously not able to produce plaques, after the "lethargy period" become competent to recover from the aPDT treatment. However, the phages were not able to recover their viability in the 120 h post-phototreatment period. This indicates that when the phages are efficiently inactivated they cannot recover from the photodynamic effect. Using a period of irradiation of 120 min, the phages were inactivated to the limits of detection only after three consecutive cycles and did not recover within 120 h. This result indicates that, although one cycle of 120 min of photodynamic treatment with Tri-Py$^+$-Me-PF is not enough to inactivate all the phages in the suspension, the phages inactivated after this time do not recover their viability. This is in accordance with what was found for hepatitis C virus, which did not recover its viability after exposure to 5,10,15,20-tetraki(3,5-dicarboxy-4,4'-biphenyl)porphyrin (Cheng et al., 2010).

In conclusion, the results of this study show that Tri-Py$^+$-Me-PF has potential use as an efficient alternative to conventional antimicrobial drugs to treat viral infections. With this PS, viral resistance

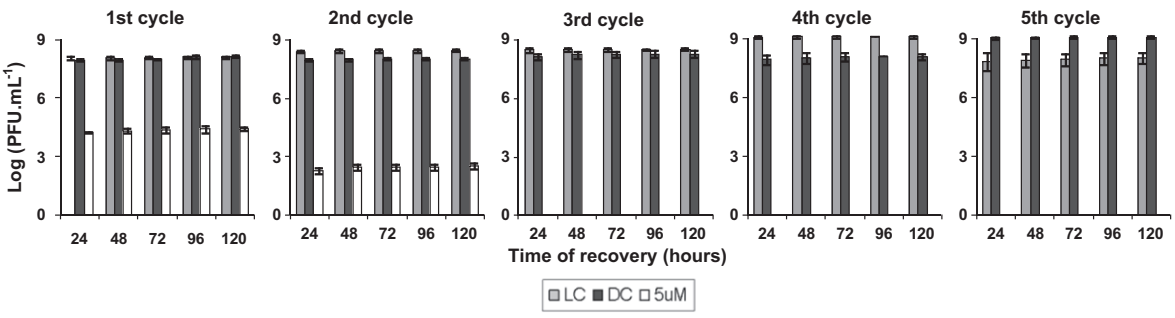

Fig. 4. Variation of the number of T4-like bacteriophages after irradiation with white light (40 W m^{-2}) during 120 min in presence of Tri-Py$^+$-Me-PF at 5.0 μM, after 24, 48, 72, 96 and 120 h, for five cycles of treatment. For each cycle five petri plates were used for both controls and for the sample. Error bars represent the standard deviation.

and the potential recovery of viral viability after photodynamic treatment are not a problem.

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