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Sensitization of Oral Bacteria to Killing by Low-Power Laser Radiation

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Abstract. Twenty-seven compounds were screened for their ability to sensitize *Streptococcus sanguis* to killing by light from a 7.3-mW Helium/Neon (HeNe) laser. Bacteria were mixed with various concentrations of the test compounds, spread over the surfaces of agar plates, and then exposed to light from the HeNe laser for various time periods. The plates were then incubated and examined for zones of inhibition. Those compounds found to be effective photosensitizers were then tested against *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans,* and *Fusobacterium nucleatum.* Toluidine blue O, azure B chloride, and methylene blue at concentrations of 0.005% (wt/vol) were effective photosensitizers of all four species, enabling killing of bacteria following exposure to laser light for only 30 s.

It has long been known that light can exert a deleterious effect on cells and that this can be exacerbated by treatment with "photosensitizing" compounds [12]. The latter phenomenon forms the basis of photochemotherapy (PCT)—the treatment of disease with light following the administration of a photosensitizer capable of absorbing light of the wavelength used. One well-established application of PCT, involving the use of UV light and psoralen as a photosensitizer, is in the treatment of psoriasis [10]. During the last 25 years considerable interest has been shown in the use of PCT for the treatment of tumors, whether superficial or deep-seated [3, 11]. This has resulted from the development of suitable lasers as light sources, efficient optical fiber light delivery systems, and appropriate photosensitizers [4, 15]. A number of studies have demonstrated that microbes, as well as mammalian cells, can be sensitized to killing by both polychromatic [14] and monochromatic light [8]. However, little consideration has been given to the potential use of PCT in the treatment of infectious diseases. This is possibly because studies have generally used photosensitizers developed for use in the treatment of tumors (e.g., the hematoporphyrins, which are reportedly ineffective against Gram-negative bacteria [2, 7]). The purpose of the present study was to screen a range of chemicals for their ability to sensitize a number of oral bacteria to killing by light from a Helium/Neon (He/Ne) laser.

Materials and Methods

Laser. The laser used was a HeNe gas laser (NEC Corporation, Japan) with a power output of 7.3 mW. This emitted radiation in a collimated beam, diameter 1.3 mm, with a wavelength of 632.8 nm.

Target organisms. The organisms used in the study were: *Streptococcus sanguis* NCTC 10904, *Porphyromonas gingivalis* W50, *Fusobacterium nucleatum* NCTC 10562, and *Actinobacillus actinomycetemcomitans* Y4. All were maintained by weekly transfer on Wilkins Chalgren (WC) blood agar (Oxoid Ltd., Basingstoke, UK) except for *S. sanguis*, which was sub-cultured every 48 h on brain–heart infusion (BHI) agar (Oxoid Ltd.).

Photosensitizers. Test compounds were obtained from Sigma Ltd. (Poole, UK) except for those listed below. Ariabel dark blue, FDC blue #2, ariavit patent blue, ariavit indigo carmine, arianor steel blue, ariavit brilliant blue FCF, and usacert FD and C blue #1 and #2 (all from Williams Ltd., Hownslow, UK); azure mixture sicc. and azure B (Fluka, Buchs, Switzerland); brilliant cresyl blue and trypan blue (BDH, Poole, UK); aluminum disulfonated phthalocyanine (a gift from Prof. D. Phillips, Chemistry Department, Imperial College, London); and hematoporphyrin ester (Paisley Biochemicals Ltd., Glasgow).

Effect of laser light on bacterial viability. Several colonies of the test organism were suspended in sterile saline and vortexed to provide a homogeneous suspension. Two milliliters of this suspension was mixed with 2.0 ml of various concentrations of the test compound in saline (or saline alone in the case of controls), and 1.0 ml was spread over the surfaces of agar plates. After 10 min, excess fluid was removed, and the plates were dried at 37°C. The plates were then exposed to the laser for various periods of time, following which they were incubated in anaerobic jars until

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growth was visible on the control plates. The plates were examined for zones of inhibition and were then re-incubated for an additional 7 days to determine whether any growth occurred within the inhibition zones.

In the case of *S. sanguis* and *A. actinomycetemcomitans*, the medium used was BHI, while for *P. gingivalis* and *F. nucleatum* this was supplemented with 0.0001% (wt/vol) menadione and 0.001% (wt/vol) hemin. Control plates in which the bacteria were not exposed to the test compound served to determine whether laser light alone had any effect on the viability of the target organisms. Any adverse effect on the viability of the bacteria by the test compound itself was ascertained by examination of unirradiated portions of those plates receiving bacteria previously exposed to the compound.

Screening of compounds for photosensitizing activity. Twenty-seven compounds were tested for their ability to inhibit growth of *S. sanguis* following exposure to HeNe laser light. Each compound was tested at concentrations of 0.1% and 0.01% (wt/vol), and exposure to the laser light was for 5, 10, 30, and 60 s.

Effect of varying the concentration of photosensitizer. Compounds shown to act as photosensitizers in the screening program were selected for further investigation. With the method described above, the effects of varying the concentration of the compound and the light exposure time on the growth of *S. sanguis* were determined. A range of concentrations from 0.00015% (wt/vol) to 0.01% (wt/vol) was used, each at an exposure time of 2, 10, and 30 s.

Photosensitization of other oral bacteria. Some of the most promising compounds were then tested for their ability to sensitize *P. gingivalis*, *A. actinomycetemcomitans*, and *F. nucleatum* to killing by HeNe light.

Results

The results of the initial screening program of the 27 test compounds with *S. sanguis* as the target organism are shown in Table 1. From this it can be seen that the following compounds were effective photosensitizers: arianor steel blue, toluidine blue O, crystal violet, methylene blue, thionin, several azure dyes, hematoporphyrin, and hematoporphyrin ester. Kill times ranged from 5 to 60 s, which represented energy doses of 2.75–33 J/cm^2. Zones of killing were not seen on control plates in which *S. sanguis* was irradiated without prior exposure to any of the test compounds. Except in the case of phthalocyanine, the test compounds themselves had no apparent effect on the growth of *S. sanguis* at the concentrations tested.

The effect on *S. sanguis* of varying the concentrations of the most promising compounds selected from the preliminary screening program was then investigated. Table 2 shows that, of the photosensitizers tested, toluidine blue O, azure A chloride, and thionin were the most effective at inducing killing of *S. sanguis*. In the case of toluidine blue O, for example, zones of killing were apparent in some experiments following irradiation for 2 s with a concentration of 0.0003% (wt/vol).

From Table 3 it can be seen that, of the photosensitizers tested, toluidine blue, methylene blue, and azure B chloride were the only ones effective against all of the target organisms. In general, *F. nucleatum* and *A. actinomycetemcomitans* appeared to be more resistant to killing than *S. sanguis* and *P. gingivalis*, under the conditions of photosensitizer concentration and exposure time employed.

Discussion

The results of this investigation have demonstrated that a number of compounds can sensitize several species of oral bacteria, both Gram-positive and Gram-negative, to killing by light from a HeNe laser. Irradiation of the bacteria in the absence of the photosensitizers had no detectable effect on the viability of these organisms and, at the concentrations tested, the photosensitizers themselves did not exert a bactericidal effect. Not surprisingly, of the compounds exhibiting photosensitizing activity, those with absorption maxima closest to the wavelength of the radiation emitted by the laser (632.8 nm) were among the most effective. These included toluidine blue (632.2 nm) and azure A chloride (632.4 nm).

While there have been no previous reports of the ability of azure dyes to act as photosensitizing agents, other investigators have shown toluidine blue to be an effective photosensitizer of bacteria. Mathews and Sistrom [9] demonstrated that a toluidine blue-sensitized colorless mutant of *Sarcina lutea* could be killed by polychromatic light from tungsten and fluorescent lamps. Macmillan et al. [6] reported that both Gram-positive (*Sarcina lutea*) and Gram-negative species (*Escherichia coli* and *Pseudomonas aeruginosa*) were killed by HeNe laser light following treatment with toluidine blue. On the other hand, Takahashi et al. [13] found that the presence of toluidine blue did not enhance the killing of *E. coli* achieved by exposure to laser light with a wavelength of 590 nm. With regard to this finding, however, it should be noted that toluidine blue shows poor absorption of light with this wavelength.

Most reported investigations of bacterial photosensitization have been concerned with sensitizers developed for use in PCT of tumors, e.g., porphyrins. These have been shown to be effective photosensitizers of Gram-positive bacteria, yeasts, and *Mycoplasma* species, but not generally of Gram-

Table 1. Effect of light from a He/Ne laser on the survival of *S. sanguis* following exposure to a range of test compounds

Test compound	Exposure time (s)	Dye concentration (% wt/vol)	Result
Brilliant blue FCF	60	0.1	−
Ariavit patent blue V	60	0.1	−
Usacert FD and C blue #1	60	0.1	−
Usacert FD and C blue #2	60	0.1	−
Arianor steel blue	60	0.1	+
Ariabel turquoise	60	0.1	−
Ariavit indigo carmine	60	0.1	−
Patent blue VRS	60	0.1	−
Toluidine blue O	5	0.01	+
Crystal violet	10	0.01	+
Methylene blue	10	0.01	+
Azure blue cert	5	0.01	+
Azure B chloride	5	0.01	+
Azure 2	5	0.01	+
Azure A chloride	5	0.01	+
Azure B tetrafluoroborate	5	0.01	+
Thionin	5	0.01	+
Azure A eosinate	5	0.01	+
Azure B eosinate	5	0.01	+
Azure mix sicc.	5	0.01	+
Azure II eosinate	5	0.01	+
Trypan blue	60	0.1	−
Bromocresol blue	60	0.01	−
Gallocyanin	60	0.01	−
Hematoporphyrin HCl	10	0.01	+
Hematoporphyrin ester	5	0.01	+
Phthalocyanine	DT		

+, bactericidal effect; −, no detectable bactericidal effect; DT, direct toxicity shown to *S. sanguis*.
In the case of a positive result (i.e., killing) the lowest concentration of dye tested is given in combination with the shortest exposure time used. For negative results (i.e., no killing) the highest dye concentration and longest exposure times are given.

negative bacteria [7, 11]. In the present investigation we have found that hematoporphyrin HCl and hematoporphyrin ester were capable of sensitizing both Gram-positive and Gram-negative bacteria to killing by HeNe light. Since these compounds absorb poorly at 632.8 nm, this finding was surprising. Other investigators have also shown that hematoporphyrins can act as bacterial photosensitizers. Martinetto et al. [8] reported killing of *E. coli* with light from a dye-Argon laser (524 nm) following sensitization with hematoporphyrin HCl, and Venezio et al. [14] showed that *Bacteroides fragilis,* sensitized with a hematoporphyrin derivative, could be killed by white light.

One of the newer generation of tumor photosensitizers, aluminum disulfonated phthalocyanine, was also found to be an effective photosensitizer of Gram-positive and Gram-negative bacteria in the present investigation. This compound has also been shown to sensitize *Helicobacter pylori* to killing by light from a copper vapor pumped dye laser [1]. In general, however, the photosensitizers developed for use in the PCT of tumors were less effective at sensitizing bacteria to killing by HeNe light than dyes such as toluidine blue, thionin, and some azure dyes.

Despite the potential usefulness of PCT in microbial infections, very few in vivo studies have been reported. Light from an Argon laser has been used in conjunction with hematoporphyrin to treat five patients who developed infections after central nervous system surgery [5]. The hematoporphyrin was applied directly into the infected cavity, which was irradiated 5 min later, resulting in sterilization of the cavity. The organisms responsible were *Peptostreptococcus anaerobius, Staphylococcus aureus,* and streptococci.

Of the 16 compounds found to be photosensitizers of *S. sanguis,* toluidine blue, methylene blue, and azure B chloride also proved to be effective sensitizers of *P. gingivalis, F. nucleatum,* and *A. actinomycetemcomitans.* At a concentration of

Table 2. Effect of irradiation time on the survival of *S. sanguis* following treatment with various concentrations of photosensitizing agents

Dye	Concentration (%, wt/vol)	Exposure time		
		2 s	10 s	30 s
Toluidine blue O	0.01	+	+	+
	0.005	+	+	+
	0.0025	v	+	+
	0.00125	v	+	+
	0.00063	v	v	+
	0.00031	v	v	v
	0.00016	−	−	−
Azure A chloride	0.01	v	+	+
	0.005	v	+	+
	0.0025	v	+	+
	0.00125	v	v	+
	0.00063	−	−	+
	0.00031	−	−	−
Crystal violet	0.01	+	+	+
	0.005	v	v	+
	0.0025	v	v	+
	0.00125	−	−	+
	0.00063	−	−	v
	0.00031	−	−	−
Thionin	0.01	+	+	+
	0.005	+	+	+
	0.0025	+	+	+
	0.00125	+	+	+
	0.00063	v	+	+
	0.00031	−	+	+
	0.00016	−	v	+
	0.00008	−	−	+
Azure B chloride	0.01	−	+	+
	0.005	−	+	+
	0.0025	−	−	v
	0.00125	−	−	−
Azure B fluoroborate	0.01	−	v	+
	0.005	−	−	+
	0.0025	−	−	v
	0.00125	−	−	−
Methylene blue	0.01	+	+	+
	0.005	−	+	+
	0.0025	−	−	+
	0.00125	−	−	−
Hematoporphyrin ester	0.01	+	+	+
	0.005	+	+	+
	0.0025	−	+	+
	0.00125	−	−	−
Phthalocyanine	0.01	NT		
	0.005	NT		
	0.0025	−	+	+
	0.00125	−	+	+
	0.00063	−	+	+
	0.00031	−	−	+
	0.00016	−	−	+
Hematoporphyrin HCl	0.01	−	+	+
	0.005	−	−	+
	0.0025	−	−	−

+, bactericidal effect; −, no detectable bactericidal effect; v, variable results; NT, not tested because of direct toxicity of dye to *S. sanguis* at these concentrations.

Table 3. Susceptibility of various oral bacteria to light from an HeNe laser following exposure to a range of photosensitizers

Photosensitizer	Exposure time (s)	*S. sanguis*	*A. actinomycetemcomitans*	*F. nucleatum*	*P. gingivalis*
Toluidine	10	+	v	+	+
blue O (0.005%)	30	+	+	+	+
Hematoporphyrin	10	+	−	−	+
HCl (0.5 mM)	30	+	−	−	+
Crystal	10	+	−	−	v
violet (0.005%)	30	+	−	−	v
Thionin (0.005%)	10	+	−	−	−
	30	+	−	−	−
Azure B chloride	10	+	v	+	+
(0.005%)	30	+	v	+	+
Methylene blue	10	+	+	+	+
(0.005%)	30	+	+	+	+
Phthalocyanine	10	+	−	−	+
(0.0025%)	30	+	−	−	+
Hematoporphyrin	10	+	−	−	+
ester (0.005%)	30	+	+	−	+

+, bactericidal effect; −, no detectable bactericidal effect; v, variable results.

0.005% (wt/vol), these compounds enabled killing of the organisms following exposure to HeNe light for only 30 s. Since these organisms are involved in a number of oral infections, including gingivitis and periodontitis, these results imply that PCT may be effective in treating such infections. Furthermore, the topical nature of such diseases renders them particularly amenable to this form of treatment, since the lesions are readily accessible to the photosensitizer and to the light.

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