Diode Laser Activated Indocyanine Green Selectively Kills Bacteria

Tobias K. Boehm[1] and Sebastian G. Ciancio[2]
[1]College of Dental Medicine, Western University of Health Sciences, Pomona, CA, and [2]School of Dental Medicine, State University of New York at Buffalo, Buffalo, NY

Abstract

Background and Objective: Commercially available photodynamic therapy for periodontal diseases utilizes methylene blue as a photosensitizer. Here we propose a novel photosensitizer dye, indocyanine green (ICG), because it can be readily activated by commercially available dental 810 nm diode lasers and has an established safety record as an intravascular agent in cardiac imaging and ophthalmologic photodynamic therapy. Therefore, we aim to characterize ICG uptake and killing of key periodontal pathogens to explore its potential as a periodontal photodynamic therapy agent. **Materials and Methods:** We tested ICG uptake by spectroscopy in *Porphyromonas gingivalis* 381 and *Aggregatibacter actinomycetemcomitans,* in addition to *Escherichia coli* DH5alpha and a human gingival epithelial cell line, HepG, in relation to ICG dose and exposure time. We then measured killing of bacteria by determining viable bacteria counts before and after exposure to ICG and 810 nm diode laser light (0-0.5 W output settings, 0-5 seconds). ICG was also applied to extracted, restored teeth, and the teeth inspected visually for staining after rinsing with saline. **Results:** We found rapid and significant uptake of indocyanine green into *P. gingivalis* 381 and *A. actinomycetemcomitans* 67, compared to *E. coli* DH5alpha and HepG gingival cell line. This correlated with significant killing of strains 381 and 67 compared to *E.coli*, with less than 10% survival. ICG does not appear to stain tooth surfaces and materials except calculus**. Conclusion:** ICG combined with an 810 nm diode laser may be useful as a photodynamic adjunct for reduction of bacterial load in periodontal pockets.

Key words: Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, non-surgical periodontal therapy

Introduction

Photodynamic therapy has been used since the 1960s with Lipson *et al.* and Schwartz *et al.* for the treatment of skin cancers, in which some photosensitizing agents are selectively taken up by cancer cells by mechanisms not fully understood (Lipson *et al.*, 1961; Sausville, 2008; Schwartz, 1955). Photosensitizing agents such as methylene blue are molecules that release radicals such as singlet oxygen upon excitation by light of an appropriate wavelength (Pass, 1993). These radicals damage membrane lipids and proteins, leading to cell death (Pervaiz and Olivo, 2006).

Photodynamic therapy has also been explored for periodontal therapy (Wilson *et al.*, 1992) using a variety of photosensitizing dyes (methylene blue and chlorins, Pfitzner *et al.*, 2004), and with lasers of various wavelengths (Chan and Lai, 2003). Methylene blue (0.01%) and a 630 nm diode laser appears to be the most commonly tested combination. In rats, studies suggest that photodynamic therapy might be a useful adjunct to conventional nonsurgical therapy (de Almeida *et al.*, 2007; 2008). One human study showed no significant benefits of methylene blue/laser alone compared to scaling and root planing (Yilmaz *et al.*, 2002), while another study in humans showed beneficial effects if combined (Andersen *et al.*, 2007). A commercial system exploiting methylene blue/630 nm diode laser is available, and a recent study (Ge *et al.*, 2008) demonstrated a possibly longer reduction in anti-inflammatory mediators when used as an adjunct to scaling and root planing.

However, methylene blue has not been characterized to our knowledge in regard to bacterial uptake, and it is unclear if bacterial and cellular uptake differ much. Here we propose the use of indocyanine green, also known as Cardiogreen for its FDA-approved cardiovascular use, because its excitation

Corresponding author: Tobias K. Boehm, DDS, PhD
College of Dental Medicine, Western University of Health Sciences, 309 East Second Street, Pomona, CA 91766. Telephone: (909) 706-3831, Fax: (909) 706-3800, E-mail: tboehm@westernu.edu

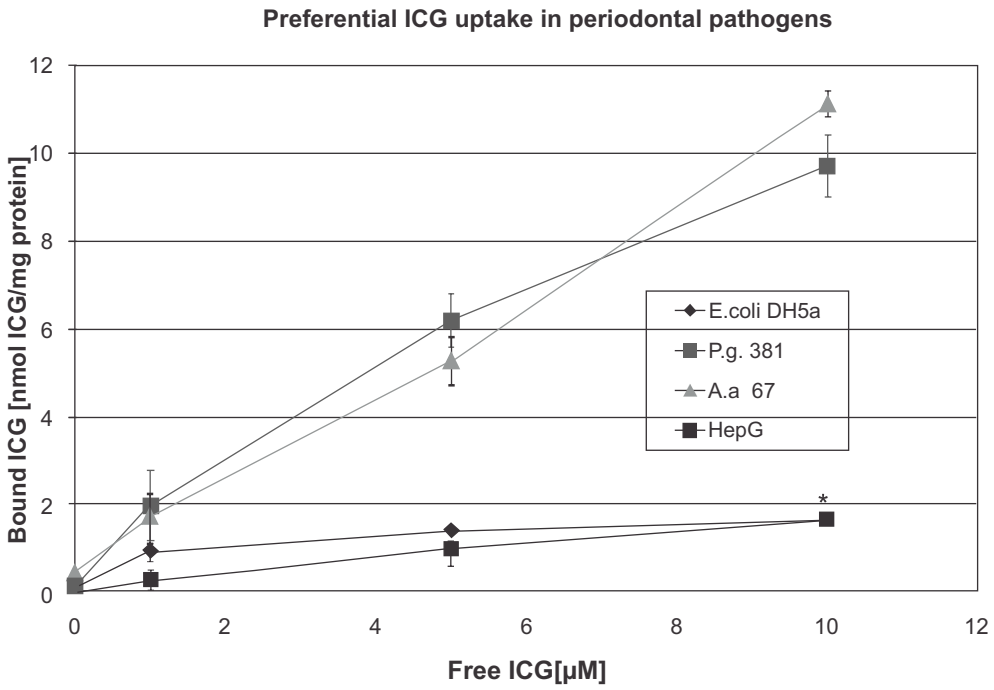

Figure 1. Indocyanine green (ICG) is preferentially taken up by periodontopathic bacteria. Standardized planctonic solutions of different bacteria (E. coli, P. gingivalis, A. actinomycetemcomitans) and cultured gingival epithelial cells (HepG) were incubated with various concentrations of ICG for 1 minute, and then washed twice with PBS. Pellets were then lysed and ICG concentration measured using spectroscopy. ICG amounts were calculated, normalized to protein content and averaged. The graph shows the average ICG concentration and standard deviation, with statistically significant differences ($p < 0.05$) highlighted by an asterisk. This figure is representative of the results we obtained.

peak coincides with the wavelength produced by 810 nm diode lasers commonly used for soft tissue surgery. Indocyanine green (ICG) has low toxicity, high absorption in the near infrared spectrum, rapid elimination, and is a good photosensitizer according to Genina *et al.* According to this group (Genina *et al.*, 2004), ICG has an absorption peak near 800 nm, which is close to the emission maximum of commercially available dental diode lasers. In addition, variants of indocyanine green are functionalized with a carboxylate group, which allows conjugation to biologically active molecules such as antibodies (Folli *et al.*, 1994; Withrow *et al.*, 2007) and possible specific targeting of bacteria or cellular components.

Because ICG appears to be a promising agent and it appears to have not been characterized for its photodynamic properties against periodontal bacteria, we aim to determine if unconjugated indocyanine green is taken up by periodontal bacteria and if uptake leads to efficient killing *in vitro*. In addition, we also aim to determine optimal conditions of dose, incubation time, irradiation level and irradiation time.

Materials and methods

Materials

Reagent grade indocyanine green (ICG) was purchased from Sigma-Aldrich (St. Louis, MO). In order to create appropriate dilutions of ICG for each experiment, ICG was dissolved first in dimethyl sulfoxide (DMSO) to a concentration of 5 mM, and then diluted with PBS to the desired concentrations of 0-10 µM. *P. gingivalis* 381 was generously provided by Dr. Kiyonuba Homma and *A. actinomycetemcomitans* 67 generously provided by Dr. Joseph Zambon. *E. coli* DH5alpha and HepG cell lines were cultivated from existing stocks in our lab. Culture media and supplies were obtained from established suppliers such as Invitrogen and VWR.

Culture

P. gingivalis 381 was cultivated in tryptic soy broth supplemented with hemin and vitamin K under anaerobic conditions at 37°C, and, to assess purity, every two weeks it was grown on tryptic soy agar plates supplemented with 5% sheep blood, hemin and

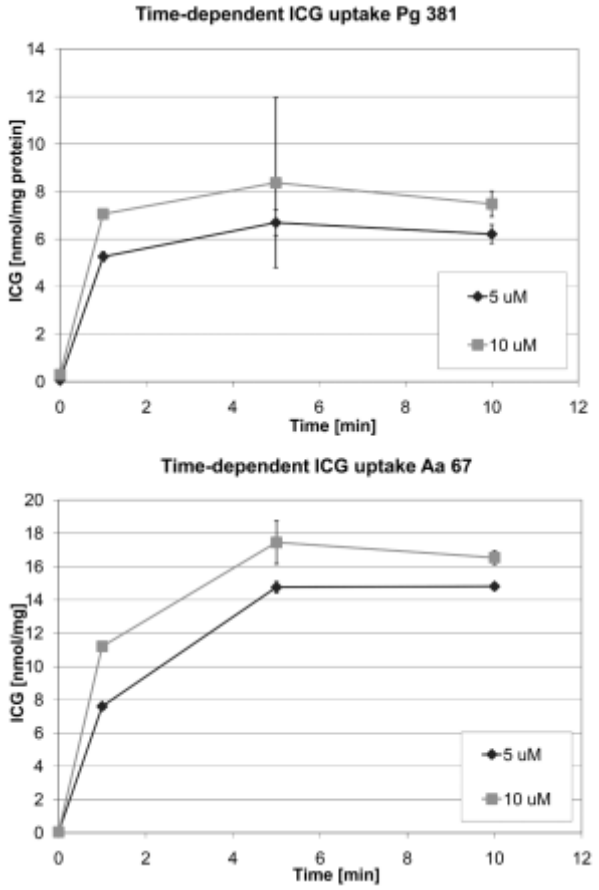

Figure 2. Indocyanine green (ICG) uptake is complete after 1-5 minutes. Standardized planctonic solutions of different bacteria (E. coli, P. gingivalis, A. actinomycetemcomitans) and cultured gingival epithelial cells (HepG) were incubated with either 5 or 10 µM concentrations of ICG over various amounts of time, and then washed twice with PBS. Pellets were lysed and ICG concentration measured using spectroscopy. ICG amounts were calculated, normalized to protein content and averaged. The graph shows the average ICG concentration and standard deviation. No significant differences were seen in these experiments. Replicate experiments showed similar time courses of ICG uptake.

vitamin K under the same conditions (Tokuda *et al.*, 1998). *A. actinomycetemcomitans* 67 was cultured in brain heart infusion broth supplemented with hemin and vitamin K at 37°C in an anaerobic chamber, and plated on brain heart infusion agar plates supplemented with hemin and vitamin K to assess purity every two weeks. *E. coli* was grown either in Luria broth or on Luria agar at 37°C in ambient air (Haraszthy *et al.*, 2002). HepG cells were grown in RPMI 1640 media supplemented with 10% fetal calf serum, streptomycin and 10 mM HEPES in 5% CO_2 at 37°C. Once the cells reached 90% confluency, cells were trypsinized and replated after washing and 10-fold dilution.

For experiments, all bacteria were grown to mid log phase and normalized with culture medium to OD_{600} = 1.000. HepG cells were grown to 90% confluency, gently scraped off the culture plate and brought to OD_{600} = 1.000.

ICG uptake

For measurement of protein content, 1 ml aliquots of suspended of bacteria or cells at OD_{600} = 1.000 were washed twice with PBS and lysed using sonication. The protein content of the resulting solution was determined through the Bradford method (Bradford, 1976).

For measurement of ICG content, 1 ml aliquots of bacteria/cell suspension were washed twice with medium and ICG was added to achieve 0, 1, 5, 10 µM final concentrations. For dose-dependent uptake, the suspension was incubated for one minute at room temperature before spinning down the pellet at 5000 x *g* for one minute, and the pellet washed twice with PBS. For time-dependent uptake, a suspension of 5 or 10 µM was incubated for 0, 1, 5, 10 minutes and then centrifuged and washed as stated before. The pellets were then lysed overnight with 1 ml 10% sodium dodecyl sulfate and 1 M sodium hydroxide, which results in complete dissolution of the bacteria and cells. We assume 100% recovery of ICG in solution, since centrifugation does not produce any pellets or residue. Indocyanine green content of the lysates is measured by absorption at 800 nm. ICG content is then converted to mmol ICG using a molar absorption coefficient of 10800 $M^{-1}cm^{-1}$, and then normalized to the pellet protein content as determined before. Experiments were repeated four times. Statistical significance was determined using one-way ANOVA and Bonferroni adjustment.

ICG killing

One ml aliquots of bacterial suspensions at OD_{600} = 1.000 were washed twice in medium and subjected to 5 µM ICG for one minute. After pelleting and washing once in medium, the resulting bacterial pellet was irradiated for 0, 0.5 s, 2 s and 5 s with an 810 nm diode laser (Ivoclar Odyssey) set to a continuous mode of action. Pellet irradiation was performed by pointing the freshly cut fiber (diameter 0.4 mm) directly over the pellet no further away than 1 mm, and irradiating at either 0.1 W or 0.5 W setting, which results in a flux of either 80 W/cm^2 or 400 W/cm2 respectively. The pellet was then resuspended in 1 ml culture medium and serially diluted 1:50 fold. Five µl aliquots were then plated and cultured, and colony counts used to determine viable bacteria before and after treatment. Four to six viable bacteria counts were performed for each sample, and bacteria counts for each sample were

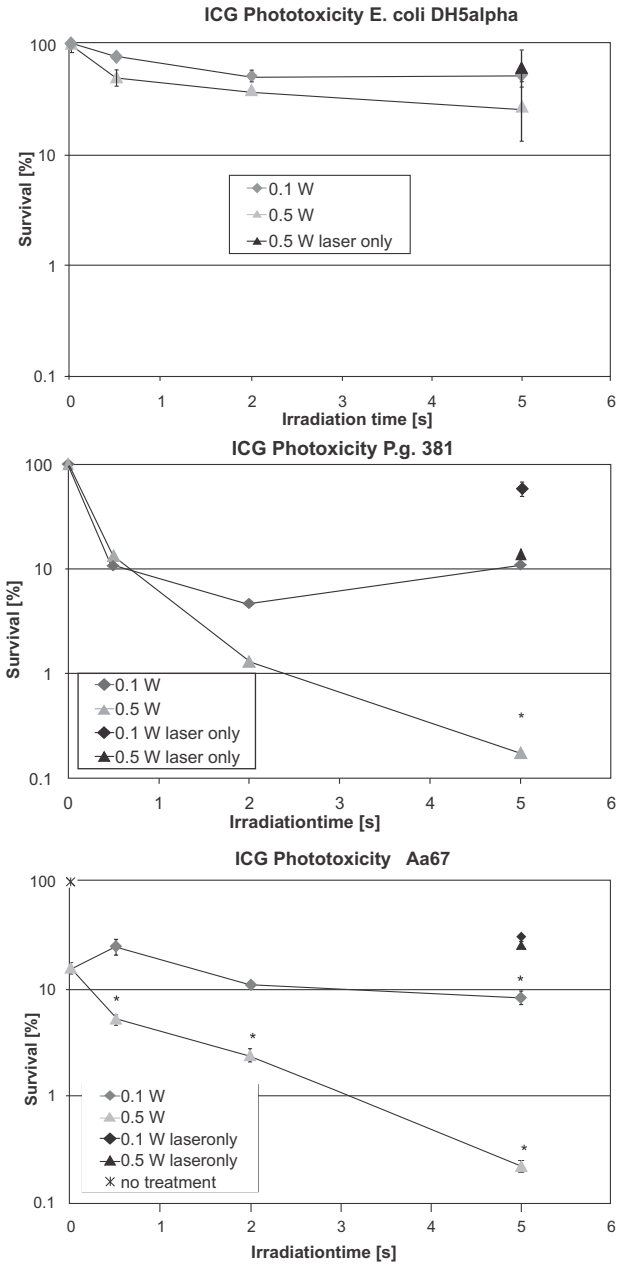

Figure 3. Indocyanine green preferentially kills periodontopathic bacteria. Standardized planctonic solutions of different bacteria (E. coli, P. gingivalis, A. actinomycetemcomitans) and cultured gingival epithelial cells (HepG) were incubated with 5 µM concentrations of ICG for 1 minute, and then washed once with PBS. Pellets were irradiated for varying times and with different doses of 810 nm laser light. The numbers of viable bacteria remaining were determined using a dilution method and counting colonies, and the percentage of killed bacteria was calculated and averaged. The graph shows the average ICG concentration and standard deviation. Statistically significant differences ($p < 0.05$) are denoted with an asterisk. Replicate experiments produced similar results period.

averaged. Statistical significance was determined using one-way ANOVA. As controls, we tested bacterial

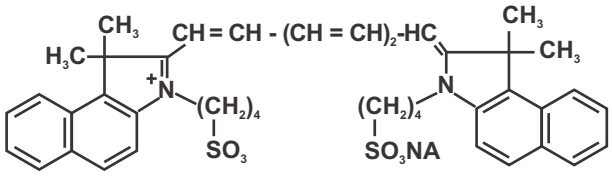

Figure 4. Chemical structure of indocyanine green (adopted from Ito S., et al., 1995)

increased exposure time. Similarly, higher levels of killing might be achieved using 10 M ICG instead of 5 μM, which would result in higher ICG uptake and presumably greater killing. Of interest might be the rapid, large uptake and killing of *A. actinomycetemcomitans*, which could make ICG useful for the treatment of *A. actinomycetemcomitans*-mediated forms of aggressive periodontitis.

At this point, it is unclear how ICG kills bacteria. It is possible that several different mechanisms exist, one based on actual phototoxicity and another based on indirect thermal effects, which are responsible for bacterial killing at higher flux levels. We noticed that at high flux levels bacterial pellets heated up significantly if ICG was present, while there was no apparent temperature increase felt in irradiated pellets in the absence of ICG. At low flux levels, no temperature increase was felt, but significant bacterial killing still took place. Therefore, it is possible that at low flux levels actual phototoxicity may result in killing, whereas at high levels of flux, thermal effects dominate killing.

It is interesting to note that the decrease in cell survival tends to level off with increased exposure time. The reasons for this are unclear, and it is possible that several mechanisms could be responsible for it. It is possible that some bacterial cells might escape destruction as they are protected by increasing amounts of debris or that fluid motion carries bacteria out of the light path. This seems plausible for the 0.5 W power setting where bacterial samples with high ICG uptake heat up significantly and form coagulated masses. As we see those thermal effects, ICG likely no longer acts as a phototoxic agent under these conditions, but probably exerts its bactericidal effect by efficiently absorbing laser energy within the cells and denaturing bacterial proteins. For low power settings, it is possible that photobleaching takes place after five seconds, or some intrinsic bacterial mechanisms such as efflux pumps are activated. Further studies might elucidate these mechanisms.

In contrast to many other photosensitizing dyes, excluding methylene blue, indocyanine green is already used clinically for other purposes in an FDA-approved formulation (NDA 11-525-S-017). It is administered as 10 ml IV solution of 2.5 mg/ml ICG (3.2 mM) with 0.125 mg/ml sodium iodide, and is used to determine cardiac output, hepatic function and liver blood flow, and ophthalmic angiography. The formulation in this study contains a much lower amount of ICG (5 M) in a smaller volume, and in a clinical setting would be applied as a topical solution, either injected into periodontal pockets or applied to exposed root surfaces. The amount of DMSO present is well below the maximum amount permissible for inactive ingredients, and possibly could be eliminated entirely since ICG dissolves reasonably well in saline. Therefore, safety issues should be comparable or less than for the existing FDA-approved formulation. In addition, we used extracted teeth to test if ICG would stain tooth surfaces and materials. We applied the 10 μM ICG solution to enamel, cementum, calculus and fissures, as well as to restorations such as composites, amalgam, porcelain fused to metal crowns and all ceramic crowns for one minute, and rinsed off the solution with saline for one minute. We did not note any staining on tooth surfaces and restorations, with the exception of calculus, which stained green, presumably due to the bacterial content.

Because indocyanine green demonstrated promising results in this *in vitro* study using planctonic bacteria, further study should test if indocyanine green in conjunction with low level laser treatment has significant clinical effects in the biofilm environment of human periodontal pockets.

Acknowledgments

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References

Andersen, R., Loebel, N., Hammond, D. and Wilson, M. Treatment of periodontal disease by photodisinfection compared to scaling and root planing. *Journal of Clinical Dentistry* 2007; **18**:34-38.

Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 1976; **72**:248-254.

Chan, Y. and Lai, C.H. Bactericidal effects of different laser wavelengths on periodontopathic germs in photodynamic therapy. *Lasers in Medical Science* 2003; **18**:51-55.

Cowan, M.M., Van der Mei, H.C., Stokroos, I. and Busscher, H.J. Heterogeneity of surfaces of subgingival bacteria as detected by zeta potential measurements. *Journal of Dental Research* 1992; **71**:1803-1806.

de Almeida, J.M., Theodoro, L.H., Bosco, A.F., Nagata, M.J., Oshiiwa, M. and Garcia, V.G. Influence of photodynamic therapy on the development of ligature-induced periodontitis in rats. *Journal of Periodontology* 2007; **78**:566-575.

de Almeida, J.M., Theodoro, L.H., Bosco, A.F., Nagata, M.J., Oshiiwa, M. and Garcia, V.G. *In vivo* effect of photodynamic therapy on periodontal bone loss in dental furcations. *Journal of Periodontology* 2008; **79**:1081-1088.

Folli, S., Westermann, P., Braichotte, D. *et al.* Antibody-indocyanin conjugates for immunophotodetection of human squamous cell carcinoma in nude mice. *Cancer Research* 1994; **54**:2643-2649.

Ge, L.H., Shu, R. and Shen, M.H. [Effect of photodynamic therapy on IL-1beta and MMP-8 in gingival crevicular fluid of chronic periodontitis]. *Shanghai Kou Qiang Yi Xue* 2008; **17**:10-14.

Genina, E.A., Bashkatov, A.N., Simonenko, G.V., Odoevskaya, O.D., Tuchin, V.V. and Altshuler, G.B. Low-intensity indocyanine-green laser phototherapy of acne vulgaris: pilot study. *Journal of Biomedical Optics* 2004; **9**:828-834.

Haraszthy, V.I., Lally, E.T., Haraszthy, G.G. and Zambon, J.J. Molecular cloning of the fur gene from *Actinobacillus actinomycetemcomitans*. *Infection & Immunity* 2002; **70**:3170-3179.

Krespi, Y.P., Slatkine, M., Marchenko, M. and Protic, J. Lethal photosensitization of oral pathogens via red-filtered halogen lamp. *Oral Diseases* 2005; **11 Suppl 1**:92-95.

Lipson, R.L., Baldes, E.J. and Olsen, A.M. Hematoporphyrin derivative: a new aid for endoscopic detection of malignant disease. *Journal of Thoracic Cardiovascular Surgery* 1961; **42**:623-629.

Ito, S., Muguruma, N., Kakehashi, Y. *et al.* Development of fluorescence-emitting antibody-labeling substance by near-infrared ray excitation. *Bioorganic & Medical Chemistry Letters* 1995; **5**:2689-2694

Pass, H.I. Photodynamic therapy in oncology: mechanisms and clinical use. *Journal of the National Cancer Institute* 1993; **85**:443-456.

Pervaiz, S. and Olivo, M. Art and science of photodynamic therapy. *Clinical Experimental Pharmacology Physiology* 2006; **33**:551-556.

Pfitzner, A., Sigusch, B.W., Albrecht, V. and Glockmann, E. Killing of periodontopathogenic bacteria by photodynamic therapy. *Journal of Periodontology* 2004; **75**:1343-1349.

Sausville, E.A., Longo, D.L. . Chapter 81. Principles of Cancer Treatment. In Fauci, A.S., Braunwald, E., Kasper, D.L., Hauser, S.L., Longo, D.L., Jameson J.L., Loscalzo, J. (Eds.): *Harrison's Principles of Internal Medicine*, 17th ed New York: McGrawHill, 2008; 514-532

Schwartz, S.K., Abolon K, Vermund H. Some relationships of porphyrins, x-rays and tumors. *University of Minnesota Medical Bullettin* 1955; **27**:7-8.

Soukos, N.S., Ximenez-Fyvie, L.A., Hamblin, M.R., Socransky, S.S. and Hasan, T. Targeted antimicrobial photochemotherapy. *Antimicrobial Agents and Chemotherapy* 1998; **42**:2595-2601.

Tadatsu, M., Ito, S., Muguruma, N. *et al.* A new infrared fluorescent-labeling agent and labeled antibody for diagnosing microcancers. *Bioorganic and Medicinal Chemistry* 2003; **11**:3289-3294.

Tokuda, M., Chen, W., Karunakaran, T. and Kuramitsu, H.K. Regulation of protease expression in *Porphyromonas gingivalis*. *Infection & Immunity* 1998; **66**:5232-5237.

Wilson, M., Dobson, J. and Harvey, W. Sensitization of oral bacteria to killing by low-power laser radiation. *Current Microbiology* 1992; **25**:77-81.

Withrow, K.P., Gleysteen, J.P., Safavy, A. *et al.* Assessment of indocyanine green-labeled cetuximab to detect xenografted head and neck cancer cell lines. *Otolaryngology Head and Neck Surgery* 2007; **137**:729-734.

Yilmaz, S., Kuru, B., Kuru, L., Noyan, U., Argun, D. and Kadir, T. Effect of gallium arsenide diode laser on human periodontal disease: a microbiological and clinical study. *Lasers in Surgery and Medicine* 2002; **30**:60-66.