Inactivation of microbial cells by photosensitized processes

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The problem of microbial infections

- The treatment of microbial infections is a challenging field because of rapid evolutionary changes and the large variety of pathogens.

- Inappropriate and irrational use of antimicrobial medicines provides favourable conditions for resistant microorganisms to emerge, spread and persist.

- The expansion of poverty areas where prophylactic measures are hard to be adequately applied, favours the transmission of infectious diseases.
Antimicrobial resistance

- Infections caused by resistant microorganisms often fail to respond to conventional treatment, resulting in prolonged illness and greater risk of death.

- About 440,000 new cases of multidrug-resistant tuberculosis (MDR-TB) emerge annually, causing at least 150,000 deaths.

- A high percentage of hospital-acquired infections are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA).

- Onset of MRSA strains with reduced susceptibility to vancomycine considered last line of defense.
The rise of antibiotic resistance has led to widespread prediction of the “end of the antibiotic era”

Search for alternative antimicrobial therapies

Examples of these relatively novel therapies are bacteriophages, naturally occurring or synthetic antimicrobial peptides and PDT.

Photodynamic Therapy as an alternative to combat Microbial Infections

Recent findings support that PDT can represent a viable alternative since the mode of action of photosensitizer on microbial cells is markedly different from most antibiotic drugs. Because the delivery of visible light is almost by definition a localized process, PDT for infections is likely to be applied exclusively to localized diseases.
Problems to be addressed in developing effective protocols for antimicrobial PDT

- Several infections are characterized by a mixed microbial flora.
- Broad heterogeneity of cell morphology and metabolic properties.
- Presence of highly organized outer walls with protective function.
**Gram-positive** bacteria are surrounded by an outer wall mainly constituted by peptidoglycan, lipoteichoic and teichoic acids. This wall displays a relatively high degree of porosity that allows the crossing of molecules having a molecular weight larger than 60,000 Dalton.

The photoinactivation is due to the translocation of Sens to the inner plasma membrane which is the critical target for the induction of photodamage.

**Gram-negative** bacteria possess an additional structural element, constituted by lipoproteins, lipopolysaccharides, teichoic and lipoteichoic acids that provide the outer surface with a quasi-continuum of densely packed negative charges. This highly organized system inhibits the penetration of compounds with molecular weight larger than 600–700 Dalton.

To make easier the photoinactivation processes is necessary to devise suitable strategies which enhance the permeability of the outer wall.
Approaches to enhance the sensitivity of Gram(-) bacteria to photodynamic action

- Addition of membrane-permeabilizing agents such as Tris-EDTA or polymixin B nonapeptide
  
  Displacement of Ca\(^{2+}\)/Mg\(^{2+}\) \(\rightarrow\) electrostatic repulsion between negative charges \(\rightarrow\) release of up to 50% lipopolysaccharides

- Use of cationic photosensitizers
  
  Binding with negatively charged groups in the outer wall \(\rightarrow\) photo-induced alteration of outer wall permeability \(\rightarrow\) traslocation of sens to the inner plasma membrane
The Photosensitizer

- Presence of positively charged functional groups in peripheral substituents

**Phenothiazines**

- Phthalocyanines

- Porphyrins

- Phthalocyanines

**Chemical Structures**

- N-alkyl-pyridine
- N,N-dialkyl-piperidine
- N,N,N-trialkyl-aniline
The Photosensitizer

- Enhancing antimicrobial effect induced by:
  - Presence of hydrophobic moieties (e.g. hydrocarbon chains)
  - Association with cationic ligands (e.g. polylysine)
  - Incorporation into cationic delivery systems (e.g. liposomes)
Typical example of porphyrins with increasing hydrophobicity

\[ \begin{align*}
R_1 &= R_2 = R_3 = \text{CH}_3 \\
R_4 &= \text{CH}_3 \\
\text{CH}_2(\text{CH}_2)_4\text{CH}_3 &\quad \text{C6} \\
\text{CH}_2(\text{CH}_2)_8\text{CH}_3 &\quad \text{C10} \\
\text{CH}_2(\text{CH}_2)_{12}\text{CH}_3 &\quad \text{C14} \\
\text{CH}_2(\text{CH}_2)_{16}\text{CH}_3 &\quad \text{C18} \\
\text{CH}_2(\text{CH}_2)_{20}\text{CH}_3 &\quad \text{C22}
\end{align*} \]

Survival of \textit{E. coli} cells upon 5 min irradiation with 350-700 nm light (150 mW/cm\(^2\)) after 5 min-dark incubation with 1 \(\mu\)M porphyrins

In vitro studies with a tetracationic phthalocyanine (RLP068)

Bacterial strains

<table>
<thead>
<tr>
<th>Gram (+)</th>
<th>Staphylococcus aureus ATCC 25923</th>
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<tbody>
<tr>
<td></td>
<td>Methicillin Resistant Staphylococcus aureus MRSA</td>
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<tr>
<td></td>
<td>Enterococcus faecalis</td>
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<td></td>
<td>Streptococcus pyogenes</td>
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<table>
<thead>
<tr>
<th>Gram (-)</th>
<th>Escherichia coli ATCC 25922</th>
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<tbody>
<tr>
<td></td>
<td>Pseudomonas aeruginosa ATCC 25988</td>
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Yeasts

| Candida albicans ATCC 10231 |

Protozoa

| Colpoda inflata |
Cellular uptake studies

Effect of phthalocyanine concentration on the recovery of RLP068 from *S. aureus* after 0 ( ), 1 ( ) and 3 ( ) washing steps.

Incubation time: 5 min.
Intracellular localization studies

Fluorescence microscope images of *S. aureus* cells

**A**: 5 min-incubated with 1 µM RLP068 in the dark

**B**: 5 min-incubated with 1 µM RLP068 in the dark, then 5 min-irradiated with 600-700 nm light at a fluence-rate of 50 mW/cm²
Fluorescence micrographs *C. albicans* cells incubated for 1 h with 1 uM RLP068 (upper panel) and irradiated for 10 min with 600-700 nm light at a fluence rate of 50 mW/cm² (lower panel).

1, 4 RLP068 fluorescence
2, 5 CMAC vacuole cell tracker fluorescence
3, 6 bright field
Survival of different microbial strains upon 5 min irradiation with 600-700 nm light (50 mW/cm²) after 5 min-dark incubation (bacteria) or 1 h-dark incubation (yeasts) with 0.01-10 µM phthalocyanine.
Photosensitization studies

Survival of *A. palestinensis* in the trophozoitic and cystic stage upon 10 min (trophozoites) and 20 min (cysts) irradiation with 600-700 nm light (50 mW/cm²) after 1 h-dark incubation with different concentration of phthalocyanine.

Photosensitization studies

Photoinactivation of *S. aureus* ATCC 25923 and MRSA110 cells surviving first treatment (5 min irradiation; 50 mW/cm²) after 5 min-dark incubation with 0.1-2.5 μM RLP068 and resubjected to phthalocyanine photosensitization up to five consecutive generations.
Survival of *S. aureus* cells, fibroblasts and keratinocytes upon 5 min. irradiation with 600-700 nm (50 mW/cm²) after 5 min- dark incubation with different concentration of RLP06.

Main favorable features of antimicrobial PDT

- **Broad spectrum of action.** One irradiation protocol efficient against many different classes of pathogenic agents: Gram-positive and Gram-negative bacteria, mycoplasmas, fungi, yeasts, and parasitic protozoa.

- **Efficacy independent of the antibiotic resistance pattern of the given microbial strain.**

- **Lack of selection of photoresistant strains after multiple treatments.** The overall process is of multi-target nature, thus it is extremely difficult for microbial cells to develop protection strategies which eventually determine the selection of strains showing an enhanced resistance to the photodynamic treatment.

- **Small probability to promote the onset of mutagenicity.** The genetic material is involved in the photooxidative reactions at later stages of the overall photoprocess, when the cell is already dead.

- **High selectivity of action.** Possibility to develop PDT protocols which lead to an extensive reduction in pathogen population with very limited damage to the host tissue.
Typical indications for topical PDT of infectious diseases

Oropharyngeal Candidiasis

- High photosensitivity of Candida albicans (the aetiological agent).
- Damage confined within the diseased area.
- Availability of irradiation protocols which are ineffective toward fibroblasts and keratinocytes.
- Particularly important for HIV-infected patients.
Typical indications for topical PDT of infectious diseases

**Periodontal Diseases**

- Efficacy of photosensitizers toward a typically heterogeneous bacterial flora.
- Extensive eradication of pathogens growing both in aqueous suspensions or as a biofilm.
- No interference from substances typical of oral environment (e.g. demineralized dentine, collagen).
- Minimally invasive and fast procedure as compared with the often laborious/unpleasant mechanical approaches which are most frequently used.
Typical indications for topical PDT of infectious diseases

Healing of infected wounds

- Limitations of many commonly adopted therapeutic strategies based on the treatment with antibiotics or silver salts.

- Broad specificity of action of photodynamic sensitizers (vs. both wild and antibiotic-resistant strains).

- PDT-promoted upregulation of the expression of growth factors.
References


References


References


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