República Federativa do Brasil
Ministério do Desenvolvimento, Indústria e do Comércio Exterior
Instituto Nacional da Propriedade Industrial

(21) BR 10 2013 008777-7 A2

* B R 1 0 2 0 1 3 0 0 8 7 7 7 A 2 *

(22) Data de Depósito: 11/04/2013
(43) Data da Publicação: 18/11/2014
(RPI 2289)

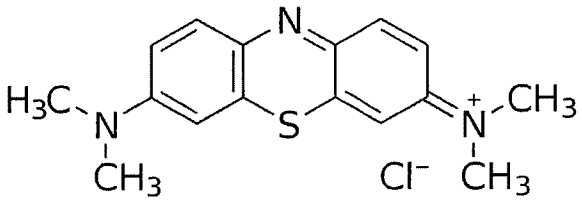
(51) Int.Cl.:
A61K 31/5415
A61N 5/067
A61P 33/02

(54) Título: USO DE TERAPIA FOTODINÂMICA PARA O TRATAMENTO DE LEISHMANIOSE TEGUMENTAR

(73) Titular(es): UNIVERSIDADE ESTADUAL DE MARINGÁ

(72) Inventor(es): ADRIANA PASSARELLA GEROLA, BRUNO HENRIQUE VILSINSKI, DIOGO SILVA PELLOSI, ELZA KIMURA, LILIAN SOMENCI PELOI, MARIA VALDRINEZ CAMPANA LONARDONI, MÔNICA RAQUEL SBEGHEN, NOBORU HIOKA, SANDRA MARA ALESSI ARISTIDES, THAÍS GOMES VERZIGNASSI SILVEIRA, WILKER CAETANO

(57) Resumo: USO DE TERAPIA FOTODINÂMICA PARA O TRATAMENTO DE LEISHMANIOSE TEGUMENTAR. A presente invenção trata da utilização do corante azul de metileno no tratamento do leishmaniose tegumentar por meio da Terapia Fotodinâmica (TFD). O mecanismo de ação da TFD em sistemas celulares consiste na irradiação de um composto fotoativo (fotossensibilizador - FS) com iradiação de comprimento de onda adequado na presença de oxigênio. As fontes de luz adotadas são LASERs, diodos emissores de luz (LED) e lâmpadas halógenas, de sódio e tungstênio, todos com emissão na região do vermelho, adequada para a fotoexcitação do FS.



USO DE TERAPIA FOTODINÂMICA PARA O TRATAMENTO DE LEISHMANIOSE TEGUMENTAR

INTRODUÇÃO

A presente invenção descreve um tratamento alternativo para a leishmaniose tegumentar, envolvendo a aplicação do Azul de Metileno (AM) (Figura 1) por meio da Terapia Fotodinâmica (TFD).

A leishmaniose tegumentar é uma doença crônica de manifestação cutânea ou visceral causada por protozoários flagelados do gênero *Leishmania*. Esta doença consiste em um problema de saúde pública no Brasil, uma vez que no período de 1980 a 2009 foram notificados cerca de 698.270 casos nas regiões endêmicas.

A TFD é uma modalidade de tratamento baseada na combinação de três elementos: luz, um composto não tóxico e sensível à luz, chamado composto fotossensibilizador (FS) e oxigênio. O mecanismo de ação da TFD em sistemas celulares consiste na irradiação do FS com radiação de comprimento de onda adequado na presença de oxigênio para gerar Espécies Reativas de Oxigênio (EROS), tais como radicais livres (como superóxido e hidroxil) e oxigênio singlete, um dos principais agentes citotóxicos, causando a morte celular por apoptose ou necrose.

O AM é um composto FS que ultimamente tem sido aplicado na TFD contra diversos tipos de doenças (tratamento de câncer e inativação de diversos tipos de micro-organismos, como bactérias, fungos e protozoários). Dentre as vantagens deste composto estão sua absortividade na região do

vermelho (região de maior penetração da luz nos tecidos), baixo custo, fácil aquisição e ausência de malefícios que comprometam a saúde do paciente.

A TFD possui diversas vantagens em relação aos tratamentos atualmente empregados contra a leishmaniose tegumentar, como a utilização dos fármacos antimoniais pentavalentes (antimoniato de N-metil-glucamina ou antimoniato de megumina – Glucantime®) e outras drogas como a anfotericina B e pentamidinas, as quais podem provocar intensos efeitos colaterais, como anorexia, mialgia, artralgia, pancreatites, trombocitopenia, leucopenia, cardiopatia, nefropatia e hepatotoxicidade, entre outros, fato que usualmente ocasiona o abandono do tratamento. Adicionalmente, a resistência primária dos pacientes aos medicamentos mencionados pode ocorrer em até 14% dos casos.

Outras formas alternativas de tratamento contra a leishmaniose têm sido aplicadas, como a crioterapia, curetagem, eletrodissecação e termoterapia. No entanto, além do alto risco de recorrência da doença, os resultados finais em termos estéticos não são satisfatórios.

Desta forma, esta invenção trata da utilização de TFD como alternativa para o tratamento de leishmaniose utilizando AM como FS. Essa técnica possui a vantagem de não apresentar efeitos colaterais, além de ter baixo custo quando se utiliza o AM (corante comercial) e dispositivos ópticos constituídos por LEDs, LASERs, bem como lâmpadas halógenas, de sódio e tungstênio, todas apresentando emissão na região do vermelho (janela terapêutica), adequada para a ativação do FS e observação dos efeitos citotóxicos.

Nossos estudos têm mostrado que o AM é eficiente no tratamento de leishmaniose por TFD, uma vez que não apresenta efeitos colaterais, não gera novos parasitos resistentes, e nem casos de recorrência da doença após o tratamento são observados.

Nas buscas de anterioridade criteriosamente realizadas foram encontradas algumas patentes com certa similaridade, porém nenhuma que impedisse o critério estabelecido de novidade da presente invenção.

A **US 20120302557 A1,** *"Biologically active methylene blue derivatives"*, propõe o uso do AM e derivados em TFD no tratamento de câncer, bem como para a prevenção de infecções microbiais e foto-desinfecção.

A **US 20090018485 A1**, *"Control of microorganisms in the sino-nasal tract"*, propõe o uso de AM no tratamento da bactéria do trato sino-nasal *Porphyromonas gingivalis* (*P. gingivalis*). Esta patente mostrou um aumento na inativação desta cultura de bactérias com a concentração do FS e em função do tempo de iluminação.

A **US 20070123520 A1**, *"Methylene blue therapy of avian influenza"*, propõe o uso de corantes de tiazina, especialmente AM, administrado por via oral duas vezes por dia com o objetivo de tratar ou prevenir o vírus da gripe aviária através da TFD.

A **US 20060264423 A1**, *"Methylene blue therapy of viral disease"*, propõe um método para a utilização do AM ou derivados numa formulação de liberação imediata ou controlada contra a infecção da hepatite. Os resultados

mostraram que o AM combinado com reduzidos níveis de luz promove a inativação da infecção da hepatite.

A **CN 20091208279**, *"New effect of methylene blue on prevention of dental caries and application therefore"*, propõe a utilização do AM na prevenção da cárie dentária.

A **CN 101648021**, *"Application of methylene blue used as cancer-philic targeting substance and nano medicine containin methylene blue"*, propõe a utilização do AM contra diversos tipos de câncer através da TFD.

A **GR 20090100147**, *"Original anti-cancerous action of sigma ligants: quinacrine and methylene blue"*, propõe a utilização de um formulado misto envolvendo a quinacrina e o AM contra o câncer. Verifica-se através deste formulado que há um aumento da ação anticancerígena do formulado misto em comparação com as drogas isoladas, além da proteção contra as dores causadas pelos medicamentos mais eficientes utilizados na terapia tradicional, como os medicamentos a base de platina, paclitaxel e vincristina.

A **KR 20080016372 A**, *"Use of methylene blue for treatment of retinal damage and protection of retina"*, propõe uma formulação de AM através da administração oral ou injeção para o tratamento de danos na retina.

A **MXPA 02002183A**, *"A methylene blue diagnostic agent and diagnostic methods for detection of epithelial cancer"*, propõe uma formulação de AM para a detecção de células cancerosas e pré-cancerosas do tecido epitelial.

A **US 6083487 A**, *"Methylene blue and toluidene blue mediated fluorescence diagnosis of cancer"*, propõe o uso de soluções de AM ou azul de toluidina para a detecção de células cancerígenas.

Assim, a presente invenção propõe a utilização do AM no tratamento da leishmaniose tegumentar através da TFD, diferindo das patentes anteriormente citadas quanto ao tipo de doença tratada. A maioria das patentes acima citadas envolve o uso do AM contra diversos tipos de micro-organismos (principalmente bactérias e fungos) e alguns tipos de câncer (inclusive como responsável pelo diagnóstico da doença), porém não foi encontrada nenhuma cujo objetivo é o tratamento da leishmaniose tegumentar.

DESCRIÇÃO DAS FIGURAS

A **Figura 1** mostra a estrutura molecular do AM, componente essencial na TFD da leishmaniose tegumentar, descrito na presente invenção.

DESCRIÇÃO DETALHADA DO INVENTO

O presente invento descreve a aplicação de AM como FS na TFD da leishmaniose tegumentar. As fontes de luz utilizadas na TFD são LEDs, LASERs e lâmpadas halógenas, de sódio e tungstênio, que emitem radiação luminosa na região do espectro de 550-750 nm, região esta que coincide com o espectro de absorção do FS em solução, necessária para a verificação do efeito fotodinâmico terapêutico. A potencialização do tratamento é alcançada pela dose fotodinâmica aplicada (D), dependente da concentração de FS e do tempo de iluminação.

Para a utilização de AM como FS aplica-se 0,5 mL de uma solução aquosa estéril a 0,5% de AM + 1% de lidocaína intralesional ou tópica. Existe a possibilidade de ampliar a faixa de concentração do AM nesta solução dependendo da gravidade e tamanho da lesão. A luz é aplicada após a administração do AM, com a fonte mantida a uma distância aproximada de até 10 cm da lesão, cobrindo-se toda a úlcera, além de cerca de 1 cm ao seu redor.

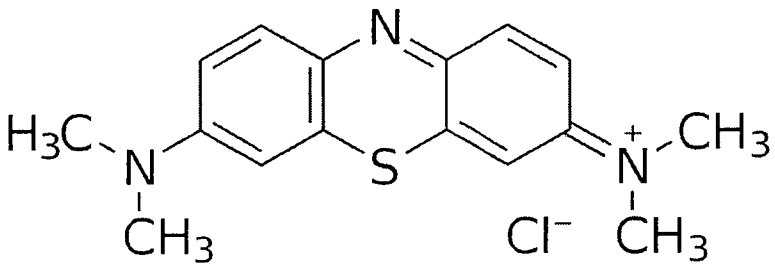
REIVINDICAÇÕES

USO DA TERAPIA FOTODINÂMICA PARA O TRATAMENTO DE LEISHMANIOSE TEGUMENTAR

1. USO DA TERAPIA FOTODINÂMICA PARA O TRATAMENTO DE LEISHMANIOSE TEGUMENTAR, **caracterizado pela** utilização do azul de metileno como composto fotossensibilizador, presente em solução aquosa estéril em diferentes proporções em massa.
2. USO DA TERAPIA FOTODINÂMICA PARA O TRATAMENTO DE LEISHMANIOSE TEGUMENTAR, de acordo com a reivindicação 1, **caracterizado pela** aplicação intralesional e tópica do azul de metileno no paciente.
3. USO DA TERAPIA FOTODINÂMICA PARA O TRATAMENTO DE LEISHMANIOSE TEGUMENTAR, de acordo com as reivindicações 1 e 2, **caracterizado pela** foto-ativação do azul de metileno utilizando luz LASER, de diodos emissores de luz (LED) e lâmpadas halógenas, de sódio e tungstênio.

DESENHO

Figura 1



RESUMO

USO DE TERAPIA FOTODINÂMICA PARA O TRATAMENTO DE LEISHMANIOSE TEGUMENTAR

A presente invenção trata da utilização do corante azul de metileno no tratamento de leishmaniose tegumentar por meio da Terapia Fotodinâmica (TFD). O mecanismo de ação da TFD em sistemas celulares consiste na irradiação de um composto fotoativo (fotossensibilizador – FS) com radiação de comprimento de onda adequado na presença de oxigênio. As fontes de luz adotadas são LASERs, diodos emissores de luz (LED) e lâmpadas halógenas, de sódio e tungstênio, todos com emissão na região do vermelho, adequada para a fotoexcitação do FS.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 April 2009 (16.04.2009)

PCT

(10) International Publication Number
WO 2009/048868 A1

(51) International Patent Classification:
A61K 31/5415 (2006.01) *A61P 31/04* (2006.01)
A61K 41/00 (2006.01) *A61K 35/00* (2006.01)

(21) International Application Number:
PCT/US2008/079050

(22) International Filing Date: 7 October 2008 (07.10.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/978,219	8 October 2007 (08.10.2007)	US
12/245,927	6 October 2008 (06.10.2008)	US

(71) Applicant *(for all designated States except US)*: **ONDINE INTERNATIONAL LTD.** [BB/BB]; Suite #5 Edghill House, Wildey Business Park, St. Michael (BB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LOEBEL, Nicholas** [GB/US]; 15211 167th Place Ne, Woodinville, WA 98072 (US). **STREET, Cale** [CA/US]; 5329 156th St. Sw, Edmonds, WA 98026 (US).

(74) Agent: **LEE, Jenny**; Dobrusin & Thennisch Pc, 29 W. Lawrence Street, Suite 210, Pontiac, MI 48342 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

WO 2009/048868 A1

(54) Title: PHOTODYNAMIC THERAPY PROCESS AND PHOTOSENSITIZER COMPOSITIONS

(57) Abstract: Use of a photosensitizer composition in the manufacture of a medicament for photodynamic therapy and a process for photodynamic therapy are provided along with a photosensitizer composition suitable for the use and the process. The photosensitizer composition comprises at least one photoactive ingredient in a chemically reduced state. According to the use, the photosensitizer composition having at least one photoactive ingredient in a chemically reduced state is provided. The photosensitizer composition is applied to tissue or other substrate such that the at least one photoactive ingredient is at or travels to a location adjacent a target medium and the at least one photoactive ingredient is then altered to a photoactive state. The at least one photoactive ingredient is then exposed to light energy for assisting in inhibiting at least one pathogen at the target medium. The prefered photosensitizer is methylene blue. It is preferably reduced by ascorbic acid.

PHOTODYNAMIC THERAPY PROCESS AND PHOTOSENSITIZER COMPOSITIONS

TECHNICAL FIELD

[0001] The present invention relates to a photodynamic therapy process and a photosensitizer composition useful for that therapy. More particularly, the present invention relates to a photosensitizer composition having photoactive ingredients in a form that is more suitable for transport to a target medium and/or provides for more efficient light absorption.

BACKGROUND OF THE INVENTION

[0002] Photodynamic therapy, particularly photodynamic disinfection, has been demonstrated to be an effective non-antibiotic antimicrobial approach *in vitro*. For the purpose of this specification, photodynamic therapy and photodynamic disinfection shall hereinafter be collectively referred to as photodynamic therapy. One exemplary advantage of photodynamic therapy as an antimicrobial treatment modality is that it is typically not subject to issues of resistance that can plague the use of antibiotics. As another exemplary advantage, it can be employed as a localized topical treatment that can be administered in areas such as the oral cavity where reliable topical antibiotic delivery can be problematic. For these reasons and others, photodynamic therapy is fast becoming a valuable tool in the treatment of infection process and/or bacterial-related conditions such as periodontal disease.

[0003] Photodynamic therapy fundamentally involves the use of light energy to activate one or more photoactive ingredients of a photosensitizer composition so that those ingredients can then either pass energy on directly to a substrate/target (type I reaction), or can interact with molecular oxygen to produce reactive oxygen species (type II reaction). These reactions mediate the non-specific inhibition of microbial, certain human (e.g. tumor) cells and other pathogens such as virus primarily via lipid peroxidation, membrane damage, and damage to intracellular components. In order for this process to generate an antimicrobial (e.g., bactericidal) or other desired effect, it is

typically desirable that the photosensitizer composition, and particularly the photoactive ingredients thereof, be internalized into or brought into very close association with the cell wall/membrane, cytoplasm, and/or inner constituents of a target cell or organism.

[0004] The molecular properties of a photosensitizer composition and/or its ingredients can play a large role in determining the site of action and therefore the antimicrobial (e.g., bactericidal) mechanism. Specifically, the charge and lipophilicity ratio of the composition and its ingredients can play a significant role. Some photoactive ingredients, such as methylene blue, are polar in nature and hence are very soluble in an aqueous environment. Ingredients or molecules of this type can remain in solution either intra- or extracellularly, and may demonstrate relatively little physical association or integration with or into a target medium (e.g., may not fractionate or associate with hydrophobic targets) such as bacterial membranes or cells. Moreover, it can often be the case that these photoactive ingredients are passed through aqueous trans-membrane channels (e.g., passive diffusion into the cytoplasm) or be brought across in aqueous vesicles via an active transport process into the intracellular environment of the bacteria or other target. Thus, it can be difficult for these ingredients to become closely associated with the cell wall/membrane of a target organism.

[0005] On the other hand, non-polar photoactive ingredients with hydrophobic properties are able to integrate into the bacterial membrane via preferential association with the hydrophobic interior of the lipid bilayer. Photoactive ingredients of this type can thus associate with the outer bacterial membrane, or if internalized, can be partitioned into a hydrophobic location within the bacterial cell. Thus, non-polar photoactive ingredients can more easily associate with and pass through the cell wall/membrane of a target organism, such as a bacterium or other microbe.

[0006] Association with cell walls or membranes can be further complicated by the fact that Gram-negative and Gram-positive bacteria differ in structure. Gram-positive strains, such as *Staphylococcus aureus ("S. aureus")*, possess a relatively permeable cell wall consisting largely of peptidoglycan. By contrast, Gram-negative strains such as *E. Coli* have two

overlapped walls, each consisting of a separate phospholipid bilayer with the outer wall or membrane containing a large proportion of lipopolysaccharide (LPS) on the outer surface (often accounts for ~40% of the mass of the outer membrane), rendering such cells much less permeable. While both strains have a net negative charge on the outer surface, the significant number of negatively charged molecular groups comprising LPS also confers a large overall negative charge on the Gram-negative strains. Thus, photosensitizer molecules or ingredients that are cationic (i.e., positively charged) exhibit enhanced uptake at bacterial surfaces (due to electrostatic or opposite charge attraction) compared to anionic or neutral species, although uptake/internalization is generally greater in Gram-positive strains than Gram-negative strains simply due to the physical barrier structure involved.

[0007] A recent study (Maisch et al, 2007) used singlet oxygen detection techniques to show that PHOTOFRIN®, a commercial photosensitizer composition, associates with and has a photocatalytic site of action at or in the bacterial membrane of *S. aureus*. Another study (Tegos and Hamblin, 2006) examined the ability of bacterial multidrug resistance pumps to remove photosensitizers such as methylene blue, toluidine blue O, and dimethylmethylene blue from the intracellular environment, thus decreasing the efficacy of photodynamic therapy. Their results suggest that these photoactive ingredients are actively taken up into the strains examined (both Gram-negative and Gram-positive) and that the site of action is within the intracellular environment. Since the intracellular environment is unique and is closely regulated across various physiological parameters (osmolarity, pH, alkalinity, charge, redox, etc), it would be desirable to control photoactive molecules or ingredients such that they behave in a certain manner in an extracellular situation but exhibit different behavior once internalized with the intent of increasing the efficacy of the photodynamic reaction. The present invention provides such differing behaviors.

SUMMARY OF THE INVENTION

[0008] The present invention provides a photosensitizer composition for use in photodynamic disinfection comprising at least one photoactive ingredient in a chemically reduced state.

[0009] The present invention also provides a method for photodynamic disinfection comprising: providing a photosensitizer composition having at least one photoactive ingredient in a reduced state; applying the photosensitizer composition to a substrate such that the at least one photoactive ingredient associates with a target medium; allowing the at least one photoactive ingredient to reach a photoactive state while at the location adjacent, contacting or taken within (collectively hereinafter referred to as "adjacent") the target medium; exposing the at least one photoactive ingredient to light at a wavelength absorbed by the at least one photoactive ingredient so that the at least one photoactive ingredient at least assists in inhibiting at least one pathogen associated with, or comprising, the target medium. The term "pathogen" as used herein shall mean undesired prokaryotic or eukaryotic cells or collections of cells, including microbes, bacteria, virus, fungi, tumor cells, or other nucleic-acid containing particles or cells or. The terms "inhibiting" "inhibition" and/or "inhibit" as used herein shall mean to inhibit, reduce, destroy, kill and/or eliminate.

[0010] The present invention further provides a use of a photosensitizer composition in the manufacture of a medicament for photodynamic therapy comprising the same elements and/or steps as described above for the method for photodynamic disinfection.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. 1 is a graph showing optical absorbance measurements for an oxidized methylene blue solution and also for several reduced methylene blue solutions using 0.05% w/v, 0.1% w/v, 0.2% w/v, 0.5% w/v, and 1.0% w/v ascorbic acid as the reducing agent as described in Example I;

[0012] FIG. 2 is a bar graph showing the antimicrobial efficacy data of a reduced methylene blue solution and a non-reduced methylene blue solution

in the photodynamic disinfection of *E. coli* versus controls as described in Example II; and

[0013] FIG. 3 is a table showing the antimicrobial efficacy data of various samples used in the experiment as described in Example III.

DESCRIPTION OF THE PREFERRED EMBODIMENT

[0014] The present invention is predicated upon provision of a process for performing photodynamic therapy upon tissue or other substrates and a photosensitizer composition suitable for use in the process. As used herein, the term "tissue" can include any substrate associated with a collection of cells (e.g., a collection of bacterial cells or a localized infection or tumor site). Typically, the process of the present invention includes provision of the photosensitizer compositions with photoactive ingredients in a chemically reduced state. The term "chemically reduced state" shall mean a partially reduced state and/or a wholly reduced state with respect to the neutral point of the molecule or composition as part of a redox couple. The reduced state of the photoactive ingredients can render the ingredient more transportable and/or suitable for passage through one or more barrier mediums such that the photosensitizer composition, particularly the photoactive ingredients, can more effectively reach a target medium. At the target medium, the photoactive ingredients are typically converted to a second state (e.g., a photoactive state) that is particularly suitable for photodynamic therapy. The reduced state can also provide the ingredients in a relatively colorless or low color state prior to conversion of the ingredients to the second state (i.e., a colored, oxidized state) such that photodynamic therapy can be more efficiently or selectively directed at a target medium without causing the same amount of undesirable non-specific staining, killing and/or unnecessary light absorption caused by other photosensitizer compositions. Moreover, this colorless or low color state can cause such efficiencies and selectivity regardless of whether the photoactive ingredients pass through a barrier medium or are placed directly in apposition to the target medium.

Photosensitizer Composition

[0015] The photosensitizer composition can include at least one photoactive ingredient and optionally a combination of the at least one photoactive ingredient and other ingredients. Such other ingredients can include, without limitation, solvents (aqueous or otherwise), buffering components (osmolarity, pH), diluent, adjuvant, excipient, and redox-potential-controlling agents, viscosity agents, surfactants, flavorants, preservatives, cell permeabilizing agents, antibiotics, bactericides/bacteriostats, and combinations thereof. The ingredients are preferably approved by a regulatory agency of the Federal or a State government, or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

[0016] Generally, the at least one photoactive ingredient is capable of exhibiting photoactive characteristics. As used herein, the term photoactive ingredient encompasses ingredients that are photoactive or that can become photoactive as will be understood further below. Preferably, the photoactive ingredients can assist in the performance of photodynamic therapy, and particularly the inhibition of undesirable pathogens, cells or the like according to one or multiple different mechanisms. According to one mechanism, the one or more photoactive ingredients are activated upon exposure to light energy such that the one or more ingredients can pass that energy to and/or engage in redox reactions with a target medium and particularly cells (e.g., bacterial cells) of that target medium in order to inhibit those cells. According to another mechanism, the one or more photoactive ingredients are activated upon exposure to light energy such that the photoactive ingredients interact with molecular oxygen to produce reactive oxygen species that are toxic to the undesired cells.

[0017] The photoactive ingredient can be any suitable art-disclosed photosensitizer. For example, the photoactive ingredient can be from the phenothiazine class (e.g., methylene blue and its derivatives, etc.). Arianor steel blue, toluidine blue O, crystal violet, azure blue cert, azure B chloride, azure 2, azure A chloride, azure B tetrafluoroborate, thionin, azure A eosinate, azure B eosinate, azure mix sicc., azure II eosinate,

haematoporphyrin HCl, haematoporphyrin ester, aluminium disulphonated phthalocyanine are also examples of suitable photoactive ingredients. Porphyrins, pyrroles, tetrapyrrolic compounds, expanded pyrrolic macrocycles, and their respective derivatives are further examples of suitable photoactive ingredients. Photofrin® manufactured by QLT PhotoTherapeutics Inc., Vancouver, B.C., Canada, is yet another example of a suitable photoactive ingredient. Other exemplary photoactive ingredients may be found in U.S. Patent Nos. 5,611,793 and 6,693,093. The photoactive ingredients mentioned above are examples are not intended to limit the scope of the present invention in any way.

[0018] Depending on the desired application, the photosensitizer composition may optionally comprise a plurality of the photoactive ingredients. The amount or concentration of the photoactive ingredient(s) may vary depending upon the desired application, the particular photoactive ingredient(s) used, and the target microbes to be destroyed. For example, concentration of the photoactive ingredient(s) in the photosensitizer composition may range from about 0.00001% to about 25% w/v, from about 0.0001% to about 10% w/v, from about 0.001% to about 1% w/v, from about 0.001% w/v to about 0.1% w/v, or from about 0.005% w/v to about 0.05% w/v.

[0019] It is contemplated that the light or light energy of the present invention can be provided by any electromagnetic radiation source including visible light and non-visible radiation or light. The light can contain any wavelength(s) that can be absorbed by the at least one photoactive ingredient of the photosensitizer composition, when the at least one photoactive ingredient is in a colored and/or photoactive state. The wavelengths include wavelengths selected from the continuous electromagnetic spectrum, ranging from below the ultraviolet ("UV") range, through the visible range, and into and beyond the near, mid and far-infrared range etc. The wavelengths are generally preferably between about 160 nm to about 1600 nm, more preferably between about 400 nm to about 800 nm, most preferably between about 500 nm to about 850 nm although the wavelengths may vary depending upon the particular photoactive ingredient(s) used and the light intensity. The light may be produced by any suitable art-disclosed light

emitting devices such as lasers, light emitting diodes ("LEDs" including organic light emitting diodes ["OLED's"], superluminescent light emitting diodes ["SLED's")], or the like), incandescent sources, fluorescent sources, or the like.

[0020] At least one and possibly all of the photoactive ingredients are capable of being altered to a state that make the ingredients more suitable for transport through one or more barrier mediums to one or more target mediums. In addition or alternatively, the altered state of the photoactive ingredients can make the ingredients less colored such that the ingredients can cause less staining, less non-specific (i.e., away from the desired treatment area) killing and/or less unnecessary light absorption (e.g., by allowing light energy to pass through the composition to the target site).

[0021] Although other alternative states may be employed for the photoactive ingredients, it is preferable for the photoactive ingredients to be reacted or otherwise brought to a chemically reduced state (i.e., a molecular state whereby the photoactive ingredient is chemically "reduced" via a gain of electrons from another substance which in turn donates said electrons, itself becomes oxidized, and thus acts as the "reducing agent"). Thus, it is preferable for the photoactive ingredient to be capable of being driven, either fully or partially, to a reduced state for transport through the barrier medium. Generally, it is contemplated that nearly any photoactive ingredient that can be reduced by virtue of a reduction reaction can be employed in the photosensitizer composition. Preferred photoactive ingredients are dyes and more preferably phenothiazine dyes or derivatives thereof such as methylene blue or toluidine blue O. It is to be understood that the term photoactive ingredients include the ingredients that are not necessarily "photoactive" in their fully reduced form but become "photoactive" after re-oxidation to a colored form.

[0022] In various biological systems, several key enzymes and major metabolic pathway reactions can mediate the oxidation or reduction of photoactive ingredients that are brought into association with a pathogen, unwanted cell or cluster of cells, organisms or pathogens. For example, there is evidence that a thiazine reductase enzyme present in the membrane of

mammalian endothelial cells can reduce methylene blue to its colorless (*leuco)* form at the cell surface. In the reduced form, a photoactive ingredient such as a thiazine dye, being substantially uncharged and relatively lipophilic in nature after such reduction, can cross the cell membrane with little difficulty by a diffusion process or otherwise. Once in the cell, the photoactive ingredient is re-oxidized to the colored (photoactive) form by, for example, exposure to molecular oxygen, heme-containing proteins, active intracellular oxidation processes or otherwise. Thus, the reduced photoactive ingredient is more capable of crossing a biological barrier medium and more closely associating with the target medium (e.g., pathogen, cell, organism or combination thereof). Moreover, the colorless or substantially colorless nature of the reduced photoactive ingredient (as compared to the intense color observed in the oxidized form) will decrease incidental staining of tissue or otherwise and will allow activating light energy to more readily penetrate into the treatment site such that photodynamic reactions can be more efficiently driven. This, in turn, may allow for less light to be used for photodynamic therapy and/or inhibition of pathogens.

[0023] To prepare the photosensitizer composition, the one or more photoactive ingredients are typically exposed to a reducing agent, which is typically added to the photosensitizer composition or otherwise associated with the photoactive ingredient[s]. The reducing agent can include one or more reducing compounds. In general, efficient reducing compounds are atoms, molecules, compounds or the like in which the atoms have relatively large atomic radii, low electronegativity, and low ionization energy.

[0024] Desirable reducing compounds generally include, without limitation, ferrous ions, lithium aluminum hydride, hydrogen, sodium dithionite, zinc amalgam, potassium ferrocyanide, sodium borohydride, stannous ion, sulfites, hydrazine, diisobutylaluminum hydride, oxalic acid, ascorbic acid, ascorbate, active metal (e.g., potassium, calcium, sodium, barium or the like), reduced glutathione, dextrose, urea and any urea derivatives, anethol, glyoxol, ethylenediamine tetraacetic acid (EDTA), any combination thereof or the like. Preferably, the reducing agent is capable of reducing the photoactive ingredient[s] to a reduced form (e.g., a leuco form) either partially,

substantially entirely (e.g., at least 90% reduced) or fully. It should be recognized, however, that it is undesirable to use excess reducing agent, which can thereafter prevent the photoactive ingredient from returning to a photoactive or oxidized state. A stoichiometric reduction can, in a preferred embodiment, be quantified visually or preferably spectrophotometrically as a percentage of color loss as the photoactive ingredient[s] transition from an oxidized form to a reduced form. It is contemplated that the photoactive ingredients can undergo a color loss of at least about 10%, more typically at least about 40% and possibly even at least about 70%, at least about 90% or even more. The amount of color loss can be determined by measuring optical density. In particular, the optical density of a photosensitizer solution or formulation can be determined non-quantitatively by visual inspection or quantitatively via a standard spectrophotometric assay. The formulation would be reduced to a condition in which the optical density is about 90% or less, more typically about 70% or less, still more typically about 50% or less an even possibly about 20% or about 10% or less of the original non-reduced form. If desirable, the solution can be reduced to the point where the optical density is essentially 0% (e.g., about 0.5% or less) of the original solution, a condition in which the formulation would appear virtually colorless.

[0025] The reducing agent can react with the photoactive ingredient[s] in a reaction (typically a reversible reaction) that causes electrons to be transferred from the reducing agent to the photoactive ingredient[s]. As one example, methylene blue and other dyes (e.g., phenothiazine dyes) can react with ascorbic acid such that electrons are taken on by the methylene blue or the like through a semiquinone intermediate, which then in turn dismutates to the reduced or leuco-form of the dye. The reducing agent is by contrast itself oxidized, for example ascorbic acid is converted to monodehydroascorbate and eventually dehydroascorbate. Neither the leuco-form of methylene blue, nor the oxidized forms of ascorbate are toxic *in vivo*, as both are commonly found intermediates in the normal metabolic breakdown of the parent compounds, are relatively stable and unreactive, and do not cause cellular damage. The reducing agent or the reducing agents contemplated can be relatively weaker or stronger in their ability to donate electrons to the

photoactive ingredient[s] based upon their relative redox potential (measured in volts relative to a standard hydrogen electrode). Generally, reducing agents are more electronegative than the photoactive ingredients.

[0026] Once reduced, it is preferable for the photosensitizer composition and/or the photoactive ingredient[s] to lose at least some degree of color. Typically, when the photoactive ingredient is a dye such as those discussed herein and has an original dye color (e.g., blue) it will lose a substantial amount of color upon reduction of that ingredient. Such color loss can be due to, for example, saturation of the central thiazine ring in a phenothiazine compound, and resulting loss of pi-electron mobility. In a preferred embodiment, the one or more photoactive ingredient[s] lose at least about 50%, more typically at least about 80% and even more typically at least about 90% or more of their optical absorbance.

Photodynamic Therapy

[0027] Photodynamic therapy can be performed according to the present invention by introducing the photosensitizer composition to tissue (e.g. human tissue, animal tissue, another substrate or otherwise) in a reduced and/or transportable state in which the at least one photoactive ingredient is typically less photo-active or substantially nonphotoactive. The photosensitizer composition, particularly the at least one photoactive ingredient thereof, is then allowed to pass through one or more barrier mediums to be adjacent, contacting or taken within ("collectively hereinafter referred to as "adjacent") a target medium, which preferably includes one or more pathogenic organisms (e.g., microbes, bacterial, virus, fungus, other nucleic-acid containing particle cells, or the like) or target cells (such as tumor cells). It should be understood that such barrier mediums can be within the target medium (e.g., cells). Once the at least one photoactive ingredient has penetrated through, or been associated with the barrier mediums, the at least one photoactive ingredient is then allowed to change form (e.g., become photoactive and/or oxidized) in the oxidizing microenvironment of the target medium. In addition or as an alternative to oxidization by the microenvironment of the target medium, or alternatively, the at least one photoactive ingredient may change form to its

photoactive state optionally with the assistance of an oxidizing agent. The oxidizing agent can be any suitable compound that is more electropositive or oxidizing than the at least one photoactive ingredient in its reduced state. Examples of such an oxidizing agent include molecular oxygen, a hypochlorite solution, an oxidizing gas (e.g., fluorine, ozone, or the like), an oxidizing ion (e.g., permanganate ion or the like), an acid (e.g., nitric acid or the like), and a combination thereof. During and/or after such change in form, the photosensitizer composition, particularly the photoactive ingredients, are exposed to light such that the photoactive ingredients can then engage in photodynamic type I and/or II reactions and at least assist in inhibiting at least one pathogen associated with the target medium.

[0028] The photosensitizer composition can be applied to tissue using a variety of techniques. As examples, it can be sprayed on, brushed on, poured on or otherwise applied to the tissue. Moreover, the tissue to which the composition is applied can be human tissue, animal tissue or other living substrates or non-living substrates (e.g. surface of instruments) and can be performed for therapeutic purposes (e.g., for the purpose of inhibiting bacteria at a wound or lesion or elsewhere) for disinfection/sterilization procedures, experimental purposes or otherwise. In situations where the photosensitizer composition and/or the photoactive ingredients (e.g., photoactive dyes) thereof are substantially in a fully reduced state and/or are rendered less colorful or substantially colorless, light energy can pass through the solution without appreciable absorbance. Thus, in this state, the photosensitizer composition can be applied to a physical treatment site (e.g. a wound, lesion or otherwise) without significant local staining or discoloration. Furthermore, the optical density of the excess solution at the site is thereby reduced such that it does not substantially preclude the penetration of light into that treatment site.

[0029] It should be understood that the advantages of the solution or photoactive ingredients in terms of having less color can be achieved in situations where the photoactive ingredients were transported into and/or through a barrier medium and in situations where the ingredients did not experience such transport. Thus, the advantages of transportability and

lesser color can be independent of each other or can depend on each other. As such, only one or the other or neither may be required for the invention, rather than both unless otherwise specified. It is also contemplated that neither advantage be required unless otherwise specified.

[0030] Generally, the photosensitizer composition and process of the present invention can be applied to a wide variety of target mediums. As examples, and without limitation, the target medium could be a wound (e.g., a surface wound), a lesion, a full thickness wound/incision, nasal mucosa, a nail bed, a cell or cells, bacteria, virus, fungus, or otherwise. For experimental purposes, the target can be almost any biological or synthetic substrate target mediums that will particularly benefit from the photosensitizer composition and process including, without limitation, periodontal pockets or other components of the oral mucosa.

[0031] Once applied, the photosensitizer composition and particularly the photoactive ingredients thereof may be transported to the target medium (e.g., a wound, lesion or other site containing bacterial cells to be inhibited) or may be applied directly to the target medium without additional transport being necessary. Generally, it is desirable for the photoactive ingredients in their transportable and/or lower color state (e.g., reduced state) to be applied to or transported to a location that is adjacent to cells of the target medium that are to be reduced and/or killed by a photodynamic reaction, as is described herein. As suggested, some of the photoactive ingredients may be directly applied to a location adjacent those cells and/or the photoactive ingredients can be transported (e.g., travel through, into or around) relative to one or more barrier mediums to the location adjacent those pathogens (e.g., cells or organisms). As used herein, a location adjacent the pathogens (e.g., cells) in this situation can mean that there is small space between the pathogens and the photoactive ingredients. It is preferable, however, that a location adjacent means that the photoactive ingredients are contacting or even more preferably taken up within the pathogens. In one preferred embodiment, the photoactive ingredients are internalized into cells (e.g., bacterial cells), either through transition into the aqueous cytosol partition, or

through sequestration in the peptidoglycan bilayer membrane or LPS component.

[0032] The barrier mediums relative to which the photoactive ingredients are transported are typically environments that the photoactive ingredients might otherwise have difficulty penetrating if they were not in their transportable (lipophilic) or reduced state. In one embodiment, a barrier medium can be a hydrophilic or aqueous environment in which a normally polar photoactive ingredient might be very soluble if that ingredient were not in a reduced or otherwise transportable state. Such barrier mediums can be intracellular or extracellular. Examples of such barrier mediums include, without limitation, extracellular fluid, aqueous trans-membrane channels, aqueous vesicles, intracellular compartments, combinations thereof or the like. Advantageously, since the photoactive ingredients are in a transportable state, they can travel into, past or through the barrier mediums, which might otherwise impede their travel. Moreover, the photoactive ingredients may, in their transportable form, be more easily internalized into the cell and/or the cell's hydrophobic membrane components. This transportable form of the photoactive ingredient(s) may explain its increased antimicrobial efficacy against Gram-negative bacteria as demonstrated in Example III below. This increased antimicrobial efficacy against Gram-negative bacteria can be used to inhibit various Gram-negative pathogens including but not limited to *E. coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, black pigmented anaerobes such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens*, *Fusobacterium nucleatum*, *Aggregetobacter actinomycetemcomitans* or the like, and a combination thereof.

[0033] During or after location of the photoactive ingredients adjacent to the pathogens (e.g., cells) of the target medium, it is typically desirable for the photoactive ingredients to reach and/or return to a second, preferably photoactive state. Generally, it is contemplated that multiple different mechanisms may be employed to change the photoactive ingredients from their reduced state to a photoactive state. For example, chemicals such as oxidizers may be applied to the target medium for initiating the change. Alternatively energy may be provided to the photoactive ingredients in order

to initiate the change. In one embodiment, photoactive ingredients that are in a reduced state are oxidized through a natural process of the target medium or cells thereof. For example, in cellular aqueous environments such as those discussed herein, the cell creates localized conditions to maintain a redox equilibrium within the cell. Such conditions can result in the oxidation of the reduced photoactive ingredients. In particular, oxidative conditions (created by intracellular atmospheric oxygen or other oxidizing agents) and/or metabolic pathways involving specific oxidative reactions act on the reduced photoactive ingredient to convert it back to its oxidized form. Where the photoactive ingredients are dyes (e.g., phenothiazine dyes or derivatives thereof such as tricyclic phenothiazines or methylene blue), the oxidation of those ingredients typically results in the ingredients becoming colorful or returning color from their colorless state. Preferably, the bulk of the photosensitizer composition that remains in the extracellular environment typically stays colorless, which can prevent staining, allow greater light penetration and can avoid undesirable photodynamic killing by photoactive ingredients that do not reach the target medium.

[0034] Once the photoactive ingredients become photoactive, light can be directed at the ingredients in order to achieve inhibition of the pathogen or cell-counts, against or within which the ingredients are located. In a preferred embodiment, the light energy activates the photoactive ingredients of a photosensitizer composition to either pass energy on directly to the cell or interact with molecular oxygen to produce reactive oxygen species for inhibiting the pathogen or cell (e.g., the bacterial cell). As used herein, light can include both visible and non-visible light. Moreover, such light can be provided by a variety of sources such as a light emitting diode (LED) a laser, incandescent source, OLED (organic light emitting diode), SLED (superluminescent light emitting diode), white light or filtered light or the like. One preferred source is a laser such as a non-thermal diode laser or HeNe laser emitting a wavelength matching one or more absorbance peaks of the partially or wholly oxidized state of the photosensitizer ingredient[s] being used. Such a laser typically has a total power output of 10 – 2000 milliwatts and preferably be in the range of 50-500 milliwatts.

[0035] It will be recognized that the process and photosensitizer composition of the present invention can provide for multiple advantages, although none of these advantages are required unless otherwise stated. As one advantage, selectivity of the photosensitized kill reaction between prokaryotes and eukaryotes can be increased. As another advantage, the lipophilicity ratio (log *P*) of the photoactive ingredient (e.g., the dye) can be adjusted such that enhanced uptake at prokaryotic biomembranes occurs, followed by internal conversion to a form more readily retained within the cell or organism. Yet another advantage of the invention is to permit selection of relatively arbitrary dye concentration, in order to maximize the number of peroxidative events at or within the prokaryotic biomembrane. Yet another advantage is to utilize a form of photosensitizer composition or photoactive ingredient that is well tolerated by humans. Other advantages inherent within the technique will become apparent to one skilled in the art of photodynamic inhibition of microbes or pathogens.

[0036] The following examples provided in accordance to the present invention are for illustrative purpose only and are not intended as being exhaustive or limiting of the invention.

Example I

[0037] A 0.01% w/v methylene blue solution in an oxidized, cationic, or non-reduced state (hereinafter referred to as "non-reduced") was prepared by adding 1 ml of methylene blue solution USP (1%, ScholAR Chem) to 99 ml of sterile ultrapure water with mixing. Reduced methylene blue solutions for absorbance measurements were prepared using 0.05% w/v 0.1% w/v, 0.2% w/v, 0.5% w/v, and 1.0% w/v ascorbic acid. The reduced methylene blue solutions were transferred to sealed plastic tubes and the reduction/oxidation reaction was allowed to proceed overnight in the dark until completion. Absorbance measurements of these reduced methylene blue solutions were taken using thin film spectroscopy (1.0 mm path length) as shown in FIG. 1. FIG. 1 shows the absorbance (optical density) on its vertical axis and the range of wavelengths on its horizontal axis. FIG. 1 also shows the absorbance measurements for the non-reduced methylene blue solution (Line

A in FIG. 1) and the reduced methylene blue solutions using 0.05% w/v (Line B in FIG. 1), 0.1% w/v (Line C in FIG. 1), 0.2% w/v (Line D in FIG. 1), 0.5% w/v (Line E in FIG. 1), and 1.0% w/v (Line F in FIG. 1) ascorbic acid. As shown in FIG. 1, the extent of reduction of methylene blue in each of the solutions was proportional to the amount of ascorbic acid added, and the data confirmed that the ending concentration of ascorbic acid in the solution was directly correlated to the reduction in optical density of methylene blue.

Example II

[0038] A 0.01% (v/v) aqueous solution of methylene blue was reduced substantially entirely to the reduced state using ascorbic acid. It was found that 0.2% (w/v) ascorbic acid was sufficient to render the solution virtually colorless in the visible range, and this was also confirmed by measuring the absorbance profile spectrophotometrically. This virtually colorless form of methylene blue solution was stable for several days when stored in a sealed container in the dark. The formulation was then tested for antibacterial efficacy against *E. Coli* (ATCC® 25922™) using two established photodynamic disinfection kill models (planktonic culture and biofilm) with a 670 nm laser energy source to provide desired illumination for 60 seconds. Referring to FIG. 2, results showed that there were substantial reductions in bacterial counts using the reduced formulation. FIG. 2 is a bar graph showing the eradication of *E. coli* expressed in $\log^{10}$ reduction in bacterial counts for the various samples used in the experiment. The "A" bar in FIG. 2 denotes the viable count of a bacterial suspension sample when treated with a phosphate buffered saline ("PBS") solution without illumination; the "B" bar represents the viable count of the bacterial suspension when treated with a solution of PBS and 0.2% w/v absorbic acid with illumination for 60 seconds; the "C" bar represents the viable count of the bacterial suspension when treated with a solution of 0.01% v/v methylene blue with illumination for 60 seconds; the "D" bar represents the viable count of the bacterial suspension when treated with a solution of 0.01% v/v methylene blue and 0.2% w/v absorbic acid without illumination; and the "E" bar represents the viable count of the bacterial suspension when treatment with the same solution as the "D"

bar (i.e., the solution of 0.01% v/v methylene blue and 0.2% w/v absorbic acid) with illumination for 60 seconds. As shown in FIG. 2, the A, B and D bars showed limited, if any, antimicrobial efficacy as there was no detectable change in the viable count of the bacterial suspension before and after the application of these controls. The experimental controls showed that 0.2% w/v ascorbic acid did not itself demonstrate any antibacterial effects (either in PBS as shown in the B bar or in methylene blue as shown in the D bar). Furthermore, the spectrophotometric data for the reduced solution of methylene blue showed that there was little or no capacity for this solution to capture photons from a 670nm laser energy source. Thus, it would be expected that there would be little or no photodynamic inhibition seen when illuminating bacterial cells in the presence of substantially reduced methylene blue, since the photons would transmit through the solution rather than be absorbed and would therefore be unable to initiate photodynamic reactions such as the formation of excited energy states of the dye. However, in contrast to expected theoretical results, our results show that there is significant inhibition of *E. coli* when illuminated with a 670nm light source in the presence of reduced, colorless, photosensitizer as shown in the E bar in FIG. 2. Without being bound by theory, it is believed that a local interaction in the bacterial microenvironment causes the re-oxidation of reduced methylene blue back to the colored, cationic, light-absorbing blue form, thus allowing light energy to be captured and Type I and II photoreactions to be produced which are in turn inhibitory to the cell.

Example III

[0039] In another in vitro experiment, inocula of each of the following microbes: methicillin-resistant *Staphylococcus aureus* or "MRSA" (ATCC® 33592™), methicillin-sensitive *Staphylococcus aureus* (ATCC® 25923™), *Staphylococcus epidermidis* (ATCC® 49461™), *Streptococcus mutans* (ATCC® 35668™), *E. Coli* (ATCC® 25922™), *Pseudomonas aeruginosa* (ATCC® 9027™), and *Serratia marcescens* (ATCC® 43862™), were prepared using PBS as the diluent and adjusted to ~10^7 CFU/ml via spectrophotometric measurement for 96-well planktonic assay. Also for the

96-well planktonic assay, mixed cultures of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were also prepared by having each inoculum individually adjusted to ~10^7 CFU/ml and then combining 10 ml from each inoculum to produce a mixed 20 ml culture.

[0040] Two reduced 0.01% w/v methylene blue solutions were prepared. The first reduced methylene blue solution was reduced substantially (e.g., to a virtually colorless state) and was prepared by adding 40 mg ascorbic acid to 20 ml of 0.01% w/v oxidized methylene blue solution while stirring, for a final concentration of 0.2% w/v ascorbic acid ("Substantially Reduced Solution"). The second reduced methylene blue solution was partially reduced (e.g., to a slightly colored state) and was prepared by adding 10 mg ascorbic acid to 20 ml of 0.01% w/v methylene blue while stirring, for a final concentration of 0.05% w/v ascorbic acid ("Partially Reduced Solution"). The two solutions were transferred to sealed plastic tubes and the reduction/oxidation reaction was allowed to proceed overnight in the dark until completion.

[0041] Thereafter, the 96-well planktonic assay was prepared by combining a 100 µl aliquot of each of the inocula described above with 100 µl of test solution for a 1:1 ratio in test/control samples. The four test solutions used were the Substantially Reduced Solution, the Partially Reduced Solution, a non-reduced 0.01% w/v methylene blue solution ("Non-Reduced Solution"), and a 0.0005% w/v non-reduced (cationic) methylene blue solution ("Diluted Non-Reduced Solution"). The Diluted Non-Reduced Solution was formulated to be approximately optically equivalent to the Partially Reduced Solution. The Diluted Non-Reduced Solution was used in order to determine whether the antimicrobial activity of the Partially Reduced Solution was due to excitation of the non-reduced methylene blue fraction. Once the samples were prepared, the 96-well planktonic assay was then illuminated using a non-thermal 670 nm diode laser at 344 mW/cm^2 for 60 seconds (total energy dose = 20.6 Joules/cm^2).

[0042] FIG. 3 is a table showing the data obtained from this experiment. The numbers provided in each column of FIG. 3 are the $\log_{10}$ reductions in bacterial counts. Referring to FIG. 3, data showed that the illuminated Partially Reduced Solution (shown as column "B" in FIG. 3) and the

Substantially Reduced Solution (shown as column "C" in FIG. 3) each exhibited a higher reduction of *E. coli* compared to the Non-Reduced Solution (shown as column "A" in FIG. 3) and the 0.0005% w/v methylene blue solution (shown as column "D" in FIG. 3). In contrast and as shown in FIG. 3, these two non-reduced methylene blue solutions (i.e., Non-Reduced Solution and Diluted Non-Reduced Solution) exhibited a higher reduction of MRSA compared to the reduced methylene blue solutions. It should be noted that the two non-reduced methylene blue solutions (i.e., Non-Reduced Solution and Diluted Non-Reduced Solution) showed similar antimicrobial activity (e.g., microbial reduction levels) against both MRSA and *E. coli*. These results suggested that the increased antimicrobial efficacy of the Partially Reduced Solution was not solely due to the fraction of non-reduced methylene blue molecule present.

[0043] Referring to FIG. 3, data also showed that the Substantially Reduced Solution provided selective antimicrobial efficacy against Gram-negative organisms (e.g., *E. coli*, *P. aeruginosa* and *S. marcescens*) when compared to Gram-positive organisms (e.g., MRSA, methicillin-sensitive *Staphylococcus aureus*, *S. epidermidis*, and *S. mutans*). In fact, the Substantially Reduced Solution provided virtually no antimicrobial efficacy against any of the Gram-positive samples. In contrast, the Non-Reduced Solution provided similar level of antimicrobial efficacy against both Gram-negative organisms and Gram-positive organisms. Furthermore, the Substantially Reduced Solution provided higher antimicrobial efficacy against Gram-negative organisms when compared to the Non-Reduced Solution. For example, as to *E. coli* and *P. aeruginosa*, the Substantially Reduced Solution samples demonstrated microbial reductions from control that were more than 2 $\log_{10}$ (e.g., a factor of 100 times) greater than the Non-Reduced Solution samples.

[0044] Finally, data from the samples containing a mixture of *P. aeruginosa* and *S. epidermidis* provided further evidence of the selectivity of the Substantially Reduced Solution in eradicating Gram-negative bacteria expressed in $\log_{10}$ reduction in bacterial counts. The data showed that the *P. aeruginosa* culture was completely eradicated (e.g., >6.8 $\log_{10}$ reduction) by

the Substantially Reduced Solution, yet the *S. epidermidis* population was left unaffected. In contrast, the Non-Reduced Solution provided similar level of antimicrobial efficacy against both *P. aeruginosa* (4.8 $\log_{10}$ reduction) and *S. epidermidis* (3.8 $\log_{10}$ reduction). The Non-Reduced Solution had a lower antimicrobial efficacy against *P. aeruginosa* (4.8 $\log_{10}$ reduction) when compared to the efficacy level provided by the Substantially Reduced Solution against *P. aeruginosa* (>6.8 $\log_{10}$ reduction).

Example IV

[0045] An in vitro experiment was undertaken to assess the antibacterial efficacy of the reduced methylene blue described in Example I in a bacterial zone of inhibition photodynamic therapy model. Aqueous 0.01% w/v methylene blue was reduced substantially (substantially colorless) when combined with 0.2% w/v ascorbic acid as described in Example I. As previous experiments had demonstrated the selectivity of the reduced methylene blue against Gram-negative organisms as opposed to Gram-positive, the Gram-negative pathogen *Pseudomonas aeruginosa* was used as the test organism for this experiment. *P. aeruginosa* is a rod-shaped bacterium that plays a role in infectious conditions including otitis externa, infectious keratitis, pneumonia, and cystic fibrosis-related respiratory disease. It is also a significant factor in opportunistic infections of compromised sites such as wounds, burns, and surgical incisions.

[0046] During the in vitro experiment, a liquid inoculum of *P. aeruginosa* (~10^7 CFU/ml, ATCC#9027) was streaked over the entire surface of tryptic soy agar plates. These plates were allowed to dry. Thereafter, a non-reduced 0.01% w/v methylene blue solution was applied in a 20 µl aliquot directly onto the agar surface in a defined treatment site on the first quadrant of each of the plates; and the reduced 0.01% w/v methylene blue solution described in Example I was applied in a 20 µl aliquot directly onto the agar surface in a defined treatment site on the second quadrant of each of the plates. Defined treatment sites within third and fourth quadrants of each of the plates were used for dark controls (i.e., no illumination) to ensure that any reductions in microbial and/or bacterial viability were not due to the presence

of methylene blue on the media surface during culture. Illumination was applied to each of the defined treatment sites of the first and second quadrants of the plates using a non-thermal 670 nm diode laser at a power density of 308 mW/cm^2 for 60 seconds (total energy dose = 18.5 Joules/cm^2). Thereafter, all of the plates were placed back in culture at 37°C for 24 hours to allow viable bacteria to grow to visible confluence.

[0047] It was expected that in the event of bactericidal activity within each defined treatment site, a zone of no observable bacterial growth would be present at such defined treatment site after culture. Bacteria inoculated outside the defined treatment site should grow as normal and form a confluent lawn around the defined treatment site. If there was no observable difference between the defined treatment site and the non-treated area, it could then be concluded that the treatment administered did not lead to a reduction in bacterial viability. Zones of growth inhibition after treatment were scored visually and measured (diameter) using a calibrated electronic caliper.

[0048] The results of this experiment showed that the defined treatment sites treated with the reduced 0.01% w/v methylene blue solution and illumination, led to complete eradication of *Pseudomonas aeruginosa* within each of the treatment sites (9.7 mm treatment site with zero growth within the treatment site). In contrast, the treatment sites treated with the non-reduced 0.01% w/v methylene blue solution and illumination only caused a partial reduction in bacterial viability with many observable colonies of *P. aeruginosa* colonies growing within each of the treatment sites. No reduction in bacterial viability was observed in the dark controls with either the reduced or non-reduced methylene blue solution. These results further demonstrate the antimicrobial efficacy of reduced methylene blue against Gram-negative pathogens upon activation with visible light. Furthermore, the data suggests that reduced methylene blue provides greater antimicrobial efficacy than the non-reduced, cationic form of methylene blue.

[0049] Unless stated otherwise, particular chemistry or biology terms used herein are not intended to be restrictive of the invention. Plural components of the composition and/or steps of the process can be provided by a single components or step. Alternatively, a single component or step may be

divided into separate plural components or steps. In addition, while a feature of the present invention may have been described in the context of only one of the illustrated embodiments, such feature may be combined with one or more other features of other embodiments, for any given application.

CLAIMS

WHAT IS CLAIMED IS:

1. A photosensitizer composition for use in photodynamic disinfection comprising at least one photoactive ingredient in a chemically reduced state.

2. The composition according to claim 1 wherein the at least one photoactive ingredient is a phenothiazine.

3. The composition according to claim 2 wherein the phenothiazine is methylene blue.

4. The composition according to claim 2 or claim 3 wherein the phenothiazine is toluidine blue O.

5. The composition according to any one of claim 1 to 4 wherein the at least one photoactive ingredient in a reduced state is achieved by using a reducing agent to reduce the at least one photoactive ingredient.

6. The composition according to claim 5 wherein the reducing agent is selected from the group consisting of ferrous ions, lithium aluminum hydride, hydrogen, sodium dithionite, zinc amalgam, potassium ferrocyanide, sodium borohydride, stannous ion, sulfites, hydrazine, diisobutylaluminum hydride, oxalic acid, ascorbic acid, ascorbate, an active metal, reduced glutathione, dextrose, urea, urea derivative, anethol, glyoxol, ethylenediamine tetraacetic acid (EDTA), and a combination thereof.

7. The composition according to claim 5 wherein the reducing agent includes ascorbic acid.

8. The composition according to claim 5 wherein reduction of the at least one photoactive ingredient by the reducing agent causes the at least one photoactive ingredient to lose a degree of color resulting in the photosensitizer composition losing at least about 10% of the photosensitizer's composition's optical density

9. The composition according to claim 8 wherein the photosensitizer composition's loss of optional density is at least about 90%.

10. The composition according to claim 5 wherein the at least one photoactive ingredient includes about 0.01% w/v methylene blue and the reducing agent includes about 0.2% w/v ascorbic acid.

11. The composition according to any one of claim 1 to 10 wherein the at least one photoactive ingredient gains a degree of color upon reaching a photoactive state.

12. The composition according to any one of claim 1 to 11 wherein the at least one photoactive ingredient becomes photoactive adjacent to the target medium through oxidation of the at least one photoactive ingredient.

13. Use of a photosensitizer composition in the manufacture of a medicament for photodynamic therapy:
providing a photosensitizer composition having at least one photoactive ingredient in a reduced state;
applying the photosensitizer composition to a substrate such that the at least one photoactive ingredient associates with a target medium;
allowing the at least one photoactive ingredient to reach a photoactive state while at the location adjacent the target medium;
exposing the at least one photoactive ingredient to light at a wavelength absorbed by the at least one photoactive ingredient so that the at least one photoactive ingredient at least assists in reducing at least one pathogen associated with the target medium.

14. The use according to claim 13 wherein the at least one pathogen includes Gram-negative bacteria.

15. The use according to claim 13 or claim 14 wherein the at least one pathogen includes tumor cells.

16. The use according to claim 14 wherein the Gram-negative bacteria are selected from a group consisting of *E. coli, Pseudomonas aeruginosa, Serratia marcescens* and a combination thereof.

17. The use according to claim 14 wherein the Gram-negative bacteria are selected from a group consisting of *Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Fusobacterium nucleatum, Aggregetobacter actinomycetemcomitans*, and a combination thereof.

18. The use according to any one of claim 13 to 17 wherein the at least one photoactive ingredient in a reduced state is transported through one or more barrier mediums to the location adjacent the target medium wherein the one or more barrier mediums optionally include an aqueous environment.

19. The use according to any one of claim 13 to 18 wherein the at least one photoactive ingredient is a phenothiazine.

20. The use according to any one of claim 13 to 19 wherein the at least one photoactive ingredient in a reduced state is achieved by using a reducing agent to reduce the at least one photoactive ingredient.

21. The use according to claim 20 wherein reduction of the at least one photoactive ingredient by the reducing agent causes the at least one photoactive ingredient to lose a degree of color resulting in the photosensitizer composition losing at least about 10% of the photosensitizer's composition's optical density

22. The use according to claim 21 wherein the photosensitizer composition's loss of optional density is at least about 90%.

23. The use according to any one of claim 20 to 22 wherein the reducing agent includes ascorbic acid.

24. The use according to any one of claim 13 to 23 wherein the at least one photoactive ingredient is selected from a group consisting of methylene blue, toluidine blue O, a phenothiazine derivative, and a combination thereof.

25. The use according to any one of claim 13 to 24 wherein the at least one photoactive ingredient gains a degree of color upon reaching a photoactive state.

26. The use according to any one of claim 13 to 25 wherein the at least one photoactive ingredient becomes photoactive adjacent the target medium through oxidation of the at least one photoactive ingredient.

27. The use according to claim 26 wherein the oxidation of the at least one photoactive ingredient is assisted by application of an oxidizing agent.

28. The use according to claim 27 wherein the oxidizing agent is selected from the group consisting of molecular oxygen, a hypochlorite solution, an oxidizing gas, an oxidizing ion, an acid, and a combination thereof.

29. The use according to any one of claim 13 to 28 wherein the light is provided by a light source selected from a group consisting of a laser, LED, incandescent source, OLED (organic light emitting diode), SLED (superluminescent light emitting diode), white light, filtered light, and a combination thereof.

30. The use according to any one of claim 13 to 29 wherein the light activates the at least one photoactive ingredient to either pass energy on directly to the at least one pathogen via Type-I photodynamic reactions or interact with molecular oxygen to produce reactive oxygen

species via Type II photodynamic reactions, in order to reduce the at least one pathogen cell counts.

31. The use according to any one of claim 13 to 30 wherein localized conditions created within each cell of the at least one pathogen in an effort to maintain a redox equilibrium results in the oxidation of the at least one photoactive ingredient to a photoactive state.

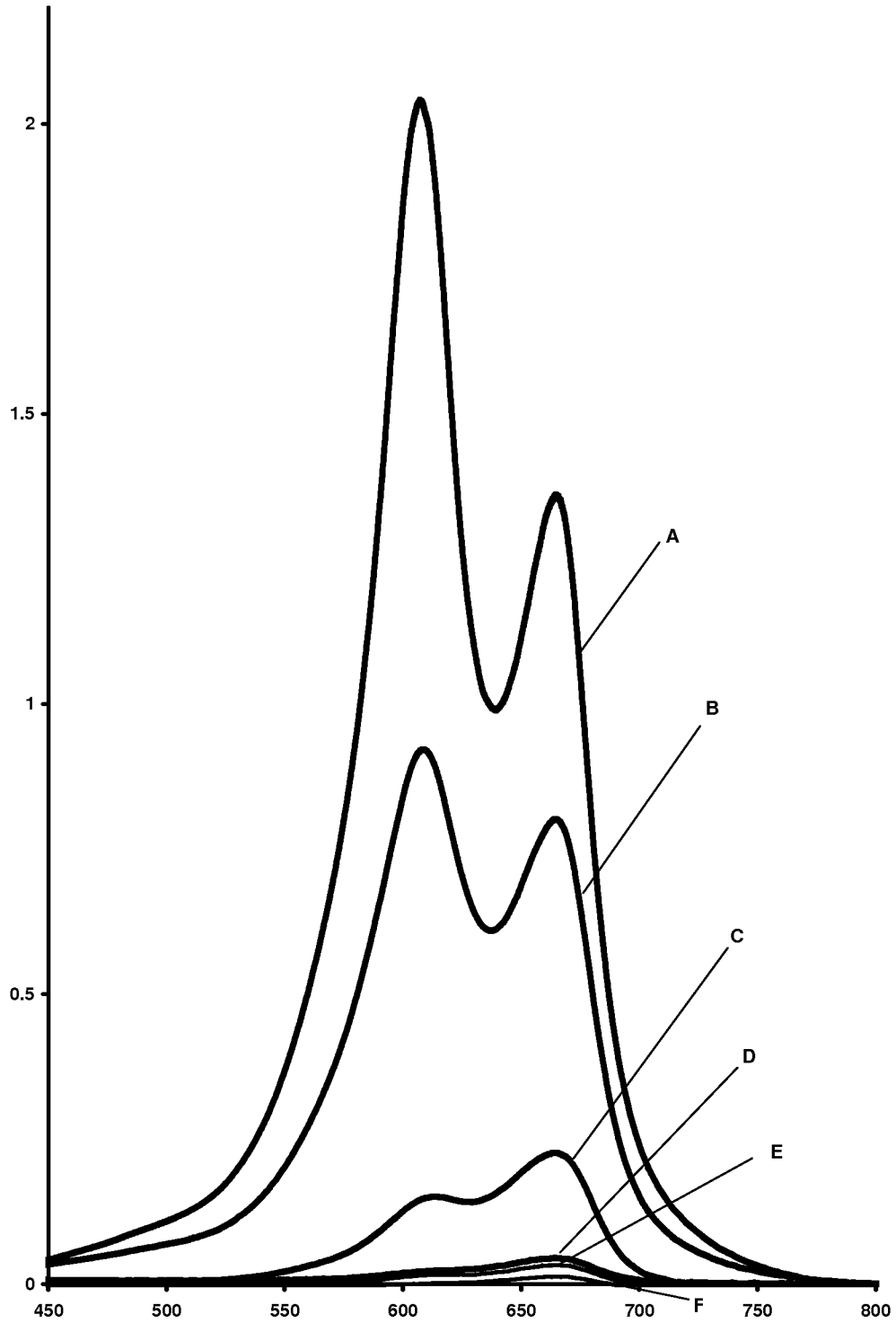


FIG. 1

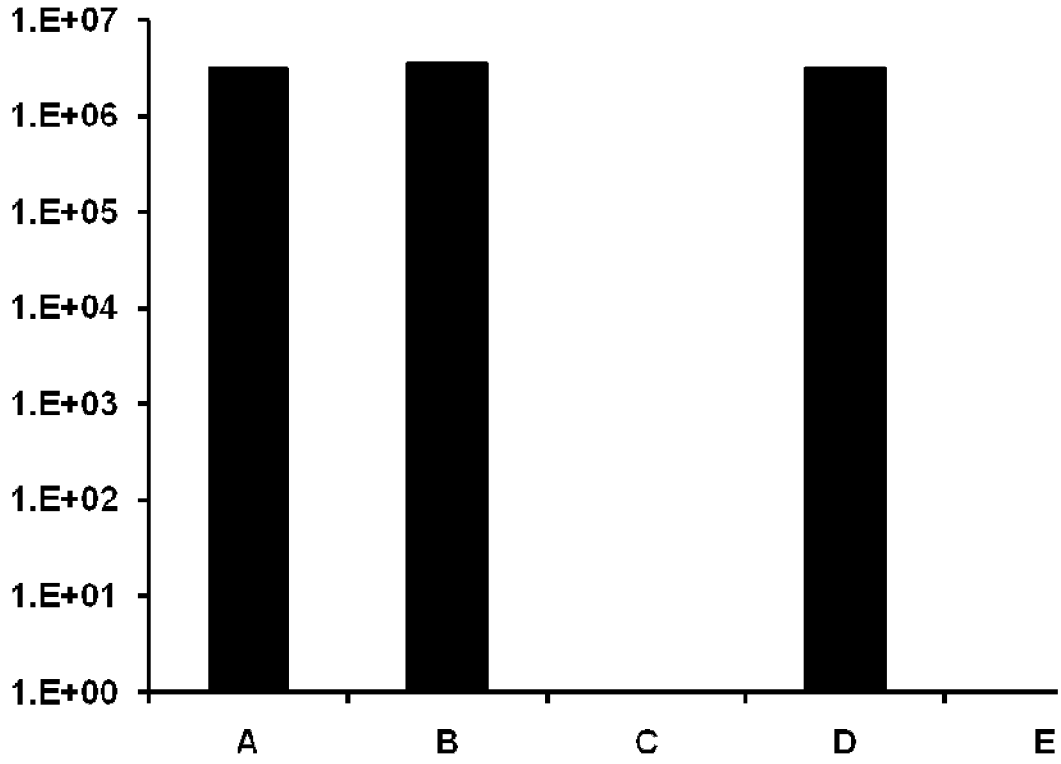


FIG. 2

	A	B	C	D
Methicillin Resistant *Staphylococcus aureus*	4.3	3.4	0.1	5.5
Methicillin Sensitive *Staphylococcus aureus*	4.1	3.2	0.1	
Staphylococcus epidermidis	3.7	2.4	0.1	
Streptococcus mutans	4.6	3.9	0.1	
Escherichia coli	3.8	5.7	6.1	4.1
Pseudomonas aeruginosa	3.6	2.9	6.6	
Serratia marcescens	4.9	2.2	5.2	

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/079050

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/5415 A61K41/00 A61P31/04 A61K35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data, EMBASE, BIOSIS, BEILSTEIN Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/135344 A (UNIV SINGAPORE [SG]; KISHEN ANIL [SG]; GEORGE SAJI [SG]; NEO CHIEW LIA) 21 December 2006 (2006-12-21) abstract; example 1 page 5 - page 10 page 46; claims 1-66; example 3	1-5,8,9, 11-14, 16-22, 24-31
X	WO 02/096896 A (PHOTOPHARMICA LTD [GB]; BROWN STANLEY BEAMES [GB]; O'GRADY CASSANDRA C) 5 December 2002 (2002-12-05) the whole document	1,2,5,8, 9,11-22, 24-31
X	WO 2006/034219 A (GEN HOSPITAL CORP [US]; HAMBLIN MICHAEL R [US]; TEGOS GEORGE P [US]) 30 March 2006 (2006-03-30) the whole document	1-5,8,9, 11-14, 16-22, 24-31

-/--

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 2008	03/02/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Jakobs, Andreas

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/079050

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/28607 A (BAXTER INT [US]) 2 July 1998 (1998-07-02) the whole document	1-3,5,6, 8,9, 11-13, 18-22, 24-31
X	WAINWRIGHT M ET AL: "A STUDY OF PHOTOBACTERICIDAL ACTIVITY IN THE PHENOTHIAZINIUM SERIES" FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY, ELSEVIER SCIENCE B.V., AMSTERDAM, NL, vol. 19, no. 1, 1 September 1997 (1997-09-01), pages 75-80, XP001042388 ISSN: 0928-8244 the whole document	1-5,8,9, 11-14, 16-22, 24-26, 29-31
X	WAINWRIGHT M ET AL: "PHOTOBACTERICIDAL ACTIVITY OF PHENOTHIAZINIUM DYES AGAINST METHICILLIN-RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS" FEMS MICROBIOLOGY LETTERS, AMSTERDAM, NL, vol. 160, no. 2, 15 March 1998 (1998-03-15), pages 177-181, XP001042387 ISSN: 0378-1097 the whole document	1-5, 8-14, 18-22, 24-31
X	MEINDL W ET AL: "[Antimycobacterial properties of phenothiazine type compounds]" ARCHIV DER PHARMAZIE MAR 1989, vol. 322, no. 3, March 1989 (1989-03), pages 133-136, XP002508934 ISSN: 0365-6233 the whole document	1,2,5,6, 8,9,11, 12
X	WO 95/32732 A (SUOMEN PUNAINEN RISTI VERIPALV [FI]; PARKKINEN JAAKKO [FI]) 7 December 1995 (1995-12-07) the whole document	1-13, 18-31
X	WO 2006/127482 A (BIOENVISION INC [US]; WOOD CHRISTOPHER [GB]; HABIB NAGY [EG]) 30 November 2006 (2006-11-30) abstract; claims 1-29	1-12, 18-31
X	JP 49 020323 A (GREEN CROSS CORP) 22 February 1974 (1974-02-22) abstract	1-12

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/079050

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; September 1994 (1994-09), EFFROS R M ET AL: "Reduction and uptake of methylene blue from rat air spaces." XP002508945 Database accession no. NLM7836153 abstract & JOURNAL OF APPLIED PHYSIOLOGY (BETHESDA, MD. : 1985) SEP 1994, vol. 77, no. 3, September 1994 (1994-09), pages 1460-1465, ISSN: 8750-7587	1-6,8,9, 11,12
T	MERKER M P ET AL: "Pulmonary endothelial thiazine uptake: separation of cell surface reduction from intracellular reoxidation." THE AMERICAN JOURNAL OF PHYSIOLOGY APR 1997, vol. 272, no. 4 Pt 1, April 1997 (1997-04), pages L673-L680, XP008099903 ISSN: 0002-9513 abstract	1-31
X	DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; July 1995 (1995-07), BONGARD R D ET AL: "Reduction of thiazine dyes by bovine pulmonary arterial endothelial cells in culture." XP002508946 Database accession no. NLM7631818 abstract & THE AMERICAN JOURNAL OF PHYSIOLOGY JUL 1995, vol. 269, no. 1 Pt 1, July 1995 (1995-07), pages L78-L84, ISSN: 0002-9513	1-31
T	MAY JAMES M ET AL: "Reduction and uptake of methylene blue by human erythrocytes." AMERICAN JOURNAL OF PHYSIOLOGY. CELL PHYSIOLOGY JUN 2004, vol. 286, no. 6, June 2004 (2004-06), pages C1390-C1398, XP002508938 ISSN: 0363-6143 abstract	1-31

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2008/079050

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006135344 A	21-12-2006	EP 1906947 A1	09-04-2008
WO 02096896 A	05-12-2002	AT 355282 T	15-03-2006
		BR 0209783 A	20-04-2004
		CA 2448303 A1	05-12-2002
		CN 1551872 A	01-12-2004
		DE 60218454 T2	27-12-2007
		DK 1392666 T3	07-05-2007
		EP 1392666 A1	03-03-2004
		EP 1790642 A1	30-05-2007
		ES 2280533 T3	16-09-2007
		HK 1063788 A1	29-06-2007
		JP 2005500271 T	06-01-2005
		MX PA03010916 A	28-10-2004
		NO 324321 B1	24-09-2007
		NZ 529682 A	31-03-2006
		US 2008261960 A1	23-10-2008
		US 2008275033 A1	06-11-2008
		US 2008268033 A1	30-10-2008
		US 2004147508 A1	29-07-2004
		ZA 200309215 A	11-03-2005
WO 2006034219 A	30-03-2006	US 2008166304 A1	10-07-2008
WO 9828607 A	02-07-1998	CA 2244952 A1	02-07-1998
		CN 1212055 A	24-03-1999
		EP 0900371 A1	10-03-1999
		JP 2000508428 T	04-07-2000
		US 5976884 A	02-11-1999
		US 5866074 A	02-02-1999
WO 9532732 A	07-12-1995	DE 69522685 D1	18-10-2001
		DE 69522685 T2	20-06-2002
		EP 0762893 A1	19-03-1997
WO 2006127482 A	30-11-2006	NONE	
JP 49020323 A	22-02-1974	JP 916231 C	21-07-1978
		JP 52047018 B	29-11-1977

República Federativa do Brasil
Ministério do Desenvolvimento, Indústria e do Comércio Exterior
Instituto Nacional da Propriedade Industrial

(11) (21) **PI 0603621-0 A**

(22) Data de Depósito: 08/08/2006
(43) Data de Publicação: **01/04/2008**
(RPI 1943)

(51) Int. Cl.:
A61K 41/00 (2008.01)
A61P 31/04 (2008.01)
G03C 1/00 (2008.01)

(54) Título: **AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO**

(71) Depositante(s): Aptivalux Bioengenharia Ltda (BR/MG)

(72) Inventor(es): Marcus Vinícius Lucas Ferreira, Luiz Fernando Lucas Ferreira

(74) Procurador: Lacerda & Taranto Advogados Associados

(57) Resumo: AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO A presente invenção tem por objetivo apresentar o Processo de preparação de AGENTE FOTOSENSIBILIZADOR para sua utilização no processo antimicrobiano ativado por luz e destinado a redução de bactérias e fungos. Podem ser utilizados para esta terapia os corantes azul de metileno, azul de toluidina, azul de trypan, azul de Evans e verde de malaquita, associados à uma fonte de luz compostas por LEDs (light emmiting diode) no processo de redução bacteriana com aplicação em odontologia e medicina.

"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO"

A presente invenção tem por objetivo apresentar o Processo de preparação de AGENTE FOTOSENSIBILIZADOR para sua utilização no processo antimicrobiano ativado por luz e destinado a redução de bactérias e fungos. Podem ser utilizados para este processo os corantes azul de metileno, azul de toluidina, azul de trypan, azul de Evans e verde de malaquita, associados à uma fonte de luz compostas por LEDs (light emmiting diode)

O uso de corantes ou agentes fotosensibilizadores na morte de células cancerígenas é conhecido na literatura científica internacional como PDT (Photodinamic Therapy), e quando esses agentes fotosensibilizadores são utilizados para morte de fungos e bactérias o processo é conhecido como PACT (Photodinamic Antimicrobial Chemotherapy) . Estes processos são extremamente seletivos e visam destruir tecidos ou células indesejadas sem afetar os tecidos normais adjacentes. Portanto, o processo de PDT é utilizado para tratamento de câncer e o PACT tem aplicação em tratamentos para redução microbiana.

O corante, ou agente fotossensibilizador, é uma droga normalmente administrada por injeção ou tópica, que sozinha, não é capaz de produzir qualquer efeito nos tecidos. No entanto, quando ativada pela luz, tem alta capacidade de destruição nas áreas irradiadas. Existem vários corantes que podem ser utilizados no tratamento para redução bacteriana, cada um com sua determinada faixa de absorção máxima para a luz.

O processo de morte celular pela luz tem sido utilizado na área da Saúde para a destruição seletiva de tumores e redução microbiana. Esse processo consiste na associação de uma fonte de luz de alta intensidade associada a um corante. A morte microbiana ocorre quando o corante absorve a energia luminosa, levando-o a produzir substâncias altamente reativas, que causam danos ao microorganismo ou à célula-alvo.

Na Odontologia, sua utilização é bastante indicada, visto que esse método mostra-se mais eficiente em infecções localizadas, de pouca profundidade e de microflora conhecida.

Em busca realizada nos bancos de patentes, foi encontrada a patente americana PI 5,611,793, cujo título é "Laser Threatment ", que se refere ao método de desinfetar e esterilizar tecidos da cavidade oral ou de feridas, utilizando um composto fotosensibilizador e laser para irradiar estes tecidos. Esta patente restringe o uso de laser para irradiação, sendo que o uso de LEDs não é mencionado.

A patente brasileira PI0211173-0 descreve uma formulação galênica injetável para a utilização em um diagnóstico ou em uma terapia fotodinâmica (PDT) e o seu processo de preparação. A formulação contém: um composto representado pela fórmula geral (I) a seguir: na qual R~ 2~ representa um grupo H, OH ou COOR~ 4~, em que R~ 4~ é um hidrogênio ou um alquila em C~ 1~-C~ 12~ ou um cicloalquila em C~ 3~-C~ 12~, R~ 3~ representa H, OH ou um alquila ou alcóxi em C~ 1~C~ 12~ e * representa um carbono assimétrico sob a forma de um sal de metal alcalino, em uma quantidade que não ultrapassa 10 mg/ml, como agente fotossensibilizador e um veículo em fase aquosa que contém pelo menos uma mistura de álcool benzílico - etanol ou propileno glicol como agente de solubilização do agente fotossensibilizador e um tensoativo em uma quantidade que não ultrapassa 20% em peso em relação ao peso total da formulação.

Os fotosensibilizadores utilizados nessa patente são totalmente diferentes dos propostos no presente pedido de patente, além da aplicação do processo descrito acima diferir do processo proposto na presente invenção.

Foi encontrada também a patente brasileira PI0305664-3 que se refere a um material para utilização em terapia fotodinâmica, compreendendo um corante com o princípio ativo camazuleno, associado a uma pasta composta de um agente oxidante, detergentes e álcoois para que não haja manchamento do tecido biológico. Esta patente também possui como agente fotossensibilizador um corante diferente dos propostos no invento aqui apresentado.

Os LEDs apresentam vantagens em relação às fontes de luz halógenas, ou de arco de plasma, ou laser, uma vez que, devido à grande eficiência desses diodos, a conversão de energia elétrica em energia eletromagnética (luz) produz significantemente menos calor.

Como conseqüência da redução significante de calor, o risco de haver algum dano térmico nos tecidos adjacentes é reduzido a zero.

O custo de manutenção dos equipamentos eletromédicos que utilizam LEDs no lugar de outras fontes de luz é menor, uma vez que a durabilidade de um LED pode chegar a 30000 horas contra pouco mais de 1000 horas das outras fontes. Além disto, por operarem sob baixa tensão,tais equipamentos geram menos calor, possibilitando que os aparelhos fiquem mais compactos, leves, portáteis e ergonômicos, uma vez que os mesmos dispensam o sistema de ventilação, o que também contribui para serem mais silenciosos, aumentando a durabilidade e reduzindo o consumo de energia.

Os LEDs também abrangem uma área luminosa maior do que os equipamentos à Laser, o que possibilita a irradiação de uma área em tratamento em um período menor de tempo quando comparado àqueles equipamentos.

Na presente invenção foi desenvolvido um processo de preparação de agente fotosensibilizador para tratamento que utiliza LEDs baseado na diluição de corantes por soluções aquosas, ou alcoólicas, ou outras substâncias metil sulfóxidas, ou ainda, diluída em tergensol, para irradiação com comprimento de onda que varia entre 510nm e 880nm emitidos por LEDs.

Os agentes fotosensibilizadores utilizados no processo para redução microbiana foram o azul de metileno, azul de toluidina, azul de trypan, azul de Evans e o verde de malaquita, pois estes corantes apresentaram bons resultados para a faixa de comprimento de onda utilizada, sem restrições quanto ao tipo de corante utilizado.

De acordo com os LEDs uti;izados, os fotosensibilizadores apresentaram a seguinte absorção: o azul de metileno possui sua faixa de absorção máxima para um comprimento de onda entre 663 e 667 nm. Para o azul de toluidina, esta faixa de máxima absorção fica entre 628 e 633nm. Para o azul de trypan, entre 590 e 600nm. Para o azul de Evans, entre 606 e 611nm. Para o verde de malaquita, entre 616 e 620nm.

A concentração dos corantes pode variar de 0,001 a 10%, diluídos em solução aquosa ou alcoólica, sem restrições. Para a utilização em tecidos

duros como esmalte e dentina, os corantes podem ser diluídos também em tergensol, sem restições.

Os corantes também podem ser misturados à uma solução de DMSO (dimetil sulfóxido) ou MSO (metil sulfóxido) na quantidade de 1% a 60% na forma líquida ou gel, sem restrições.

O PH da solução pode variar de 4 a 9, de acordo com a concentração do corante e das condições do tecido biológico ao qual será aplicado,sem restrições.

Os melhores resultados de redução bacteriana que foram alcançados em pesquisas realizadas no desenvolvimento do presente invento mostram que a concentração ideal para utilização dos corantes aqui apresentados como antimicrobianos ativados por luz emitida por LEDs é de 0,01%. Quando os corantes são associados ao DMSO ou ao MSO o ideal é que esses compostos químicos estejam em uma concentração de 25%. O pH ideal dos compostos fotosensibilizadores é 7, por ser um pH neutro e que não influi na absorção desses corantes pelas bactérias e fungos.

De acordo como o meio em que a solução será aplicada, como por exemplo pele, unhas e cascos, mucosa, esmalte e dentina, glândulas mamárias, porém não limitados a estes, a mesma poderá ser aquosa ou alcólica, ou ainda, preparada no solvente tergensol, que permite a utilização do produto sem que haja manchamento de tecidos duros como esmalte e dentina.

Para o processo aqui descrito, obteve-se resultados na faixa de 95% de redução bacteriana para as seguintes bactérias e fungos: *Actinomyces georgiae, Actinomyces gerencseriae, Actinomyces israelli, Actinomyces odontolyticus, Actinomyces naeslundii 1 e 2, sp subgengival atípico, Actinomyces israelli sorotipo I e II, Actinomyces naeslundii sorotipo I II e III, Actinomyces viscosus sorotipo II, Actinomyces meyeri, Streptococcus mutans, Streptococcus sobrinus, Streptococcus cricetus, Streptococcus rattus, Streptococcus ferus, Streptococcus macacae, Streptococcus downei, Streptococcus salivarus, Streptococcus vestibularis, Streptococcus constellatus, Streptococcus intermedius, Streptococcus anginosus, Streptococcus sanguis, Streptococcus gordonii, Streptococcus parasanguis,*

Streptococcus oralis, Streptococcus mitis, Streptococcus crista, Eubacterium, Lactobacillus casei, Lactobacillus fermetum, Lactobacillus acidophilus, Lactobacillus salivarus, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus cellobiosus, Lactobacillus buchneri, Lactobacillus oris Peptostreptococus, Stomatococus, Moraxella, Neisseria, Veilonella, Corynebacterium, Bifidobacterium, Eubacterium, Lactobacillus, Propionobacterium, Pseudoramibacter, Rothia, Actinobacillus, Cantonella, Capnocytoiphaga, Eikenella, Fusobacterium, Haemophillus, Johnsonni, Leptotrichia, Porphyromonas, Prevottlella, Selemonas, Treponema, Wolinella, Porphyromonas gingivalis, Prevotella intermédia, Fusobacterium nucleatum, Actinomyces actinomicetencomitans, Staphylococcus aureus, Corynebacterium bovis, Corynebacterium pyogenes, Pasteurella bovis, Pseudomonas sp, Staphylococcus hyicus, Staphylococcus chromogenes, Staphylococcus haemolyticus, Staphylococcus simulans, Staphylococcus intermedius, Actinobacillus spp, Fusobacterium necrophorus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Staphylococcus aureus, Staphylococcus sp coagulase negativa, *Streptococcus faecium, Klebsiella pneumoniae, Enterecoccus faecalis, Bacillus subtillis, Trichophyton rubrum, não restritos.*

O presente invento inova em relação ao estado da técnica na utilização de LEDs na utilização de fotosensibilizador. A sua aplicação apresenta solução para vários problemas existentes e ainda não solucionados pelo estado da técnica.

Para aplicação na área de odontologia, a presente invenção garante eficiencia em sua utilização. Tal processo implica no tratamento antimicrobiano em periodontite; endodontia; cárie de esmalte, dentina e raiz; e demais tratamentos odontológicos, sem limitar.

Para aplicação em medicina, a tecnologia descrita implica no tratamento antimicrobiano em infecções de pele, úlceras de decúbito, úlceras de diabéticos, úlceras venosas, micoses e demais infecções, sem restringir.

Para aplicação em veterinária, a tecnologia apresentada implica no tratamento antimicrobiano em mastite, infecções dermatológicas em pequenos

e grandes animais, otite, infecções de casco em pequenos e grandes animais e demais tratamentos veterinários, sem restringir.

A presente tecnologia, portanto, apresenta uma amplitude de aplicações na área de saúde com grande potencial de utilização para garantia da saúde publica de uma população.

.REIVINDICAÇÕES

1. **"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO" caracterizado pelo** fato de que a diluição dos corantes como azul de metileno, azul de toluidina, azul de trypan, azul de Evans e o verde de malaquita, e estas diluições formam uma solução fotosensibilizadora e estas apresentam uma taxa de absorção característica.

2. **"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO"** de acordo com a reivindicação 1, **caracterizado pelo** fato de que corantes luminecentes são diluídos em solução aquosa ou alcoólica com concentrações entre 0,001 a 10% formando uma solução fotosensibilizadora.

3. **"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO",** de acordo com a reivindicação 1, **caracterizado pelo** fato de os corantes poderem ser misturados à uma solução de DMSO (dimetil sulfóxido), MSO (metil sulfóxido) ou em tergensol, na quantidade de 1% a 60% , na forma de líquido ou de gel.

4. **"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO",** de acordo com a reivindicação 1, 2 e 3, **caracterizado pelo** fato de que o corante diluído ser transforma em um antimicrobiano quando ativado por um bactericida com utilização de LED's para irradiação com comprimento de onda entre 510nm e 880nm.

5. **"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO",** de acordo com as reivindicações 3 e 4, **caracterizado pelo** fato de que o PH da solução fotosensibilizadora pode variar de 4 a 9, de acordo com a concentração do corante e das condições do tecido biológico ao qual será aplicado.

6. **"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO" caracterizado pelo** fato de que a solução fotosensibilizadora quando aplicadas e iluminadas com leds produz 95% de redução bacteriana para as bactérias e fungos seguintes, *Actinomyces georgiae, Actinomyces gerencseriae, Actinomyces israelli, Actinomyces odontolyticus, Actinomyces naeslundii 1 e 2, sp subgengival atípico, Actinomyces israelli sorotipo I e II, Actinomyces naeslundii sorotipo I II e III, Actinomyces viscosus sorotipo II, Actinomyces meyeri, Streptococcus mutans, Streptococcus sobrinus, Streptococcus cricetus, Streptococcus rattus, Streptococcus ferus, Streptococcus macacae, Streptococcus downei, Streptococcus salivarus, Streptococcus vestibularis, Streptococcus constellatus, Streptococcus intermedius, Streptococcus anginosus, Streptococcus sanguis, Streptococcus gordonii, Streptococcus parasanguis, Streptococcus oralis, Streptococcus mitis, Streptococcus crista, Eubacterium, Lactobacillus casei, Lactobacillus fermetum, Lactobacillus acidophilus, Lactobacillus salivarus, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus cellobiosus, Lactobacillus buchneri, Lactobacillus oris Peptostreptococus, Stomatococus, Moraxella, Neisseria, Veilonella, Corynebacterium, Bifidobacterium, Eubacterium, Lactobacillus, Propionobacterium, Pseudoramibacter, Rothia, Actinobacillus, Cantonella, Capnocytoiphaga, Eikenella, Fusobacterium, Haemophillus, Johnsonni, Leptotrichia, Porphyromonas, Prevottlella, Selemonas, Treponema, Wolinella, Porphyromonas gingivalis, Prevotella intermédia, Fusobacterium nucleatum, Actinomyces actinomicetencomitans, Staphylococcus aureus, Corynebacterium bovis, Corynebacterium pyogenes, Pasteurella bovis, Pseudomonas sp, Staphylococcus hyicus, Staphylococcus chromogenes, Staphylococcus haemolyticus, Staphylococcus simulans, Staphylococcus intermedius, Actinobacillus spp, Fusobacterium necrophorus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Staphylococcus aureus, Staphylococcus sp* coagulase negativa, *Streptococcus faecium, Klebsiella pneumoniae, Enterecoccus faecalis, Bacillus subtillis, Trichophyton rubrum*

7. **"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO" caracterizado pela** aplicação do fotosensibilizador em periodontite; endodontia; cárie de esmalte, dentina e raiz; e demais tratamentos odontológicos.

8. **"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO" caracterizado pelo** fato de que agente fotosensibilizador ser aplicado em infecções de pele, úlceras de decúbito, úlceras de diabéticos, micoses e demais infecções.

9. **"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO" caracterizado pelo** fato do agente fotosensibilizador ser aplicado em mastite, infecções dermatológicas em pequenos e grandes animais, otite, infecções de casco em pequenos e grandes animais e demais usos veterinários.

RESUMO

Patente de invenção: **"AGENTE FOTOSENSIBILIZADOR: PROCESSO DE PREPARAÇÃO E SUA UTILIZAÇÃO"**

A presente invenção tem por objetivo apresentar o Processo de preparação de AGENTE FOTOSENSIBILIZADOR para sua utilização no processo antimicrobiano ativado por luz e destinado a redução de bactérias e fungos. Podem ser utilizados para esta terapia os corantes azul de metileno, azul de toluidina, azul de trypan, azul de Evans e verde de malaquita, associados à uma fonte de luz compostas por LEDs (light emmiting diode) no processo de redução bacteriana com aplicação em odontologia e medicina.

Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 146 (2007) 175–181
veterinary parasitology
www.elsevier.com/locate/vetpar

Short communication

Ultrastructural changes in *Tritrichomonas foetus* after treatments with $AlPcS_4$ and photodynamic therapy

Newton Soares da Silva *, Claudia de Mello Ribeiro, Aline Helena Araujo Machado, Cristina Pacheco-Soares

Laboratório de Biologia Celular e Tecidual, Instituto de Pesquisa e Desenvolvimento, Universidade do Vale do Paraíba, Av. Shishima Hifumi 2911, 12244-000 São José dos Campos, SP, Brasil

Received 11 September 2006; received in revised form 5 February 2007; accepted 8 February 2007

Abstract

The *Tritrichomonas foetus* is an amitochondrial parasitic protist which causes bovine trichomoniasis, a major sexually transmitted disease in cattle. No effective drugs for this disease have been approved to this date. Photodynamic therapy (PDT) is an experimental treatment that shows great potential for treating bacteria, fungi, yeasts, and viruses. However, the cytotoxic effect of PDT on protozoan has been poorly studied. In this study, PDT with aluminum phthalocyanine tetrasulfonated ($AlPcS_4$) photosensitizer was efficient in killing *T. foetus*. The mode of cell death in *T. foetus* after PDT was investigated by transmission electron microscopy. Morphological changes, such as membrane projections, nucleus fragmentation with peripheral masses of heterochromatin, endoplasmic reticulum proliferation, intense cytoplasmic vacuolization, fragmented axostyle–pelta complex, and internalized flagella could be observed. This is the first report to demonstrate cell death in *T. foetus* after PDT, and thus will open up new lines of investigation to develop new treatments for bovine trichomoniasis.

Keywords: *Tritrichomonas foetus*; Photodynamic therapy; $AlPcS_4$; Cytotoxic effect; Cell death

1. Introduction

The parasitic protozoan *Tritrichomonas foetus* causes bovine trichomoniasis, a major sexually transmitted disease in cattle. Bovine trichomoniasis amounts to considerable economic losses in the United States, Canada, and South America, as it leads to miscarriage and infertility in bovines (Cobo and Campero, 2002). Treatment is difficult because no effective drugs to be used in cattle have been approved to this date (Bondurant, 1997). Thus, it is necessary to develop and test new therapies against this disease.

Photodynamic therapy (PDT) is an experimental treatment, which shows great potential for treating neoplastic and nonneoplastic diseases. In PDT, visible light activates a photosensitizing drug accumulated in cells or tissue. The interaction between the excited photosensitizer and molecular oxygen produces singlet oxygen ($^1O_2$) as well as other reactive oxygen species (ROS) (Dougherty et al., 1998). The generation of singlet oxygen is supposed to play a major role in photodynamic cytotoxicity (Gomer et al., 1989). PDT can induce cell death by necrosis or apoptosis, both *in vitro* and *in vivo* (Fabris et al., 2001). Various cell organelles could be postulated as target for PDT, such as plasma membrane, mithocondria, endoplasmic reticulum, and cytoskeletal structure (Ferreira et al., 2004).

* Corresponding author. Tel.: +55 12 39471143.
E-mail address: nsoares@univap.br (N.S.d. Silva).

0304-4017/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2007.02.006

Oscar Raab (1900) published the first paper about the photodynamic effects of the activities of chemical compounds against microorganisms. He observed that low concentrations of acridines in the presence of light could be lethal to the protozoan *Paramecium*. Antimicrobial PDT research has increased in the last 20 years. Bacteria, as well as fungi, yeasts, and viruses, treated with photosensitizers were shown to be successfully killed by visible light (Wainwright, 1998). However, the cytotoxic effect of PDT on protozoan has been poorly studied.

Evidence supports the involvement of mitochondria during cell death of many different cell types, including unicellular organisms, caused by PDT (Wainwright, 1998; Lam et al., 2001). *T. foetus* does not contain mitochondria but has characteristic membrane-bound organelles that are termed hydrogenosomes, which contain enzymes that participate in the metabolism of pyruvate and are the site of formation of ATP and molecular hydrogen (Muller, 1993).

Cell death by PDT in amitochondrial organisms is still to be elucidated. The aim of this study was to evaluate the cytotoxic effect of PDT with aluminum phthalocyanine tetrasulfonated ($AlPcS_4$) photosensitizer, in culture of *T. foetus*.

2. Materials and methods

2.1. T. foetus

T. foetus, K strain, was kindly provided by Dr. Fernando Costa e Silva Filho, from the Institute of Biophysics Carlos Chagas Filho, Brazil (UFRJ-RJ). The parasite was kept in TYM Diamond's medium (Diamond, 1957) supplemented with 10% of fetal calf serum for 48 h at 37 °C in a humidified atmosphere containing 5% of CO_2. The number of parasites was standardized at a number of 1×10^6 cell mL^{-1}.

2.1.1. Photosensitizer

The drug chloroaluminum phthalocyanine tetrasulfonate ($AlPcS_4$) (Porphyrin Products, Inc.), was dissolved in PBS to a stock concentration of 1 mM, and stored in the dark at 4 °C until used.

2.1.2. Treatment of T. foetus with $AlPcS_4$

Parasites were distributed at a number of 1×10^6 cells mL^{-1} as follows: four vials without treatment (control and for light treatment only) and four vials with $AlPcS_4$ (10 μM) were incubated for 60 min in the dark at 37 °C. After this period, they were washed with PBS twice in order to remove the photosensitizer that had not been taken up by cells. Furthermore, 500 μL of fresh TYM Diamond's medium without serum was added for irradiation.

2.1.3. Irradiation

Two vials with parasites that had undergone treatments with $AlPcS_4$ or not were subjected to irradiation in the dark with a semiconductor laser (Thera Lase-DMC), ($\lambda = 685$ nm; $P = 26$ mW; D.E. = 0.5 J cm^{-2}; $t = 35$ s).

The parasite was kept in TYM Diamond's medium (Diamond, 1957) supplemented with 10% of fetal calf serum for 48 h at 37 °C in a humidified atmosphere containing 5% of CO_2. Parasites were counted in Neubauer hemocytometer 24 and 48 h after treatment, using a Leica DMLB photomicroscope.

2.1.4. Transmission electron microscopy

T. foetus was fixed for 1 h at room temperature in 4.0% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After fixation, parasites were washed in PBS and postfixed in 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.2 with 1% potassium ferricyanide, and 5 mM $CaCl_2$ at room temperature, in the dark. Cells were then washed in PBS, dehydrated in acetone and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and observed in a Jeol 1210 Transmission Electron Microscope or Zeiss EM10 Transmission Electron Microscope.

2.1.5. Statistical analysis

A one-way ANOVA (Microcal™Origin™ 5.0, Microcal Software Inc.) was used. Values are given as mean ± S.E. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Cellular death of T. foetus after incubation with $AlPcS_4$

In this experiment, we investigated the action of $AlPcS_4$ in culture of *T. foetus*. To determine the effect of aluminum phthalocyanine tetrasulfonated photosensitizer alone (regardless of light) on the number of parasites, *T. foetus* was analysed 24 and 48 h after incubation with $AlPcS_4$ (10 μM). The cytotoxic effect of treatment was investigated by counting the parasites in the Neubauer hemocytometer, 24 and 48 h after incubation. The action of $AlPcS_4$ on *T. foetus* resulted in a sharp fall in the number of parasites (~46%), after 24 and 48 h of their incubation with the drug (Fig. 1).

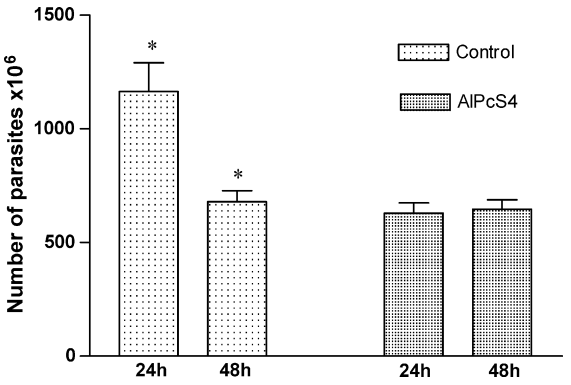


Fig. 1. Number of *T. foetus* 24 and 48 h after incubation with $AlPcS_4$. The means are significantly different: $^{*}P < 0.05$ (mean ± S.E., $n = 12$).

3.2. Cellular death of T. foetus after treatment with $AlPcS_4$ and exposed to laser irradiation (PDT)

We investigated the effect of ROS generated by PDT on *T. foetus*. The parasites were submitted to treatment with $AlPcS_4$ (10 μM) photosensitizer and subjected to irradiation. We evaluated the cytotoxic effect of PDT on *T. foetus* culture by counting the parasites in the Neubauer hemocytometer, 24 and 48 h after treatment. No significant difference was observed between control and parasites treated with light only (Fig. 2A and B). PDT resulted in sharp reduction in the number of these parasites (∼82%) 24 h after treatment (Fig. 2A). Increased number of *T. foetus* was observed 48 h after PDT in relation to what was observed 24 h after treatment (Fig. 2B).

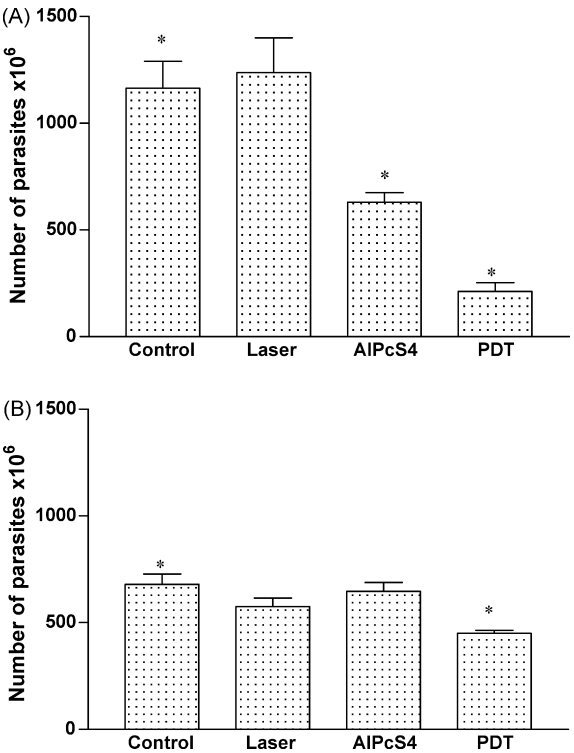


Fig. 2. Number of *T. foetus* 24 h (A) and 48 h (B) after treatment with PDT. The means are significantly different: $^{*}P < 0.05$ (mean ± S.E., $n = 12$).

3.3. $AlPcS_4$ induces morphological changes in T. foetus after incubation in the dark

Parasites were studied by transmission electron microscopy in order to analyze the cytotoxic effect of treatment with $AlPcS_4$ in *T. foetus*. An untreated parasite presents an elongated or pear shape, three anterior flagella and a recurrent flagella, one nucleus, several spherical hydrogenosomes, a prominent Golgi apparatus, and a microtubular axostyle–pelta complex (Fig. 3).

Change in the elongated shape of *T. foetus* was not observed 24 h after treatment with $AlPcS_4$. However, cellular disorder was observed in several cells, such as nucleus fragmentation with peripheral masses of heterochromatin, internalized flagella, fragmented axostyle–pelta complex, and intense vacuoles surrounded by membrane with different contents (Fig. 4).

Analysis 48 h after treatment with $AlPcS_4$ showed that the photosensitizer induced a change in the shape of parasites. *T. foetus* presented rounded shape and some vacuoles with different contents, internalized flagella, and fragmented axostyle–pelta complex were observed (Fig. 5).

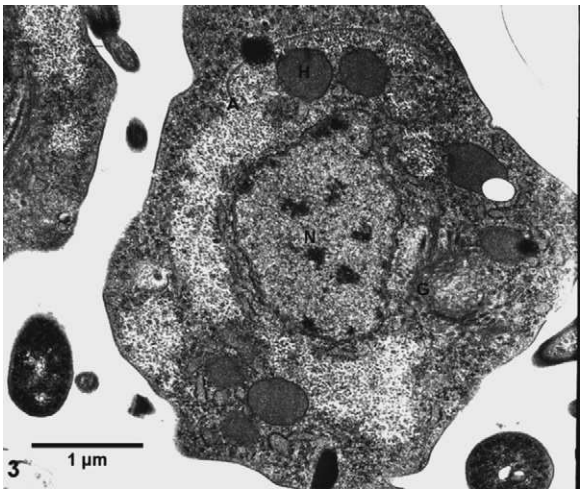


Fig. 3. Transmission electron micrographs of thin sections of untreated *T. foetus* (control): nucleus (N), hydrogenosomes (H), microtubular axostyle–pelta complex (A), and Golgi apparatus (G).

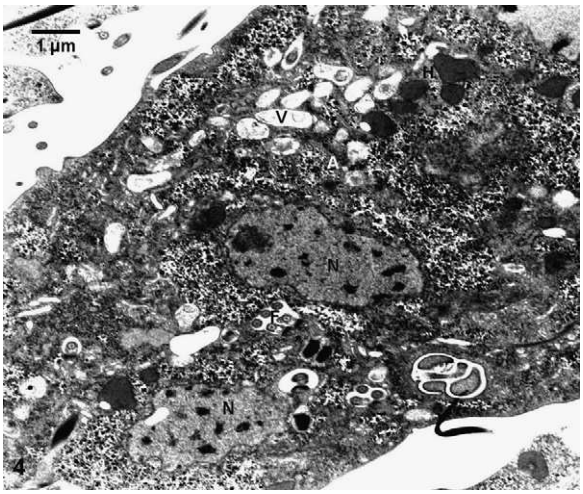


Fig. 4. Section of photosensitizer-treated *T. foetus* 24 h after incubation. Nucleus fragmentation (N), large-sized vacuoles with different contents (V), flagella inside vacuoles (F), fragmented axostyle–pelta complex (A), and hydrogenosomes (H).

3.4. PDT induces morphological changes in *T. foetus*

T. foetus presented morphological changes after treatment with PDT. Some parasites (50–70%) presented rounded shape 24 h after treatment. Cells presented cellular disorder, nucleus fragmentation with peripheral masses of heterocromatin, internalized flagella, intense cytoplasmic vacuolization with different contents, and fragmented axostyle–pelta complex (Fig. 6).

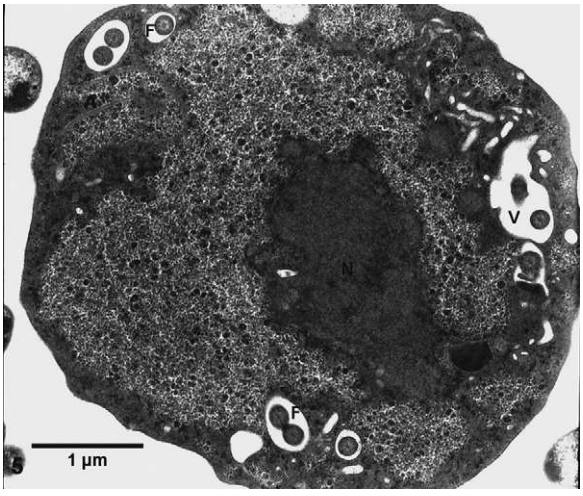


Fig. 5. *T. foetus* 48 h after incubation with photosensitizer. Parasites present rounded shape, nucleus (N), internalized flagella (F), vacuoles with different contents (V), and fragmented axostyle–pelta complex (A).

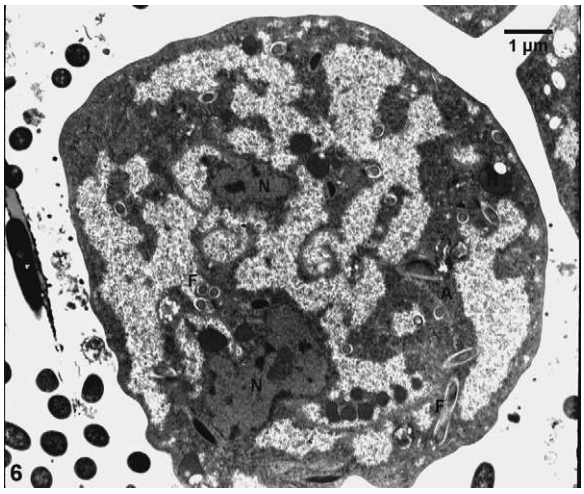


Fig. 6. *T. foetus* 24 h after PDT. The parasites display rounded shape, nucleus fragmentation (N), flagella inside vacuoles (F), and fragmented axostyle–pelta complex (A).

Forty-eight hours after PDT, morphological changes in *T. foetus* were similar to the ones in other cells studied in this experiment, which included nucleus with peripheral masses of heterocromatin, internalized flagella, membrane projections, cytoplasmic vacuoles with different contents, and hydrogenosomes with highly electron dense matrix (Fig. 7).

4. Discussion

Several drugs have been used to investigate the cytotoxic effect on *T. foetus*, such as colchicine,

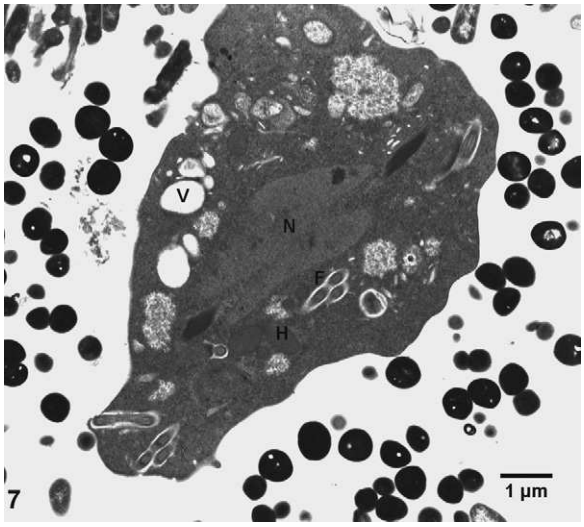


Fig. 7. *T. foetus* 48 h after PDT. Observe nucleus (N), vacuoles (V), internalized flagella (F), and hydrogenosomes (H).

vinblastine, cytochalasin B (Silva-Filho and De Souza, 1986), taxol, nocodazole, griseofulvin, lactacyst, and hydrogen peroxide (Vancini and Benchimol, 2005). In this experiment, we analyzed the cytotoxic effect produced by PDT with aluminum phthalocyanine tetrasulfonated ($AlPcS_4$) photosensitizer on *T. foetus* culture.

At first, we investigated the effects of the photosensitizer without exposing this protist to light. A decrease in the number of *T. foetus* was observed when counting the parasites in the Neubauer hemocytometer 24 h after incubation. This data suggests that $AlPcS_4$ in the dark is toxic to parasites. Similar results have been observed in protozoa *Trypanosoma cruzi* and *Plasmodium falciparum*. The silicon phthalocyanine (Pc4) (2 μM) photosensitizer, without illumination, inactivates *T. cruzi* trypomastigote in fresh frozen plasma after 30 min of incubation (Gottlieb et al., 1995). The parasitemia in culture of *P. falciparum* incubated with *d*-aminolevulinic acid (ALA), decreased after 3 days (Smith and Kain, 2004). Hence, current evidence suggests that $AlPcS_4$ without light exposure might be useful against bovine trichomoniasis for chemotherapeutic purposes.

ROS generated by antiparasitic agents or macrophages can kill intracellular parasites and are therefore important regulators of protozoal infection (Das et al., 2001). *In vitro* studies revealed decreased viability *of Trichomonas vaginalis* exposed to various concentrations of hydrogen peroxide (H_2O_2) (Davis and Lushbaugh, 1993). Inhibited motility and increased cellular death incidence were observed after treating cultures of *T. foetus* with H_2O_2, both of which depended on the H_2O_2 concentration (Mariante et al., 2003). Treatment of *Leishmania donovani* promastigotes with H_2O_2 resulted in a dose-dependent inhibition of cell motility and cell death. Nevertheless, loss of motility did not coincide with cell death, as membranes were intact even in immotile cells, suggesting that H_2O_2 was the source of stress that caused metabolic changes within the cells (Das et al., 2001).

In PDT, interaction between the excited photosensitizer and molecular oxygen caused ROS to induce cell death (Dougherty et al., 1998). Nonetheless, information concerning the effect of ROS generated by PDT in parasites protozoan is scant.

Photodynamic activation derivatives of ALA are able to inactivate *P. falciparum in vitro* (Smith and Kain, 2004). PDT with silicon phthalocyanine (Pc4) photosensitizer inactivates *T. cruzi* trypomastigote in fresh frozen plasma and red blood cell concentrates (Gottlieb et al., 1995).

In order to better analyze the cytotoxic effect of ROS generated by PDT on *T. foetus*, parasites were treated with $AlPcS_4$ and exposed to laser irradiation. When counting *T. foetus* in the Neubauer hemocytometer 24 h after treatment, a decrease in the number the parasites was observed, demonstrating that this type of therapy is efficient against *T. foetus in vitro*. *T. foetus* count 48 h after PDT demonstrated that there was an increase in the number of parasites. This fact can be explained by the life cycle of these protozoan, which lasts around 4–6 h. Although the number of parasites decreased, some protozoan demonstrated not to be affected 24 h after PDT, and thus had food and space available in the culture medium. Due to this fact, it is thought that these parasites divided themselves, increasing their number.

The mode of *T. foetus* cell death after treatments with photosensitizer and PDT was also investigated by transmission electron microscopy. Similar morphological changes in parasites after these treatments, such as cellular disorder, membrane projections, nucleus fragmentation with peripheral masses of heterochromatin, intense vacuoles with different contents, fragmented axostyle–pelta complex, and internalized flagella were observed through ultrastructural analyses.

T. foetus submitted to stressful conditions internalized their flagella to form pseudocysts which could be a defensive strategy against environmental changes (Mariante et al., 2006). The induction of pseudocysts by chemicals can lead to an irreversible process that ends up with the death of cells, depending on the exposure time and/or the concentration of the drug employed (Mariante et al., 2003).

The mode of cell death by PDT may be either apoptosis or necrosis, depending on the nature and concentration of the photosensitizer and the amount of irradiation (Moor, 2000). However, cell death after PDT is often by apoptosis, and substantial evidence supports the involvement of mitochondria in this process. It has been proposed that ROS induce the opening of the mitochondrial membrane permeability transition pores, which results in the dissipation of the mitochondrial membrane potential ($\Delta\psi_m$) and mitochondrial swelling, essential conditions for the escape of cytochrome *c* from mitochondria into the cytosol (Chiu and Oleinick, 2001; Zeiss, 2003).

Several trichomonad species, including *T. foetus*, contain no mitochondria but have another type of membrane-bound organelle, which was termed hydrogenosomes. The hydrogenosomes are bound by a double membrane, generating ATP and molecular hydrogen. However, this organelle does not contain genome, oxidative phosphorylation, tricarboxylic acid

cycle, cytochromes, cardiolipin, or F0F1 ATPase activity (Muller, 1993; Bui et al., 1996).

In this study, after treatment with photosensitizer or PDT, *T. foetus* presented some morphological aspects similar to apoptosis such as nuclear fragmentation and chromatin condensation; aspects of paraptosis were also present, a form of programmed cell death that is distinct from apoptosis by the criteria of morphology, biochemistry, and response to apoptosis inhibitors (Sperandio et al., 2000) such as cytoplasmic vacuolization and chromatin condensation.

Nevertheless, another form of cell death, autophagy, presents features of cellular components degradation that start in the autophagic vacuoles of the dying cell. The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation. The autophagic pathway begins with the sequestration of cytoplasmic material in double-membrane vesicles known as autophagosomes. Autophagosomes then fuse with lysosomes in a process that depends on the microtubules, and the contents are then degraded (Fink and Cookson, 2005).

T. foetus presented intense cytoplasmic vacuolization after treatment with photosensitizer and PDT. The vacuoles are surrounded by membranes with different contents including membrane profiles, and disorganized flagellar-axonemes. The same results were observed by Mariante et al. (2006) in *T. foetus* treatment with griseofulvin. These authors also found positive reaction for tubulin in these vacuoles, possibly corresponding to internalized flagella and/or the axostyle–pelta complex and detected autophagic vacuoles with acridineorange, suggesting the presence of an autophagic process.

Interestingly, similar morphological aspects of cell death in trichomonads have been described after treatments with drugs. Chose et al. (2002) observed morphological aspects of *T. vaginalis* treated with pro-apoptic drugs such as rounded shape, nuclear fragmentation, apoptotic-like bodies, and cytoplasmic vacuolation. After treatment with different H_2O_2 concentrations, Mariante et al. (2003) observed spherical shape *T. foetus*, nucleus with peripheral masses of heterocromatin, hydrogenosomes elongated with an electron dense matrix, myelin-like figure, large-sized vacuoles with different contents, endoplasmic reticulum proliferation, internalized flagella, and disorganized axostyle.

The data obtained in this study is not sufficient to understand cell death in *T. foetus*. Additional molecular studies are necessary to determine the overall mode of cell death in *T. foetus* after PDT.

In conclusion, we demonstrated that PDT killed an amitochondrial organism such as *T. foetus*. In this parasite, PDT induces a type of cell death other than necrosis. Apoptosis or autophagic cell death after PDT in *T. foetus* may benefit bovines by limiting the inflammatory response, which is detrimental and could even be lethal to these animals.

Acknowledgements

The authors thank Pedro Duarte Novaes, PhD, FOP-Unicamp, for letting us use the Zeiss EM10 Electron Microscope. We also thank Elisa Aparecida Gregório, PhD, CME-UNESP for letting us use the Jeol 1210 Electron Microscope.

References

Bondurant, R.H., 1997. Pathogenesis, diagnosis, and management of Trichomoniasis in cattle. Vet. Clin. N. Am. Food Anim. Pract. 13, 345–361.

Bui, E.T.N., Bradley, P.J., Johnson, P.J., 1996. A common evolutionary origin for mitochondria and hydrogenosomes. Proc. Natl. Acad. Sci. U.S.A. 93, 9651–9656.

Chiu, S.M., Oleinick, N.L., 2001. Dissociation of mitochondrial depolarization from cytochrome *c* release during apoptosis induced by photodynamic therapy. Br. J. Cancer 84, 1099–1106.

Chose, O., Noel, C., Gerbod, D., Brenner, C., Viscogliosi, E., Roseto, A., 2002. A form of cell death with some features resembling apoptosis in the amitochondrial unicellular organism *Trichomonas vaginalis*. Exp. Cell. Res. 276, 32–39.

Cobo, E.R., Campero, C.M., 2002. Nuevos aspectos inmunologicos y vacunales de la tricomoniasis Bovina. Rev. Med. Vet. 83, 203–208.

Das, M., Mukherjee, S.B., Shaha, C., 2001. Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. J. Cell. Sci. 114, 2461–2469.

Davis, S.R., Lushbaugh, W.B., 1993. Oxidative stress and *Trichomonas vaginalis* effect of hydrogen peroxide in vitro. Am. J. Trop. Med. Hyg. 48, 480–487.

Diamond, L.S., 1957. The establishment of various trichomonads of animals and man in axenic cultures. J. Parasitol. 43, 488–490.

Dougherty, T.J., Gomer, C.J., Henderson, B.W., Jori, G., Kessel, D., Korbelik, M., Moan, J., Peng, Q., 1998. Photodynamic therapy. J. Nat. Cancer Inst. 90, 889–905.

Fabris, C., Valduga, G., Miotto, G., Borsetto, L., Jori, G., Garbisa, S., Reddi, E., 2001. Photosensitization with zinc(II) phthalocyanine as a switch in the decision between apoptosis and necrosis. Cancer Res. 61, 7495–7500.

Ferreira, S.D.R.M., Tedesco, A.C., Sousa, G., Zângaro, R.A., Silva, N.S., Pacheco, M.T.T., Pacheco-Soares, C., 2004. Analysis of mitochondria, endoplasmic reticulum and actin filaments after PDT with $AlPcS_4$. Lasers Med. Sci. 18, 207–212.

Fink, S.L., Cookson, B.T., 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect. Immun. 73, 1907–1916.

Gomer, C.J., Rucker, N., Ferrario, A., Wong, S., 1989. Properties and application of photodynamic therapy. Radiat. Res. 120, 1–18.

Gottlieb, P., Shen, L., Chimezie, E., Bahng, S., Kenney, M.E., Horowitz, B., Ben-hur, E., 1995. Inactivation of *Trypanosoma cruzi* trypomastigote forms in blood components by photodynamic treatment with phthalocyanines. J. Photochem. Photobiol. 62, 869–874.

Lam, M., Oleinick, N.L., Nieminen, A.L., 2001. Photodynamic therapy-induced apoptosis in epidermoid carcinoma cells. J. Biol. Chem. 276, 47379–47386.

Mariante, R.M., Guimarães, C.A., Linden, R., Benchimol, M., 2003. Hydrogen peroxide induces caspase activation and programmed cell death in the amitochondrial *Tritrichomonas foetus*. Histochem. Cell. Biol. 120, 129–141.

Mariante, R.M., Vancini, R.G., Benchimol, M., 2006. Cell death in trichomonads: new insights. Histochem. Cell. Biol. 125, 545–556.

Moor, A.C.E., 2000. Signalling pathways in cell death and survival after photodynamic therapy. J. Photochem. Photobiol. 57, 1–13.

Muller, M., 1993. The hydrogenosome. J. Gen. Microbiol. 193, 2879–2889.

Raab, O., 1900. Ueber die Wirkung fluorizierender Stoffe auf Infusorien. Z. Biol. 39, 524–546.

Silva-Filho, F.C., De Souza, W., 1986. Effect of colchicine, vinblastine, and cytochalasin b on cell surface anionic sites of *Tritrichomonas foetus*. J. Protozool. 33, 6–11.

Smith, T.G., Kain, K.C., 2004. Inactivation of *Plasmodium falciparum* by photodynamic excitation of Heme-cycle intermediates derived from d-aminolevulinic acid. J. Infect. Dis. 190, 184–191.

Sperandio, S., Belle, I., Bredesen, D.E., 2000. An alternative, non-apoptotic form of programmed cell death. Proc. Natl. Acad. Sci. U.S.A. 97, 14376–14381.

Vancini, R.G., Benchimol, M., 2005. Appearance of virus-like particles in *Tritrichomonas foetus* after drug treatment. Tissue Cell 37, 317–323.

Wainwright, M., 1998. Photodynamic antimicrobial chemotherapy (PACT). J. Antimicrob. Chemother. 42, 13–28.

Zeiss, C.J., 2003. The apoptosis–necrosis continuum: insights from genetically altered mice. Vet. Pathol. 40, 481–495.

Experimental Parasitology 128 (2011) 353–356

Contents lists available at ScienceDirect

Experimental Parasitology

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

Photodynamic therapy for American cutaneous leishmaniasis: The efficacy of methylene blue in hamsters experimentally infected with *Leishmania* (*Leishmania*) *amazonensis*

Lílian Somenci Peloi [a], Carlos Eduardo Guerino Biondo [a], Elza Kimura [b], Mario José Politi [c], Maria Valdrinez Campana Lonardoni [d], Sandra Mara Alessi Aristides [d], Regina Coeli Cunha Dorea [e], Noboru Hioka [a], Thaís Gomes Verzignassi Silveira [d,*]

[a] *Departamento de Química, Universidade Estadual de Maringá, Maringá, Paraná, Brazil*
[b] *Departamento Farmácia e Farmacologia, Universidade Estadual de Maringá, Maringá, Paraná, Brazil*
[c] *Instituto de Química, Universidade de São Paulo, São Paulo, Brazil*
[d] *Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, Paraná, Brazil*
[e] *Departamento de Ciências Biológicas, Universidade Estadual Paulista, Assis, São Paulo, Brazil*

ARTICLE INFO

Article history:
Received 23 September 2010
Received in revised form 4 April 2011
Accepted 28 April 2011
Available online 7 May 2011

Keywords:
Cutaneous leishmaniasis
Photodynamic Therapy
Leishmania amazonensis
Methylene Blue
Light-Emitting-Diode

ABSTRACT

The aim of this study was to investigate the effectiveness of Photodynamic Therapy (PDT) using Methylene Blue (MB) as the photosensitizing compound and a Light-Emitting Diode (LED) in American cutaneous leishmaniasis (ACL). Hamsters were experimentally infected with *Leishmania* (*Leishmania*) *amazonensis*. After the development of the lesions in the footpad, the animals were treated with MB three times a week for 3 months. Ten minutes after each application of MB, the lesions were irradiated with LED for 1 h. The lesions were evaluated weekly by the measurement of the hamster footpad thickness. At the end of the treatment the parasitic load was quantified in the regional lymph node of the hamsters. The treatment promoted a decrease in the thickness of infected footpad ($P = 0.0001$) and reduction in the parasitic load in the regional lymph node ($P = 0.0007$) of the animals from group treated with MB + LED. PDT using MB + LED in ACL caused by *L. amazonensis* shows a strong photodynamic effect. This therapy is very promising, once it is an inexpensive system and the own patient can apply it in their wound and in their house without the need of technical assistance.

1. Introduction

Leishmaniasis, is one of commonest parasitic diseases in the world and according to the World Health Organization more than 12 million people in 88 countries are infected and around 350 million people are at risk (WHO, 2009). The annual incidence is estimated as 1–1.5 million cases of cutaneous leishmaniasis and 500,000 cases of the visceral form (WHO, 2009).

Cutaneous leishmaniasis (CL) is characterized by single or multiple lesions in the skin or in the mucosa tissues which develop into oral, nasal and pharyngeal destruction (Neves et al., 2000). American cutaneous leishmaniasis (ACL) is caused by *Leishmania* of the subgenus *Leishmania* and *Viannia*, the most common being *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania panamensis*, and *Leishmania amazonensis* (Neves et al., 2000).

The treatment of leishmaniasis is based, mainly, on injection of pentavalent antimony derivatives, as first choice, and also amphotericin B, paromomycin and pentamidine isothionate (Brazil, 2007). Because the high incidence of collateral effects (arthralgia, myalgia, anorexia, nausea, vomiting, swelling and local pain) and the discomfort of daily injections, patients tend to interrupt treatment and the disease may evolve with serious complications and may develop drug resistance (Brazil, 2007). Therefore, application of a topical formulation is seen as more desirable than local injections or systemic therapy, resulting in better treatment compliance.

Photodynamic Therapy (PDT), using a photosensitizing compound (PS) and visible light producing reactive oxygen species (Macdonald and Dougherty, 2001; Mang, 2004), has been successfully applied for treatment of local and topical diseases such as macular degeneration, skin lesions and cancer (Levy, 1995; Machado, 2000; Sternberg and Dolphin, 1996; Sternberg et al., 1998; Gollnick et al., 2003) and *Leishmania* eradication with low scar formation (Akilov et al., 2007a; van der Snoek et al., 2008).

* Corresponding author. Address: Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Avenida, Colombo, 5790, 87.020-900, Maringá, Paraná, Brazil. Fax: +55 55 44 3263 6044.
E-mail address: tgvsilveira@uem.br (T.G.V. Silveira).

0014-4894
doi:10.1016/j.exppara.2011.04.009

Photosensitizers such as tetracationic porphyrins (Bristow et al., 2006), cationic and anionic PS (Akilov et al., 2006), aminolevulinic acid (ALA) and protoporphyrin IX (Akilov et al., 2007b), and two cationic PS (Akilov et al., 2007a) have produced encouraging results in *Leishmania* treatement. All were tested against *Leishmania major* and to a lesser extent *Leishmania donovani* and *Leishmania tropica* (Gardlo et al., 2003; Enk et al., 2003; Asilian and Davami, 2006; Ghaffarifar et al., 2006; Sohl et al., 2007) which are more common in Europe.

Few PDT studies have been carried out against *Leishmania* species causing ACL and we could only find reports of phthalocyanine derivatives used against *L. amazonensis* (Dutta et al., 2005), *L. panamensis* and *Leishmania chagasi* (Escobar et al., 2006). As ACL are endemic diseases in forest areas and, usually, the native population from these areas cannot afford expensive treatments, low cost treatments are desirable and may help to control this disease. Additionally, it is well known that the combination of Methylene Blue (MB) and different light sources such as Light-Emitting-Diodes (LED) or low potency lasers are effective against prokaryote and eukaryote cells (Tardivo et al., 2004; Tardivo et al., 2005; Wainwright et al., 1997; Zeina et al., 2001; Floyd et al., 2004; Huang et al., 2004; ÓRiordan et al., 2005; Peloi et al., 2008). However there are no reports of effectiveness of this combination against *Leishmania* species. Therefore, the aim of our study was to investigate the photodynamic effectiveness of MB and Light-Emitting Diode (LED) against ACL in hamsters experimentally infected with *Leishmania* (*L.*) *amazonensis*.

2. Materials and methods

2.1. Chemical and LED device

Methylene Blue (MB, Vetec, $C_{16}H_{18}ClN_3S{\cdot}3H_2O$, MW 373.90 g mol^{-1}) and all other chemicals were analytical grade and were used without purification. The LED (EverLight Co.) light system was constructed using 6 units (in series) that emit red light, and their individual output was determined using a Handheld Laser Power Meter (Edmund Optics Inc.). The LED emission (maximum at 663 nm) and MB absorption (maximum at 665 nm) spectra were recorded in a SPEX Fluorolog2 model 1680 and in a Varian Cary 50 spectrophotometer, respectively.

2.2. Parasite

Leishmania (*L.*) *amazonensis* (MHOM/BR/73/M2269) was isolated from experimentally infected hamsters and cultured in medium 199 supplemented with 10% of fetal calf serum, 20 mM L-glutamine, 1% human urine and antibiotics (100 UI/mL penicillin G and 100 μg/mL streptomycin) at 26 °C, by weekly passages.

2.3. Experimental infection

Thirty-eight male golden hamsters (90 days old) from the Animal Laboratory of Universidade Estadual Paulista (UNESP, Assis – SP, Brazil) were intradermically inoculated with 100 μL of physiological saline (PS) containing 5×10^6 promastigote forms of *L. amazonensis* in the posterior right footpad. The posterior left footpad, inoculated with 100 μL of uninfected PS was used as a vehicle control. The development of the lesions in the footpads were observed and measured for up to 12 weeks.

2.4. Photodynamic Therapy of MB/LED-PDT

The animals were separated in three groups: Control group ($n = 8$) treated with MB half in lotion and half in water without LED, group A ($n = 14$) treated with oil/water (O/W) lotion with 10 nM MB and group B ($n = 15$) treated with 10 nM MB aqueous solution (15 animals), and treatment started 90 days after infection. Before each treatment, the animals were anaesthetized with a combination of xylazine (10 mg/kg – Rompum®) and ketamine chlorohydrate of (50 mg/kg – Ketamina®). The footpad of the infected animals were treated with MB three times a week for 3 months. 10 min after the each application of MB, LED (665 nm, 5 mW cm^{-2}) was used for 1 h (12 J cm^{-2}). The control group received only MB without any irradiation.

2.5. The evaluation of lesions

The lesions were evaluated weekly by measurement of footpad thickness using a dial thickness gauge (Mitutoyo®, Japan). The lesion thickness was expressed as the difference between the infected footpad and the contralateral uninfected footpad.

2.6. Quantification of parasites in lymph node and spleen

Parasites were quantified in the lymph node and spleen of the hamsters after the end of the treatment, following the method described by Buffet et al. (1995), with slight modifications. The animals were euthanized under deep anesthesia. From each animal the popliteal lymph node and the spleen were aseptically removed and weighed. Each organ was macerated and the suspension was passed through a needle (insulin type) several times to disrupt the cells. The suspensions were diluted 1:4 and transferred to 96-well plates containing medium 199 (Gibco, Invitrogen Corporation) and incubated at 26 °C for 30 days. Next, each well was examined in an inverted light microscope searching for promastigote forms and the titer was considered as the highest dilution where least one parasite could be found. The incubation was extended for a further 15 days and re-examined. The parasitic load in each organ was calculated as geometric mean of the titers multiplied by 16.7 and divided by the total mass of the organs (in grams), where 16.7 represents the fraction of the suspension of each organ inoculated in the first well of the culture plate.

2.7. Statistical analysis

All results were analyzed by Shapiro Wilk's normality tests followed by Student' *t* test using the StatSoft® software; significant differences were considered at the 5% level.

2.8. Ethical aspects

All procedures were performed according to protocols approved by the Committee on Research Animal Care of the State University of Maringá (report 028/2006 on 11/Jul/2006).

3. Results

The development of the lesions in experimentally infected hamster were measured weekly for 12 weeks. After this, the hamster footpads were treated with 10 nM MB dissolved in lotion (group A) or water (group B) and irradiated with LED for 1 h three times a week for 12 more weeks (Fig. 1).

The control group treated with MB in lotion or water without LED irradiation, presented a continuous increase in thickness throughout the treatment, showing that MB alone was not able to prevent the development of lesions. In contrast, the thickness of the footpad in the groups treated with MB + LED was significantly decreased ($P = 0.0001$; $P = 0.0001$), indicating a strong

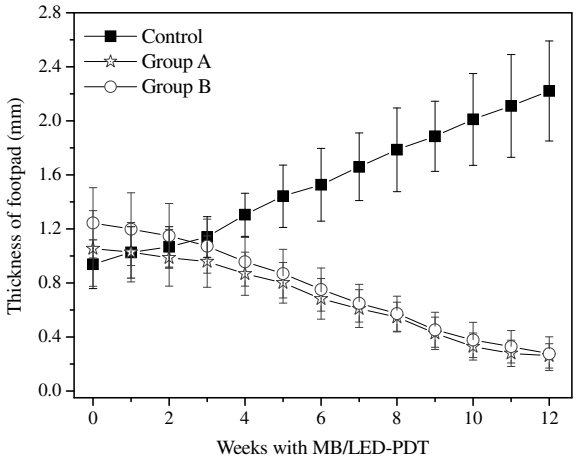


Fig. 1. Effect of MB/LED-PDT on lesions of hamsters infected with *Leishmania* (*L.*) *amazonensis*. The thickness of the footpad of hamsters infected with 5x106 promastigotes of *Leishmania* (*L.*) *amazonensis* was measured weekly for 12 weeks until lesion development and the treatment with MB or MB + LED started and followed weekly for 12 more weeks. The lesion was evaluated by the difference between the thickness of footpad and its contralateral footpad. Half of the control group was treated with MB in lotion and half MB in water ($n = 8$) and group A ($n = 14$) were treated with MB in lotion and group B ($n = 15$) received MB in water. The values represent mean ± SD of the total number of animals of the each group. $P < 0.001$ compared to control group.

photodynamic effect. No significant difference was found between groups A or B ($P = 0.470$).

The morphology of the lesions was also evaluated. Before MB + LED treatment, all animals had lesions in the infected footpad and more than 40% of them had ulcerated lesions. After 6 weeks of treatment with MB + LED, only 13.3% of the animals in group B and none in group A, had ulcerative lesions. At the end of treatment (12 weeks) 40% and 50% of the lesions in animals in groups A and B, respectively, were completely healed, while all animals in the control group, treated with MB only, had ulcerative lesions.

Fig. 2 shows a sequence of photographs of the infected footpad treated with the combination of MB + LED during and after 12 weeks of treatment, where re-epitelization, regression and healing can be seen.

The parasitic load in the spleen and in the popliteal lymph node of the infected hamsters was also investigated (Fig 3). No parasites were found in the spleens of any treated animal showing that the parasites did not migrate into spleen in the animals treated with MB + LED. In the popliteal lymph node, parasites were found in 87.5% of the control animals, while in the groups treated with MB + LED the percentage was smaller (28.6% for group A and

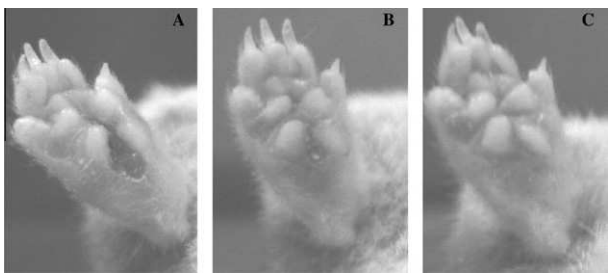


Fig. 2. Development of the lesion in hamster infected with *Leishmania* (*L.*) *amazonensis* and treated with MB/LED-PDT. Hamsters were infected with 5×10^6 promastigotes forms of *Leishmania* (*L.*) *amazonensis* and after the development of the lesion, the animals were treated with MB/LED-PDT. (A) Before treatment (90 days of infection); (B) After 6th weeks of treatment; (C) After 12 weeks of treatment.

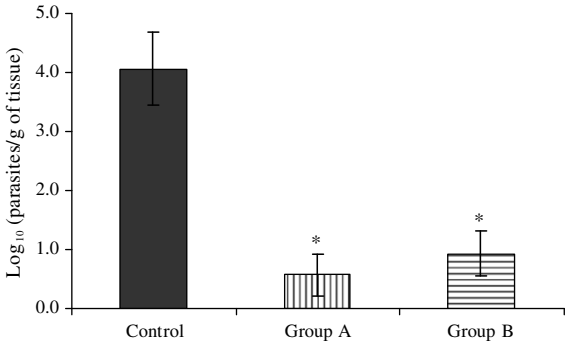


Fig. 3. Effect of MB/LED-PDT on parasite load in hamsters infected with *Leishmania* (*L.*) *amazonensis*. Hamsters were infected with 5×10^6 promastigotes forms of *Leishmania* (*L.*) *amazonensis* and treated with MB/LED-PDT. Control group ($n = 8$) was treated with MB but was not irradiated with LED. After the treatment for 12 weeks, the parasite load in the popliteal lymph node of infected footpad was evaluated. $^*P < 0.05$ compared to control group.

13.3% for group B). The parasite load in the groups treated with MB + LED was smaller compared to the control group ($P = 0.0007$ for group A and $P = 0.0001$ for group B). There was no significant difference in the parasitic load between groups A and B ($P = 0.53$) showing that MB in either water or O/W did not interfere in the permeability of MB into parasite membrane.

4. Discussion

Little effort has been made to improve the treatment of leishmaniasis around the world and the medicines recommended for treatment have been used for more than 90 years (Rath et al., 2003). Despite the high incidence of side effects and discomfort for the patient from daily subcutaneous injection, the pentavalent antimonials remain the first choice for treatment (Brazil, 2007; Rath et al., 2003). New therapies which ally lower costs, lower side effects and higher efficiency are urgently needed.

PDT has produced some success in treating superficial diseasesLevy, 1995; Machado, 2000; Sternberg and Dolphin, 1996; van der Snoek et al., 2008) and the approval of protoporphyrin has fueled the search for new photosensitizers (Machado, 2000; Sternberg et al., 1998; van der Snoek et al., 2008).

Several reports have demonstrated the efficacy of PDT for Old World CL using different photosensitizers. Among them, Gardlo et al. (2003) had treated five cases of CL with PDT using Metvix® (Photocure, Oslo, Norway), 75 J cm^{-2} of red light, applied twice a week, during 12 weeks followed by application once a week for a further 4 weeks. All patients involved in the study were healed, with excellent cosmetic results and the lesions were clinically and histologically free of parasites with no recurrence throughout the observation time (10 months). Enk et al. (2003) treated 32 lesions in 11 patients, of whom 10 had lesions healed after two PDT applications. Another study (Asilian and Davami, 2006), involving 20 patients with 31 lesions, was carried out with aminolevulinic acid (ALA-PDT), 100 J cm^{-2}, once a week, for 4 weeks. The results were satisfactory with 29 lesions completely healed and the other two being partially cure.

The main limitation to widespread use of PDT is the price of the PS and the light source, since leishmaniasis is mainly found in undeveloped countries where expensive treatments are unaffordable. MB is an inexpensive dye and has been shown to be a good PS for PDT (Peloi et al., 2008) and the combination of MB + LED was successful in our study in treating lesions caused by *L. amazonensis* in experimentally infected hamsters. After 12 weeks, half of the treated animals had their lesion healed

and, from visual analysis, the appearance of the lesions was better and the tissue around the lesions was undamaged. Furthermore, no parasites were found in the popliteal lymph node in more than 79.3% of the treated animals and the mean parasitic load was smaller in treated animals than in control animals.

Studies carried out with animals infected with *L. major* and treated topically with ALA-PDT or PPA904-PDT also had a significant reduction in the parasitic load, although local tissue damage was still observed (Akilov et al., 2007b).

Clinical cure of human patients is considered when the lesions are completed healed (Brazil, 2007). Despite results that showed clinical cure, this was not associated with the clearance of parasites, this being a hallmark of leishmaniasis. It is known that a low number of viable organisms still persist within lymphoid tissue and/or in the site of the former skin lesion after selfcure or successful chemotherapy (Ramírez and Guevara, 1997) and the clinical cure does not always coincide with histopathologic cure (Mendonça et al., 2004). It is possible that healing/cure depends on both the immune response of the host and on genotypic and phenotypic features of the parasite and play a role in the persistence of parasites (Mendonça et al., 2004).

Special and expensive equipment and time-consuming procedures have been emphasised as disadvantages of PDT treatment (van der Snoek et al., 2008), but the system used for this study is simple and cheap. The LED device is inexpensive, easy to handle and to transport, a MB is available in any local market and treatment can be carried out in the patient́s house without any special technical training.

5. Conclusions

PDT with MB + LED, a very inexpensive system, promoted significant reduction in the size of the lesion, in the parasitic load in the draining lymph node and healed the lesions in hamsters experimentally infected with *L. amazonensis*. Although the MB/LED-PDT provided promising results, further studies are necessary to better understand its mechanism of action and assure the safety and efficacy for use in humans.

Acknowledgments

This work was sponsored by the Brazilian funding agencies, Fundação Araucária – Seti/Paraná, CNPq (process 400618/2004-4 Rede Biomats) and CAPES. The authors also wish to thank Sérgio C. Silva (Physics Department – UEM) for the light device construction.

References

Akilov, O.E., Kosaka, S., O'Riordan, K., Song, X., Sherwood, M., Flotte, T.J., Foley, J.W., Hasan, T., 2006. The role of photosensitizer molecular charge and structure on the efficacy of photodynamic therapy against leishmania parasites. Chemistry and Biology 13, 839–847.

Akilov, O.E., Kosaka, S., O'Riordan, K., Hasan, T., 2007a. Photodynamic therapy for cutaneous leishmaniasis: the effectiveness of topical phenothiaziniums in parasite eradication and Th1 immune response stimulation. Photochemical and Photobiological Sciences 6, 1067–1075.

Akilov, O.E., Kosaka, S., O'Riordan, K., Hasan, T., 2007b. Parasiticidal effect of daminolevulinic acid-based photodynamic therapy for cutaneous leishmaniasis is indirect and mediated through the killing of the host cells. Experimental Dermatology 16, 651–660.

Asilian, A., Davami, M., 2006. Comparison between the efficacy of photodynamic therapy and topical paromomycin in the treatment of Old World cutaneous leishmaniasis: a placebo-controlled, randomized clinical trial. Clinical Experimental Dermatology 31, 634–637.

Brazil. Ministério da Saúde. Manual de Vigilância da Leishmaniose Tegumentar Americana. 2ª edição. 2007. Brasília. DF. Brasil.

Bristow, C.A., Hudson, R., Paget, T.A., Boyle, R.W., 2006. Potential of cationic porphyrins for photodynamic treatment of cutaneous leishmaniasis. Photodiagnosis and Photodynamic Therapy 3, 162–167.

Buffet, P.A., Sulahian, A., Garin, Y.J.F., Nassar, N., Derouin, F., 1995. Culture microtitration: a sensitive method for quantifying Leishmania infantum in tissues of infected mice. Antimicrobial Agents and Chemotherapy 39, 2164–2167.

Dutta, S., Ray, D., Kolli, B.K., Chang, K.P., 2005. Photodynamic sensitization of *Leishmania amazonensis* in both extracellular and intracellular stages with aluminum phthalocyanine chloride for photolysis in vitro. Antimicrobial Agents and Chemotherapy 49, 4474–4484.

Enk, C.D., Fritsch, C., Jonas, F., Nasereddin, A., Ingber, A., Jaffe, C.L., Ruzicka, T., 2003. Treatment of cutaneous leishmaniasis with photodynamic therapy. Archives of Dermatology 139, 432–434.

Escobar, P., Hernández, I.P., Rueda, C.M., Martínez, F., Paez, E., 2006. Photodynamic activity of aluminium (III) and zinc (II) phthalocyanines in leishmania promastigotes. Biomédica 26 (suppl 1), 46–49.

Floyd, R.A., Schneider, J.E., Dittme, D.P., 2004. Methylene blue photoinactivation of RNA viruses. Antiviral Research 61, 141–151.

Gardlo, K., Horska, Z., Enk, C.D., Rauch, L., Megahed, M., Ruzicka, T., Fritsch, C., 2003. Treatment of cutaneous leishmaniasis by photodynamic therapy. Journal of the American Academy of Dermatology 48, 893–896.

Ghaffarifar, F., Jorjani, O., Mirshams, M., Miranbaygi, M.H., Hosseini, Z.K., 2006. Photodynamic therapy as a new treatment of cutaneous leishmaniasis. Eastern Mediterranean Health Journal 12, 902–908.

Gollnick, S.O., Evans, S.S., Baumann, H., Owczarczak, B., Maier, P., Vaughan, L., Wang, W.C., Unger, E., Henderson, B.W., 2003. Role of cytokines in photodynamic therapy-induced local and systemic inflammation. British Journal of Cancer 88, 1772–1779.

Huang, Q., Fu, W.L., Chen, B., Huang, J.F., Zhang, X., Xue, Q., 2004. Inactivation of dengue virus by methylene blue/narrow bandwidth light system. Journal of Photochemistry and Photobiology B: Biology 77, 39–43.

Levy, J.G., 1995. Photodynamic therapy. Trends in Biotechnology 13, 14–18.

Macdonald, I.J., Dougherty, T.J., 2001. Basic principles of photodynamic therapy. Journal of Porphyrins and Phthalocyanines 5, 105–129.

Machado, A.E.H., 2000. Photodynamic Therapy: principles, potential of application and perspectives. Química Nova 23, 237–243.

Mang, T.S., 2004. Lasers and light sources for PDT: past, present and future. Photodiagnosis and photodynamic therapy 1, 43–48.

Mendonça, M.G., Brito, M.E.F., Rodrigues, E.H.G., et al., 2004. Persistence of *Leishmania* parasites in scars after clinical cure of American Cutaneous Leishmaniasis: is there a sterile cure? Journal of Infectious Diseases 189, 1018–1023.

Neves, D.P., Melo, A.L., Linardi, P.M., Vitor, R.W.A., 2000. Leishmaniose tegumentar Americana. In: Neves, D.P. (Ed.), Parasitologia Humana, 10a Ed. Editora Atheneu, São Paulo, pp. 36–53.

ÓRiordan, K., Akilov, O.E., Hasan, T., 2005. The potential for photodynamic therapy in the treatment of localized infections. Photodiagnosis and Photodynamic Therapy 2, 247–262.

Peloi, L.S., Soares, R.R.S., Biondo, C.E.G., Souza, V.R., Hioka, N., Kimura, E., 2008. Photodynamic effect of light-emitting diode light on cell growth inhibition induced by methylene blue. Journal of Biosciences 33, 231–237.

Ramírez, J.L., Guevara, P., 1997. Persistent Infections by *Leishmania* (*Viannia*) *braziliensis*. Memmórias do Instituto Oswaldo Cruz 92, 333–338.

Rath, S., Trivelin, L.A., Imbrunito, T.R., Tomazela, D.M., Jesús, M.N., Marzal, P.C., 2003. Antimoniais empregados no tratamento da leishmaniose: Estado da arte. Quimica Nova 26, 550–555.

Sohl, S., Kauer, F., Pasch, U., Siman, J.C., 2007. Photodynamic treatment of cutaneous leishmaniasis. Journal der Deutschen Dermatologischen Gesellschaft. 5, 128–130.

Sternberg, E.D., Dolphin, D., 1996. Pyrrolic photosensitizers. Current Medicinal Chemistry 3, 293–324.

Sternberg, E.D., Dolphin, D., Bruckner, C., 1998. Porphyrin-based photosensitizers for use in Photodynamic Therapy. Tetrahedron 54, 4151–4202.

Tardivo, J.P., Giglio, A.D., Oliveira, C.S., Gabrielli, D.S., Junqueira, H.C., Tada, D.B., Severino, D., Turchiello, R.F., Baptista, M.S., 2005. Methylene blue in photodynamic therapy: From basic mechanisms to clinical applications. Photodiagnosis and Photodynamic Therapy 2, 175.

Tardivo, J.P., Giglio, A.D., Paschoal, L.H.C., Ito, A.S., Baptista, M.S., 2004. Treatment of melanoma lesions using methylene blue and RL50 light source. Photodiagnosis and Photodynamic Therapy 1, 345.

van der Snoek, E.M., Robinson, D.J., van Hellemond, J.J., Neumann, H.A.M., 2008. A review of photodynamic therapy in cutaneous leishmaniasis. Journal of the European Academy of Dermatology and Venereology 22, 918–922.

Wainwright, M., Phoenix, D.A., Marland, J., Wareing, D.R.A., Bolton, F.J., 1997. A study of photobactericidal activity in the phenothiazinium series. FEMS Immunology and Medical Microbiology 19, 75–80.

World Health Organization – WHO 2009. Leishmaniasis. Available from <http://www.who.int/leishmaniasis/en/> (accessed 01.15.09).

Zeina, B., Greenman, J., Purcell, W.N., Das, B., 2001. Killing of cutaneous microbial species by photodynamic therapy. British Journal of Dermatology 144, 274–278.