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Antimicrobial photodynamic inactivation: a bright new technique to kill resistant microbes

Michael R Hamblin[1,2,3,*]

[1] Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA, USA

[2] Department of Dermatology, Harvard Medical School, Boston, MA, USA

[3] Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA, USA

Abstract

Photodynamic therapy (PDT) uses photosensitizers (non-toxic dyes) that are activated by absorption of visible light to form reactive oxygen species (including singlet oxygen) that can oxidize biomolecules and destroy cells. Antimicrobial photodynamic inactivation (aPDI) can treat localized infections. aPDI neither causes any resistance to develop in microbes, nor is affected by existing drug resistance status. We discuss some recent developments in aPDI. New photosensitizers including polycationic conjugates, stable synthetic bacteriochlorins and functionalized fullerenes are described. The microbial killing by aPDI can be synergistically potentiated (several logs) by harmless inorganic salts via photochemistry. Genetically engineered bioluminescent microbial cells allow PDT to treat infections in animal models. Photoantimicrobials have a promising future in the face of the unrelenting increase in antibiotic resistance.

1. Photodynamic therapy

Photodynamic therapy (PDT) was discovered over one hundred years ago (in the year 1900) by the serendipitous observation that microorganisms (*Paramecia*) were killed when exposed to both a photosensitizing dye (acridine) and sunlight at the same time [1]. However for most of the time since then, PDT has been studied and developed as an anti-cancer therapy, and not as an antimicrobial therapy [2]. The mechanism of action has been investigated in some detail, but still is not completely understood. It involves the absorption of a photon of light (with a wavelength that matches the absorption band of the dye) leading to excitation of the dye (also called a photosensitizer, PS) to its short-lived (nanoseconds) excited singlet electronic state. This singlet-state PS can undergo an electronic transition (spin flip) to a much longer-lived (microseconds) triplet state. The longer lifetime allows the triplet PS to react with ambient (ground state) oxygen by one of two different photochemical pathways, called Type 1 and Type 2. Type 1 involves an electron transfer to produce superoxide radical

* hamblin@helix.mgh.harvard.edu.

and then hydroxyl radicals (HO•), while Type 2 involves energy transfer to produce excited state singlet oxygen ($^1O_2$). Both HO• and $^1O_2$ are highly reactive oxygen species (ROS) that can damage nearly all types of biomolecules (proteins, lipids and nucleic acids) and kill cells [3]. Figure 1 shows a Jablonski diagram illustrating the photochemical production of different ROS during PDT and their broad-spectrum antimicrobial properties. A Jablonski diagram is a graphical depiction of the PS energy levels in the ground state, excited singlet state and triplet state (top of Figure 1).

2. Antimicrobial photodynamic inactivation

As mentioned above, for many years PDT was studied as a cancer therapy by designing PS that could be administered either systemically (by intravenous injection) or applied topically (e.g. aminolevulinic acid), and some time later the tumor would be irradiated with light (either as a surface spot or by insertion of interstitial fiber optics) [4]. However starting in the 1990s it was realized that PDT could also exert a powerful antimicrobial effect, if PS could be designed that could selectively bind to microbial cells, while not binding to host mammalian cells [5]. The best way of achieving this goal of antimicrobial photodynamic inactivation (aPDI) was to ensure that the PS had a pronounced cationic charge, as it was realized that microbial cells in general have a more pronounced negative charge compared to mammalian cells and cationic PS will bind selectively. Moreover the binding of the PS to the microbial cells is relatively rapid, while uptake of the cationic PS by mammalian cells is slow, thus giving good selectivity when a short drug-light interval (few minutes) is employed [6]. The advantages of aPDI as a potential clinical antimicrobial therapy were bolstered when it was realized that aPDI works equally well regardless of the antibiotic resistance status of the microbial cells [7] and moreover, that aPDI has (so far) not been shown to produce resistance in bacteria [8] even after 20 successive cycles of partial killing followed by regrowth [9]. Another advantage of aPDI is that the PS is applied topically or locally into the infected area. Many chronic infections involve a build up of microbial biofilms, into which it is now well-recognized, that systemically administered antibiotics fail to penetrate. However, aPDI has been shown to kill biofilm-grown cells both in vitro and in vivo [10]. This anti-biofilm application has found particular application in dental infections such as periodontitis [11] and peri-implantitis [12]. Moreover, infections in burns or damaged tissue suffer from a compromised blood supply, so systemically administered antibiotics fail to reach the site of infection in sufficient concentrations. The killing of microbial cells with aPDI is rapid (seconds) while the action of antibiotics can take hours or days, giving a potential advantage against fast-spreading infections such as necrotizing fasciitis. Moreover the broad-spectrum nature of aPDI means that treatment can be instituted before the infectious agents have been identified [13]. Although many infections can occur deep inside the body, it is now possible to deliver both PS and light to almost any anatomical region, via endoscopes and narrow-diameter interstitially-inserted needles and fiber optics [14].

3 New photosensitizers

The optimal molecular design of an antimicrobial PS (aPS) should have several particular features [15]. First of all the aPS should be non-toxic in the dark, especially towards mammalian cells. Secondly they should have good quantum yields of ROS and a high molar

absorption coefficient at a wavelength where light penetration of tissue is good (red and near infrared). Thirdly aPS should show selectivity for microbial cells over host mammalian cells particularly at short incubation times (short drug-light interval) [16]. Fourthly and most importantly an aPS should have cationic charges ideally provided by quaternary nitrogen atoms or basic amino groups [17].

3.1 Polycationic conjugates

It was previously established that an overall cationic charge was necessary for an efficient antimicrobial PS (especially for one that is required to kill many logs of Gram-negative bacteria) [18]. Therefore it made sense to attach a photochemically efficient PS (such as chlorin(e6) that did not possess any intrinsic cationic charges) to a polycationic polymer that had a large number of them. Proof of principle (in vitro and animal studies) was obtained using two broad classes of polycationic polymers, poly-L-lysine (pL-ce6) [19] and polyethylenimine (PEI-ce6) [20] (see Figure 1). The latter compound progressed to a clinical trial in patients suffering from endodontic infections [21].

3.2 Fullerenes

Fullerenes are closed-cage carbon allotropes with a roughly spherical shape and a diameter of about 1 nm. Due to their highly conjugated double bonds they have good absorption of visible light, and a high quantum yield of triplet state and ROS generation upon illumination. Although pristine fullerenes are highly hydrophobic, insoluble in water and prone to aggregation, when they are functionalized with cationic groups they can become water soluble and rather specific for binding to microbial cells. We have tested a variety of cationic fullerenes both in vitro [22,23] (See Figure 3 for two examples) and in vivo in animal models of localized infections [24,25]. The advantages of fullerenes as antimicrobial PS is that they are very photostable and can generate the highly toxic hydroxyl radicals, while the disadvantage is the relatively short wavelength excitation light which does not penetrate tissue very well. However for topical applications to infected areas this may not be a major limitation [26].

3.3 Bacteriochlorins

Bacteriochlorins (BCs) are tetrapyrroles that have had two double bonds (1 in each of two opposing pyrrole rings) reduced, so they can be thought of as tetrahydroporphyrins. When one double bond in a porphyrin is reduced to form a chlorin, the long wavelength band is red-shifted and increased in size, and when a second double bond is reduced to form a BC the effects on the absorption are even more pronounced. Strong long wavelength absorption bands are needed for good penetration of light into living tissue. The only remaining requirement is to have quaternary cationic groups present on the BC to allow binding and penetration of the bacterial cells [27]. Interestingly we found that an asymmetric dicationic BC was actually significantly more active against Gram-positive bacteria and fungi than a symmetrically substituted tetracationic BC (that only had high activity against Gram-negatives) presumably due to the molecular asymmetry allowing better penetration into the bacterial cell [28] (see Figure 4). This difference was particularly pronounced with eukaryotic fungal cells.

3.4. Innovative antimicrobial PS from other laboratories

The most widely used antimicrobial PS are without doubt the phenothiazinium dyes, methylene blue and toluidine blue O [29]. Both of these compounds have received regulatory approval in various countries throughout the world for aPDI. Nevertheless these compounds are not highly active, and many laboratories have attempted to introduce compounds that have much higher activity, or alternatively are naturally occurring dyes that are supposed to be easier to get thought regulatory barriers. Notable examples of the synthetic high activity compounds are the cationic zinc phthalocyanine RLP068 [30], the cationic phenalenone derivative known as SAPYR [31], and the porphyrin known as Sylsens B [32]. Examples of compounds derived from natural products are a cationic hypericin compound derived from St John's Wort [33], a cationic riboflavin compound derived from vitamin B2 [34], and a cationic derivative of curcumin (a yellow spice found in turmeric) known as SACUR-3 [35].

4. Potentiation by inorganic salts

4.1 Azide

Sodium azide has been widely used to quench the singlet oxygen produced during PDT . We discovered that in contradiction to what we originally expected, addition of azide to methylene blue (MB) aPDT, did not quench the bacterial killing, but rather potentiated it [36] (see Figure 5A). Potentiation of killing by addition of azide also applied to other phenothiazinium dyes [37] and to cationic fullerenes [38]. Although potentiation of microbial photo-killing by azide is interesting from a mechanistic viewpoint, the toxicity of azide precludes clinical application.

4.2 Iodide

Since the potentiation by azide ion was shown to involve the photochemical production of azidyl radicals by an electron transfer mechanism, we tested the possibility that antimicrobial PDI mediated by MB could be potentiated by addition of the non-toxic salt, potassium iodide [39]. This was shown to proceed by photoproduction of reactive iodine radicals. Potentiation by addition of iodide also applied to aPDI mediated by cationic fullerenes such as LC16 (see Figure 5B) [25].

4.3 Bromide

Neither phenothiazinium dyes nor cationic fullerenes were able to show any potentiation of microbial killing by addition of bromide anion. However antimicrobial photocatalysis mediated by UVA excited titanium dioxide nanoparticles (a large band-gap semiconductor) could indeed by potentiated by addition of bromide, involving the intermediate production of hypobromite [40] (see Figure 5C).

5. PDT of infections in animal models

5.1 Bioluminescent microbial imaging

In order to allow real time non-invasive monitoring of the progress and treatment response of localized infections, Contag and colleagues invented the use of genetically engineered

bioluminescent microbial cells (that “glow in the dark”) with a low-light imaging camera [41]. This technology was the forerunner of the now widely-distributed bioluminescence imaging protocols that are used in different fields of biomedical research with the ability to perform real-time, non-invasive, longitudinal imaging of many different disease states [42].

5.2 PDT of infections

We have employed this bioluminescence imaging approach to demonstrate the effectiveness of aPDT in many different animal models of localized infections [43]. When the bacterial strain was particularly virulent and invasive (such as *P. aeruginosa)*, appropriate delivery of aPDT could save mice from a certain death due to systemic sepsis [44] (see Figure 6).

6 Conclusion and future directions

In 2015 the O'Neill report published dire forecasts that by 2050 the acceleration of antibiotic resistance could cause 300 million additional deaths and cost an extra US$100 trillion [45]. Since the antibiotic era is now widely supposed to be on the verge of ending [46], and the prospect of discovering novel classes of antibiotics is considered to be rather low [47] (although incremental improvements are still being made [48]), it is necessary to discover alternative antimicrobial technologies to which bacteria will not be able to develop resistance, and which will work equally well regardless of present resistance status [49]. aPDT will have a significant role to play in this new armamentarium that is perforce being developed in the 21st century. New PS, innovative photochemical potentiation strategies, and useful animal models for testing will all be useful in this grand endeavor.

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PDT uses light-activated photosensitizers to produce reactive oxygen species that kill microbial cells.

Polycationic chlorin(e6) conjugates, stable synthetic bacteriochlorins and functionalized fullerenes are examples.

The microbial killing by aPDI can be synergistically potentiated by harmless inorganic salts via photochemistry.

Genetically engineered bioluminescent microbial cells allow PDT to treat infections in animal models.

Photoantimicrobials have a promising future in the face of the unrelenting increase in antibiotic resistance.

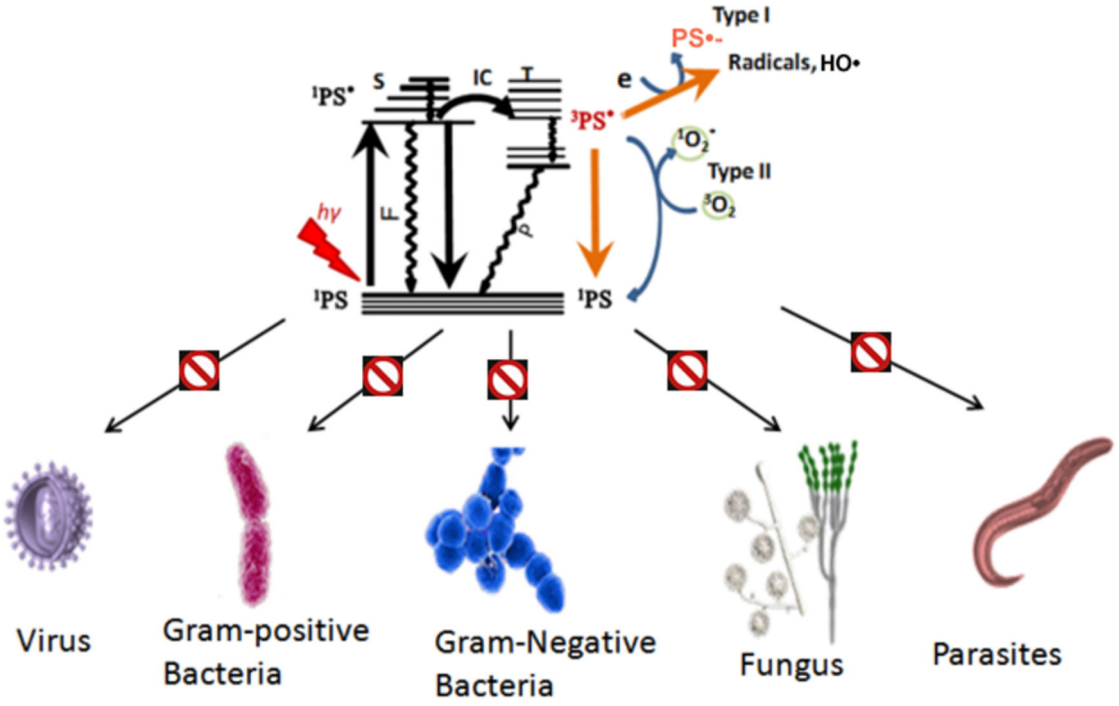

Figure 1. Jablonski diagram showing photochemical pathways in aPDI

The ground state ^{1}PS absorbs a photon to form excited singlet state ^{1}PS* that can undergo intersystem crossing (IC) to form the triplet state ^{3}PS*. This long-lived species can undergo energy transfer (Type II) to form singlet oxygen $^{1}O_{2}$* or elkectron transfer (Type I) to form hydroxyl radicals HO•. Both these ROS are capable of killing a broad spectrum of pathogens.

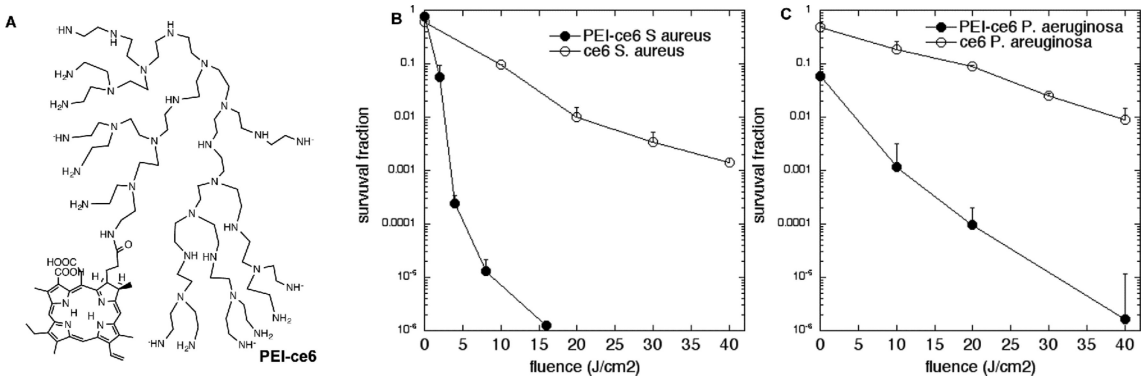

Figure 2. aPDI with PEI-ce6 and free ce6

(A) Chemical structure of PEI-ce6. (B) Killing of Gram-positive *Staphylococcus aureus* incubated for 10 min with 10 μM PEI-ce6 or free ce6 and illuminated with increasing fluences of 660 nm light. (C) Same as B but with Gram-negative *Pseuodomonas aeruginosa.*

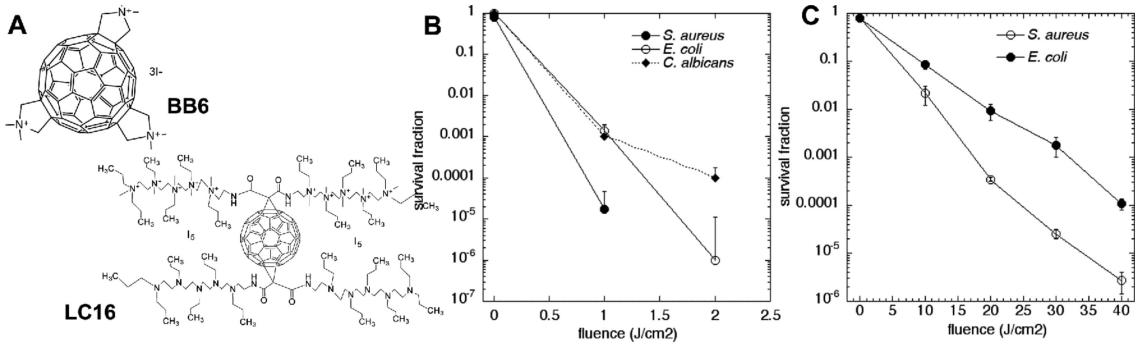

Figure 3. aPDI with cationic fullerenes (bucky-balls)

(A) Chemical structures of BB6 (3 cationic charges) and LC16 (10 cationic charges). (B) Killing of *S. aureus,* Gram-negative *Escherichia coli,* and fungal yeast *Candida albicans* incubated for 10 min with 10μM BB6 and illuminated with increasing fluences of broad-band white light (400-700 nm). (C) Killing of *S. aureus,* and *E. coli* incubated for 10 min with 10μM LC16 and illuminated with increasing fluences of UVA light (360±20 nm).

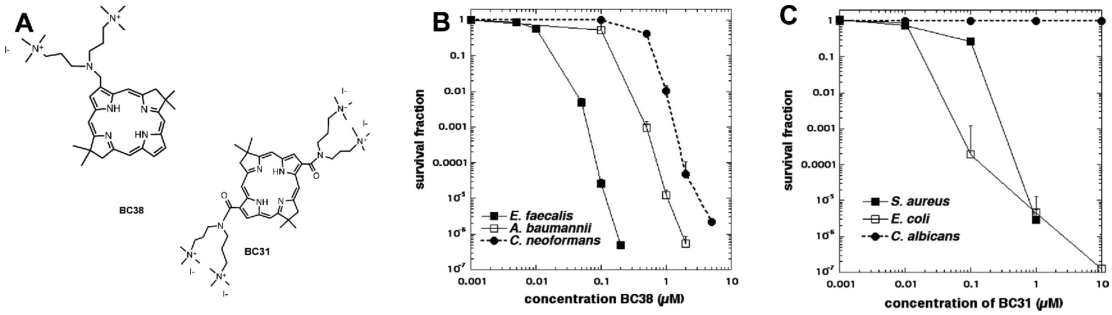

Figure 4. aPDI with cationic bacteriochlorins

(A) Chemical structures of asymmetrical dicationic BC38 and symmetrical tetracationic BC31. (B) Killing of *Enterococcus faecalis,* Gram-negative *Acinetobacter baumannii,* and fungal yeast *Cryptococcus neoformans* incubated for 10 min with increasing concentrations of BC38, and illuminated with 10 J/cm^2 of 732 nm laser. (C) Killing of *S. aureus, E. coli,* and *C. albicans* incubated for 10 min with increasing concentrations of BC31, and illuminated with 10 J/cm^2 of 732 nm laser.

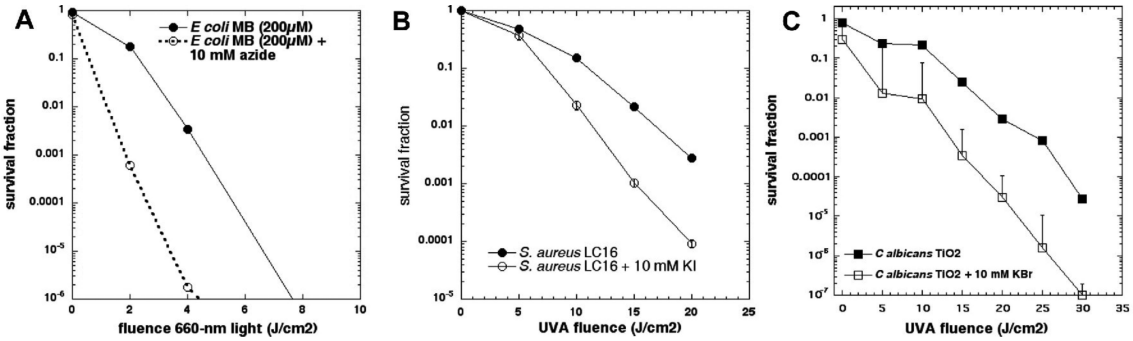

Figure 5. Potentiation of aPDI by addition of inorganic salts

(A) *E. coli,* methylene blue and red light is potentiated by sodium azide. (B) *S. aureus,* cationic fullerene LC16 and UVA light is potentiated by potassium iodide. (C) *C. albicans,* titanium dioxide nanoparticles and UVA light is potentiated by sodium bromide.

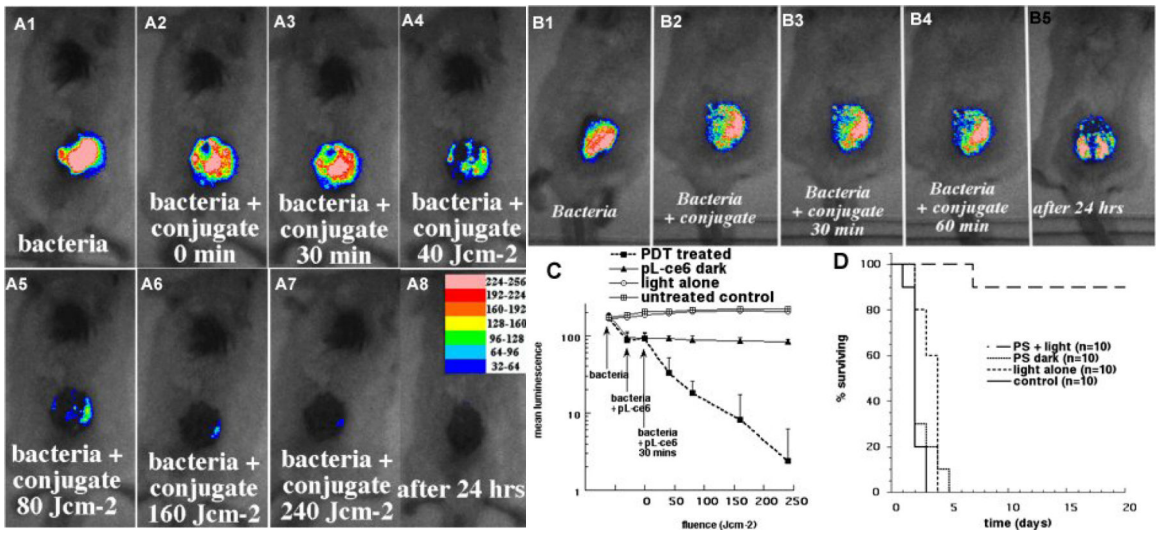

Figure 6. aPDI for wound infections in vivo

Mice with excisional wounds received 5X10(6) CFU bioluminescent *P. aeruginosa,* followed after 30 min by pL-ce6 conjugate (50 μL of 200 μM ce6 equivalent) and after 30 min by successive exposures to aliquots of 660 nm laser. (A1-A8) Representative bioluminescence images of PDT treated mice. (B1-B5) Representative bioluminescence images of dark control mice (conjugate no light). (C) Quantification of bioluminescence signals from the four groups of mice. (D) Kaplan-Meier survival curves of the four groups of mice.