Journal of Photochemistry & Photobiology, B: Biology 186 (2018) 107–115

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Side effects of intra-gastric photodynamic therapy: an *in vitro* study

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ARTICLE INFO

Keywords:
Photodynamic therapy
Helicobacter pylori
Side effects
Intragastric illumination device
Oxidative stress
Antibiotic resistance

ABSTRACT

Since many years it has been acknowledged that some bacterial species, among which *H. pylori*, *P. aeruginosa*, *P. acnes* accumulate endogenous photosensitizers (PS) in the form of porphyrins. This makes antibacterial photodynamic therapy (PDT) easier to perform due to the possible avoidance of external PS. In this study, we focus on gastric infections associated with the presence of *Helicobacter pylori* (*H. pylori*), known to accumulate and release both protoporphyrin IX (PPIX) and coproporphyrins. PDT versus *H. pylori* can be carried out by modified endoscopes or by new ingestible luminous devices under development.

In both cases of *in vitro* and *in vivo* applications, either for therapy (PDT) or diagnosis, scientific literature lacks studies on the possible side-effects of light treatments on the surrounding tissues. To this aim we evaluated *in vitro* side-effects due to a possible intrinsic photosensitivity of gastric mucosa or to a photosensitization by the PS released from the bacterium itself. Photo-toxicity studies were conducted on the AGS cell line (ATCC® CRL-1739™), commonly used as a model for the stomach mucosa tissue, considering PPIX as the photosensitizing agent. After first evaluations of PPIX dark toxicity, its uptake and accumulation sites, photo-toxicity tests were conducted using a LED light source peaked at 400 nm, by varying both PPIX concentration (50 nM – 2 μM) and light dose in the range 0.6–13 J/cm^2, representing different treatment procedures found in literature. The oxidative stress consequent to irradiation was investigated both in terms of ROS production and assessment of the activity of enzymes involved in ROS-related biological mechanisms. A significant phototoxic effect was found only for PPIX concentration > 100 nM for all tested light doses. This indicates that the evaluated photo-treatments do not cause side effects even with the sensitization due to PPIX released by the bacteria.

1. Introduction

Microbial resistance is a topic of great interest for biomedical research being it a threat to public health due to infections persistence, the increasing spreading and development of antibiotic resistance [1]. Therefore antibacterial therapies alternative to antibiotics are becoming a very important issue in the medical field. Among them, photodynamic therapies (PDT) are getting an increased interest having the advantage of being highly selective because their effects are mainly local [2].

PDT involves absorption of light by an adequate endogenous or exogenous photosensitizer leading to formation of reactive oxygen species (ROS) which can destroy biological molecules causing cell death and tissue necrosis. Besides cancer, atherosclerosis and skin diseases [3, 4], PDT is being investigated also as an antimicrobial therapy, for the treatment of infectious diseases including those caused by both Gram-negative and Gram-positive bacteria. It has been demonstrated that some bacteria, i.e. *P. acnes*, *H. pylori*, and others naturally synthesize or accumulate photosensitizing molecules: protoporphyrin IX (PPIX, a precursor of heme) and coproporphyrin (CP) which allow PDT without the need of any exogenous photosensitization [5–16]. *H. pylori* accumulates a mixture of CP and PPIX with a principal absorption peak at 415 nm and other absorption bands at wavelengths between 550 nm and 630 nm [16]. The function of PPIX as an exogenous photosensitizer for PDT is well known and in many cases its synthesis and accumulation in tissues or microorganisms are stimulated by the administration of 5-aminolaevulinic acid, 5-ALA (precursor of PPIX) [4, 17–19].

In this work, we concentrate on antimicrobial PDT applied to *H. pylori* infection. This bacterium is a pathogenic agent of group 2 responsible for severe gastric diseases such as atrophic gastritis, peptic ulcer, MALT lymphoma and adenocarcinoma demanding new therapeutic strategies for its large diffusion and its increasing antibiotic resistance rates [20].

For this reason, investigations have already been carried out on the

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https://doi.org/10.1016/j.jphotobiol.2018.07.010
Received 7 June 2018; Received in revised form 5 July 2018; Accepted 11 July 2018
Available online 12 July 2018
1011-1344/

efficacy of PDT against *H. pylori* in vitro [13, 21, 22] and preliminary clinical studies have also been conducted in patients infected by *H. pylori*: in 2002 Wilder-Smith [23] illuminated the gastric antrum of 13 patients, those have received orally 5-ALA, through an endoscope with a blue laser and after the treatment a greater reduction of *H. pylori* in biopsies from illuminated areas compared to control zones was evident. In 2005 Ganz [14] and in 2009 Lembo [24] showed remarkable reduction of antrum bacterial load after endoscopic treatment with a violet light source only. This treatment modality is invasive and associated with a poor patient compliance. In this framework alternative devices are under study and development [25–27], among which an ingestible LED-based pill for intragastric *H. pylori* phototherapy developed by our group [25, 26].

The novelty, efficacy and also the variety of the PDT therapeutic approaches require studies, now practically lacking, on the possible side effects due to the photosensitization of healthy gastric tissue by exogenous photosensitizers [2, 23] or by the ones released in the surrounding tissues from *H. pylori*. To this aim, we have evaluated the effects of 400 nm photo-treatments on AGS cell line (ATCC CRL-1739, derived from a gastric adenocarcinoma and commonly used as model of normal stomach mucosa) previously sensitized with PPIX. In particular we set the following purposes: 1) to find the minimal harmful dose of the photo-treatment in the presence of photosensitizer both in terms of irradiated energy and photosensitizer concentration; 2) to verify whether we could expect a toxic side-effect when intragastric PDT treatments, including for example the one of the luminous capsule presented in [25, 26], are performed; 3) to carry out a preliminary analysis of the cell response to the induced oxidative stress. First, the cytotoxicity of PPIX alone was evaluated by means of viability assays and proliferation assessment. PPIX uptake and intracellular accumulation sites were investigated by fluorescence microscopy and fluorimetry. The photo-treatment dose-effect curve as a function of the photosensitizer concentration and irradiated energy was built. Cell loss and viability after irradiation were evaluated by standard tests and the oxidative stress due to irradiation was investigated both in terms of ROS production and assessment of the enzymatic activity related to their detoxification.

2. Materials and Methods

2.1. Cell Line

AGS cells (ATCC CRL-1739) were grown in F-12 Kaighn's modification medium (Hyclone, GE Healthcare Lifesciences, USA) supplemented with 10% fetal bovine serum (Biowest, France) at 37 °C in a 5% CO_2 humidified atmosphere. Cells (1.0×10^6) were seeded in 100 mm Falcon dishes and propagated every 2 days by incubation for ~5 min with a 0.25% trypsin/EDTA solution (SIGMA, Italy). Cultures were periodically tested for Mycoplasma contamination.

2.2. PPIX Uptake and Intracellular Accumulation Sites

Evaluation of PPIX uptake and intracellular accumulation was performed by fluorescence microscopy (both confocal and wide field) and fluorimetry. Cells were cultivated on a slide, incubated overnight with 2 μM PPIX (SIGMA, Italy), then washed and fixed with 4% formalin. Slides were then mounted with an aqueous mounting medium (VectaMount AQ, Vector Laboratories, Burlingame, USA). Slide observations were conducted with a Leica Sp8 confocal microscope (Leica, Mannheim, Germany) and with a Nikon inverted microscope (Nikon eclipse Ti) by exploiting the PPIX fluorescence (excitation: ~405 nm, emission: ~650 nm). The fluorimetric determination of PPIX uptake was realized by overnight cell incubation in a 96 multiwell plate with various PPIX concentrations in the range 50 nM-2 μM (prepared diluting in colture medium a 5 mM PPIX standard solution in dimethylsulfoxide, DMSO) and successive measurement of the average fluorescence signal by a multiplate reader (Infinite M200PRO, Tecan, Switzerland). Fluorescence was measured either leaving the incubation medium in cell cultures or after PBS washing.

2.3. Photo-treatment

All light irradiation experiments were conducted using a fiber-coupled LED lamp (Nikon CoolLED) peaked at $\lambda = 400$ nm (corresponding to the main PPIX absorbance peak) with a beam expander and diffuser that guaranteed a ~7 cm circular spot with average intensity of 4.8 mW/cm^2 and a uniformity of ~15%, measured as maximum intensity variation respect to the mean value inside the spot surface. Irradiation times ranged from 2′ to 45′ giving an irradiated energy (hereafter "light dose" = D) in the range 0.6–13.0 J/cm^2.

2.4. Dark PPIX Toxicity

Both Trypan Blue assay and MTT test were performed. After incubation with PPIX, cells were PBS washed and detached with trypsin/EDTA. Trypan Blue assay was carried out on a portion of the collected cells, with dilution factor of 2 and after 5 min of incubation, using the Bürker hemocytometer. MTT test: cells were seeded in a 96 multiwell plate at a density of 10^4cells/well two days before treatment. Cell culture medium with PPIX (concentration ranging from 100 nM to 500 μM) was prepared as reported above. For each PPIX concentration three samples were prepared: a control sample with no PPIX, PPIX-incubated sample and a third sample incubated with the same amount of DMSO of the sample with that PPIX concentration. After PPIX incubation the cells were incubated with 1 mM thiazolyl blue tetrazolium bromide (SIGMA, Italy) in culture medium for 40 min in the dark. The medium was then removed and DMSO was added to dissolve formazan crystals. The absorbance signal at 570 nm was read on the multiplate reader, the background absorbance at 630 nm was subtracted from signal absorbance to obtain normalized absorbance values.

2.5. Cell Viability after PPIX Sensitization and Light Treatment

AGS cells were incubated overnight with PPIX concentration ranging from 50 nM to 2 μM and then exposed to photo-treatments with wavelength, intensity and treatment times previously indicated. For each PPIX concentration two samples were prepared: irradiated and non-irradiated (control). Trypan Blue assay and MTT test were performed after a delay time of 2 h from the end of irradiation. This delay time was chosen as a timescale to investigate the cell damage and was fixed to this value for all subsequent tests.

2.6. Cytofluorimetric Analysis of Cell Cycle

Collected cells after trypsinization were stained with propidium iodide according to the method of Vindeløv [28]. Samples were analysed using a FACScan flow cytometer (Becton Dickinson). Quantification of cells in the different cycle phases was performed by CellFit software (Becton Dickinson).

2.7. Apoptosis/Necrosis Assay

Cell death was quantified by means of cytofluorimetric analysis, using Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (ThermoFisher SCIENTIFIC). This assay was performed with a 500 nM PPIX sensitization and 5′ irradiation at 4.8 mW/cm^2 and a delay time between photo-treatment and test of both 2 and 4 h. This treatment was chosen, among the investigated ones, because it causes a neither too low nor to high viability decrease.

2.8. Intracellular ROS Production Assay

The level of intracellular ROS was quantified by fluorescence with