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REVIEW

Methylene blue in photodynamic therapy: From basic mechanisms to clinical applications

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Onychomicosis

Summary Methylene blue (MB) is a molecule that has been playing important roles in microbiology and pharmacology for some time. It has been widely used to stain living organisms, to treat methemoglobinemia, and lately it has been considered as a drug for photodynamic therapy (PDT). In this review, we start from the fundamental photophysical, photochemical and photobiological characteristics of this molecule and evolved to show in vitro and in vivo applications related to PDT. The clinical cases shown include treatments of basal cell carcinoma, Kaposi's Sarcoma, melanoma, virus and fungal infections. We concluded that used together with a recently developed continuous light source (RL50®), MB has the potential to treat a variety of cancerous and non-cancerous diseases, with low toxicity and no side effects.

Contents

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Introduction

Photodynamic therapy (PDT) is a promising modality for the management of various tumors and non-malignant diseases, based on the combination of a photosensitizer that is selectively localized in the target tissue and illumination of the lesion with visible light, resulting in photodamage and subsequent cell death [1—3]. Numerous worldwide clinical trials have shown that PDT represents an effective and safe modality for various malignant conditions [1—3].

A photosensitizer absorbs energy directly from a light source, which it may then transfer to molecular oxygen to create an activated form of oxygen called singlet oxygen ($^1O_2$). $^1O_2$ is extremely electrophilic and can oxidize directly electron-rich double bonds in biological molecules and macromolecules and it is believed to be the main cytotoxic agent related with PDT [4]. The photosensitizer can also get involved in electron-transfer reactions initiating radical-induced damage in biomolecules [5]. It is difficult to establish without doubt which of these mechanisms is more prevalent in vivo. It is likely that both processes can ultimately lead to cell death [6].

PDT has several advantages in comparison with other cancer therapies [1—3,7—9]. First, it works on virtually all types of cancers, lacking the specificity of chemo-therapeutics and radiation. The procedure can be repeated several times, if needed, because there are no cumulative toxic effects, and it is usually an outpatient procedure [7]. Adjuvant intraoperative PDT may be a safe and effective method of destroying residual tumor, thereby preventing loco-regional tumor recurrence [7]. PDT as an adjuvant modality to surgical resection of colon cancer is feasible and does not affect healing of the anastomosis [8]. When combined with surgery and/or chemotherapy it enhances the effect and prevents metastasis. Multiple sessions are very useful and excellent cosmetic results can be achieved [1—3]. Moreover, because of its lower risk profile, PDT can be used even in the elderly or in people who are too sick for surgery [9].

A limitation of PDT is that it cannot cure advanced disseminated diseases because light irradiation of the whole body with appropriate doses is not yet possible. Also, there are practical limitations including the difficulty in establishing the optimum variables for a specific treatment, clinician and hospital resistance to a new therapeutic approach, the capital cost of setting up a PDT center, and the lack of inexpensive and convenient light sources [2].

In 1978, Dougherty et al. [10] published results of 113 tumors from 25 patients treated with hematoporphyrin derivate with complete response in 98 tumors (86.72%). Cutaneous and subcutaneous recurrent and metastatic tumors which had not responded to conventional treatment like basal cell, malignant melanoma, chondrosarcoma, colon adenocarcinoma, prostate, mycosis fungoides, endometrial carcinoma, breast carcinoma, angiosarcoma and squamous cell carcinoma, were submitted to PDT. In general, a single treatment or fractionated treatments were used [10,11]. Worldwide thousands of patients have been given PDT over the past 20 years, but most trials have involved only a few patients and have not been sufficiently convincing of the benefits of PDT as a standard treatment [1—4,12].

Early preparations of photosensitizers for PDT were based on a complex mixture of porphyrins called haematoporphyrin derivative. Extensive chemical and biological research has been carried out over the past 20 years to identify new photosensitizers that belong to the different classes of compounds including porphyrins, chlorins, phthalocyanines, texafrins and phenothiaziniums [3]. Several drugs are approved for oncological indications: Porfirmer sodium, Temoporfin, aminolevulinic acid and methyl aminolevulinate. They are indicated for advanced and early lung cancer, superficial gastric cancer, esophageal adenocarcinoma, cervical cancer and bladder cancer, palliative head and neck cancer, actinic keratosis, superficial basal cell carcinoma and basal cell carcinoma [1—3,13].

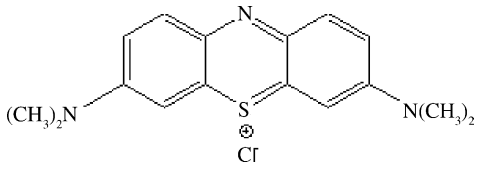
The drugs used in PDT are generally given systemically, but both intra-tumoral injection and topical application are possible [1—3,12,13]. For a photosensitizer to be clinically useful it should be non-toxic, selectively taken up and/or retained in malignant tissue, activated by penetrating light (>600 nm), and relatively photochemically efficient [1,13].

In the past 10 years, substantial advances have been made in the understanding of the behavior of light in human tissues and in the development of equipment for light delivery for PDT [1–3,14]. Initially, PDT was performed with the use of conventional gas discharge lamps. The introduction of lasers equipped with optical fibers enabled the endoscopic delivery of light to almost every site of the human body. Many non-laser light sources have also been developed, especially for treatment of skin lesions [1–3,14].

The penetration of light through the tumor depth is dependent on the characteristics of the treated tissue besides the absorption by endogenous chromophores and by the photosensitizing drug. Wavelengths shorter than 600 nm are absorbed mainly by hemoglobin, whereas vibration overtones of water and other molecules induce absorption at wavelengths longer than 950 nm, restricting the ''therapeutic window'' of the skin to 600–950 nm. Tissue scattering, which decreases as a fourth power with the wavelength increase, is another factor (perhaps the most important) that hinders light penetration. The greatest penetration depth observed in the therapeutic window is around 10 mm [15]. The light dose usually given in joules per square centimeter is empirical and varies widely. For interstitial applications, radiant exposures between 100 and 400 J/cm^2 are needed [3].

Methylene blue (MB) (Scheme 1) has shown in vivo activity against several types of tumors when locally injected and illuminated with red laser light [9,12,16–18]. Orth and coauthors have demonstrated that intratumoral injection of 1% methylene blue followed by illumination by an argon-pumped dye laser that emitted at 665 nm with an irradiance of 100 mW/cm^2 and a fluence of 100 J/cm^2 was able to kill xenotransplanted tumors in animals and recurrent esophageal tumors in patients [12,16–18]. We extended this application by showing the treatment of melanoma lesions using MB and polychromatic light for excitation [9].

The facility to obtain MB and the possibility of using non-laser polychromatic light sources [9] makes MB a potential PDT sensitizer that could be used in underserved populations for the treatment of a variety of diseases. This possibility has motivated interdisciplinary research in our labs that goes from the basic aspects of the photodynamic activity of MB to its application in medical treatments of a variety of diseases.



Scheme 1 Molecular structure of methylene blue (MB^+).

Methylene blue: photophysics and photochemistry

Methylene blue is a widely known histological dye that has been in use for many years [19]. It belongs to the phenothiazinium class of compounds. The characteristic color of MB is caused by the strong absorption band in the 550–700 nm region with maximum molar absorptivity of 85,000 $M^{-1}\,cm^{-1}$ at 664 nm [20]. MB absorption spectrum is concentration-dependent due to dimerization, whose equilibrium constant is $3.8 \times 10^3\,M^{-1}$ in water [20]. The dimerization increases with the increase in ionic strength and may increase or decrease in the presence of charged interfaces, depending on the ratio between dye and interface [20,21]. Its fluorescence quantum yield is low (ca. 0.04) in water, and it may change slightly as a function of the solvent. Solute–solute interactions (ion pair formation and dimerization) further decrease the fluorescence quantum yield [20,21].

MB monomers and dimers have distinct absorbance spectra (Fig. 1A) [20]. Monomers have maximum at 664 nm and dimers at 590 nm. The difference in absorption between monomers and dimers facilitate the calculation of each species concentrations present in solution. Note that in 20 μM aqueous solution only MB monomers are present (Fig. 1B). However, after in vivo local injection of 2% MB solution in a basal cell epidermal tumor at the arm of a patient, maximum absorption at 580 nm was observed (Fig. 1B), indicating the presence of MB dimers in the tumor tissue. As it will be mentioned below, monomers and dimers may be involved in different kinds of photochemical reactions, which may affect the mechanism and efficiency of cell kill (Scheme 2 [20,21]).

MB has been extensively used for photooxidation of natural and synthetic molecules. Two major photochemical pathways are usually observed: type II where the triplet energy is transferred to oxygen forming singlet oxygen ($^1O_2$, Reaction (2), Scheme 2) and type I where reducing agents donate an electron to the MB triplet, forming the semi-reduced radical (MB, Reaction (3), Scheme 2). At high dye concentrations ground state MB molecules can work themselves as reducing agents ($D - D^*$ mechanism) [20,21]. In homogeneous solution where no dimers are present (ethanol or

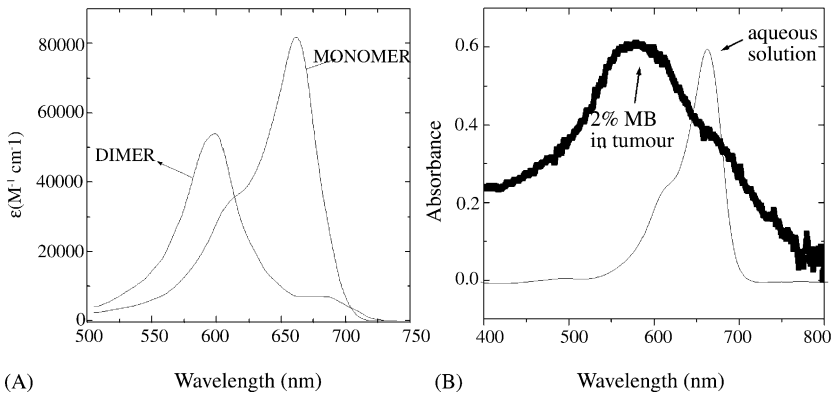
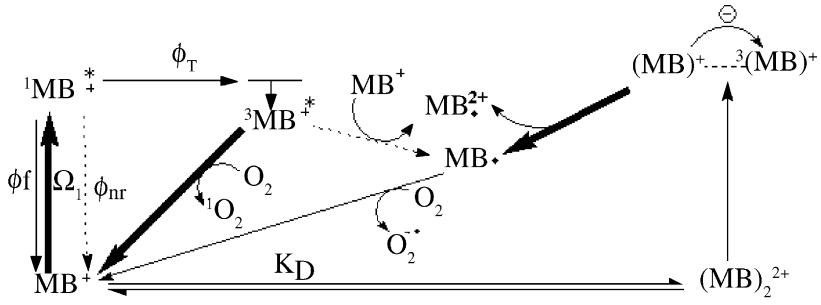


Figure 1 (A) Absorbance spectra of dimer and monomer species of MB. (B) Dark line: absorption spectrum obtained with a reflectance spectrophotometer (Ocean Optics USB2000) equipped with pulsed xenon light source, of a skin cancer in which a 2% MB aqueous solution was injected locally; thin line: absorbance spectra of a diluted MB aqueous solution ([MB] = 20 μM).



$$^3MB^{+*} \xrightarrow{k_1} MB^+ \quad (1)$$

$$^3MB^{+*} + O_2 \xrightarrow{k_2} MB^+ + {}^1O_2 \quad (2)$$

$$^3MB^{+*} + R \xrightarrow{k_3} MB^{\bullet} + R^{\bullet+} \quad (3)$$

$$MB^{\bullet} + O_2 \xrightarrow{k_4} O_2^{\cdot -} + MB^+ \quad (4)$$

$$MB^+ + MB^+ \underset{}{\overset{k_D}{\rightleftharpoons}} (MB)_2^{2+} \quad (5)$$

$$(MB)_2^{2+} \xrightarrow{k_6} MB^{\bullet} + MB^{2+}_{\bullet} \quad (6)$$

$$O_2^{\cdot -} + Fe^{+3} \longrightarrow O_2 + Fe^{2+} \quad (7)$$

$$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (8)$$

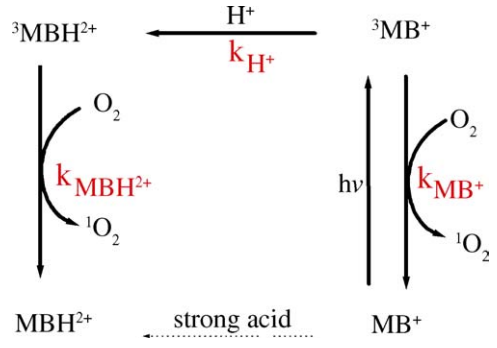
$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{+3} + OH^- + OH^{\cdot} \quad (9)$$

Scheme 2 Methylene blue photochemical reaction routes where MB^+, $^1MB^{+*}$, $^3MB^{+*}$ are methylene blue ground state, singlet and triplet excited states, respectively, $MB^{\bullet}$ and $MB^{\bullet 2+}$ are methylene blue semi-reduced and semi-oxidized radicals, respectively, Ω_1 is light absorption, ϕ_f, ϕ_{nr}, ϕ_T, are fluorescence, nonradiative and triplet quantum yield. Reactions (1—4) represent the deactivation routes of MB^+ excited state and radical species where (1) is the $^3MB^{+*}$ spontaneous decay, (2) is the reaction of $^3MB^{+*}$ with molecular oxygen, (3) is the redox suppression of $^3MB^{+*}$ by reducing agents, (4) is the oxidation of $MB^{\bullet}$ by molecular oxygen returning the ground state dye and forming superoxide, (5) is the ground state dimerization constant, (6) is the redox suppression of $(^3MB^+ - - - - MB)^*$ after exciting ground state dimers, (7—9) are Fenton reactions. The relative position of the species presented in this scheme do not represent their actual energy level. Modified from Ref. [20].

diluted aqueous solutions) MB produces triplets with high quantum yield ($\phi_T = 0.52$), working as a $^1O_2$ photogenerated source ($\phi_\Delta \sim 0.5$) [20,21].

In aqueous solution the efficiency of $^1O_2$ is dependent on the pH [22]. MB triplets are excited state bases, so that its pK_a increases from a negative value in the ground state to around 7.5 in the triplet state (Scheme 3) [21]. We have measured the excited state processes in our lab in detail [Junqueira HC, Baptista MS. Protonation of MB in different microenvironments, in preparation]. Upon excitation of MB to the triplet state at pH ~4, the protonation reaction ($k_{H^+} = 4 \times 10^6\ s^{-1}$, second order rate constant (k_2) of $2 \times 10^{10}\ M^{-1}\ s^{-1}$) is one order of magnitude faster than the reaction with oxygen ($k_{MB^+} = 7 \times 10^5\ s^{-1}$, $k_2 \sim 1 \times 10^9\ M^{-1}\ s^{-1}$). At pH ~4 just after the laser pulse, MB triplets are observed; however, after hundreds of nanoseconds the main species present in solution are MBH^{2+} triplets (Fig. 2A, Scheme 3). MBH^{2+} triplets have a higher energy level and consequently they react with oxygen with a smaller rate constant ($k_{MBH^{2+}} = 2.9 \times 10^5\ s^{-1}$, $k_2 \sim 3 \times 10^8\ M^{-1}\ s^{-1}$). They live longer in homogeneous solution and they may produce less $^1O_2$ in the presence of other reaction pathways. Therefore, the pH of the solution may certainly affect the efficiency of type I and type II photosensitization mechanisms. This is a fact to be considered when treating tumors tissues that may have a lower than neutral pH due to the anaerobic metabolism.

MB binds to negatively charged interfaces and depending on the concentration of interfacial binding sites to MB, MB aggregates are observed (Equilibrium (5), Scheme 2). Upon electronic excitation of dimers, electron-transfer reactions are observed, forming semi-reduced and semi-oxidized



Scheme 3 Scheme of the proton reactions of methylene blue where MB^+ and $^3MB^+$ are unprotonated methylene blue and MBH^{2+} and $^3MBH^{2+}$ are protonated MB in the ground and excited triplet state, respectively. k_{H^+}, k_{MB^+} and $k_{MBH^{2+}}$ are the observable (s^{-1}) rate constants of the reactions of $^3MB^+$ with H^+, of $^3MB^+$ with oxygen and of $^3MBH^{2+}$ with oxygen, respectively.

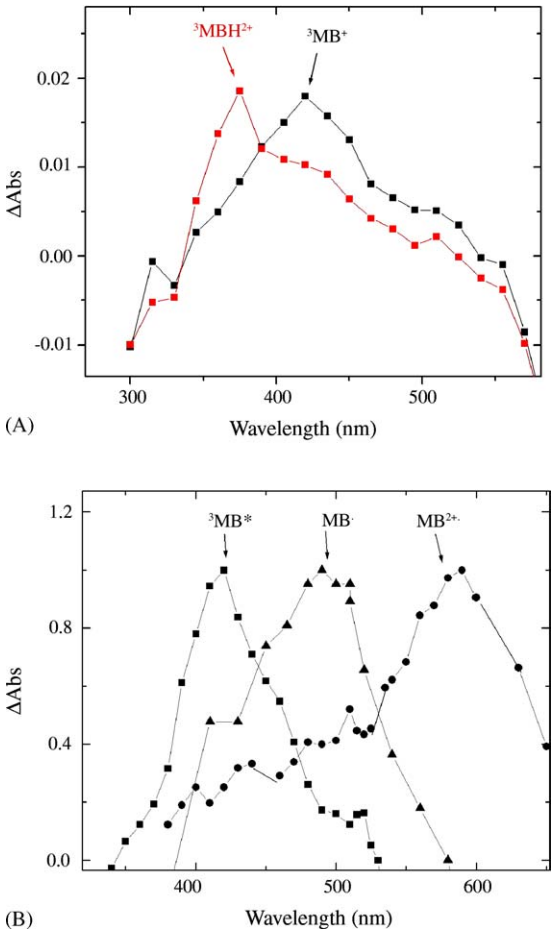


Figure 2 (A) Normalized transient spectra obtained 0.1 μs ($^3MB^*$) and 2 μs ($^3MBH^{2+}$) after the laser excitation of a 10 μM MB^+ solution at pH 3.8 (borate buffer = 50 μM), showing the characteristic spectra of the unprotonated (MB^+) and protonated (MBH^{2+}) triplet. (B) Characteristic transient spectra of ^3MB, $MB^\bullet$ and $MB^{\bullet 2+}$. The laser flash photolysis equipment that was used for the measurement is described in Ref. [20,21]. Laser pulse = 10 ns, λ_{exc} = 532 nm.

radicals (Fig. 2B). In the condition of large concentration of dimers, type II is shifted to type I reaction, practically abolishing $^1O_2$ generation (Reaction (6), Scheme 2). The semi-reduced dye radicals can react with oxygen forming superoxide (Reaction (4), Scheme 2, Fig. 2B), which can lead to several other reactive oxygen species (ROS), including the highly reactive hydroxyl radical through Fenton reaction (Reactions (7–9), Scheme 2) [20,21].

These findings are of relevance to the mechanism of action of MB in PDT, because its photoaction occurs in direct contact with membranes, polyelectrolytes and in the presence of reducing agents. The charges of the membranes and polyelectrolytes can vary from the slightly negative cytoplasmatic membranes to the highly negatively charged inter-

nal mitochondrial membrane and polyelectrolytes (nucleic acids and polysaccharides) [23,24]. The fact that MB may induce either the formation of radical (type I) or singlet oxygen (type II) species, may extend the application of MB to tumors that have areas of hypoxic tissues where type II mechanisms do not occur efficiently [5,25,26].

One of the biopolymers for which MB has high affinity is melanin. The selective binding of MB to melanin has been shown [27—30]. This is especially relevant in terms of its high affinity to pigmented cancer tissues like those found in melanoma lesions [31,32].

Photosensitization reactions induced by MB excitation are known to cause damage to several biomolecules. Damage to nucleic acids, proteins and lipids have been described and rationalized in the literature. This damage is thought to be triggered both by type I and type II processes. This subject has been studied over many years and it is comprehensively described in the review by Tuite and Kelly [19].

In type II processes, proteins are more reactive ($\sim 10^4\,L\,g^{-1}\,s^{-1}$) compared with lipids ($\sim 10^2\,L\,g^{-1}\,s^{-1}$) and nucleotides ($\sim 10^3\,L\,g^{-1}\,s^{-1}$) [33]. Using these numbers and the average concentration of isolated biomolecules, Baker and Kanofsky [33] estimated that the lifetime of singlet oxygen within the cell cytoplasm is between 0.2 and 0.3 μs and that most of the singlet oxygen is quenched by protein and only 0.1% by lipids. However, the fact that the isolated molecules react more efficiently in vitro, does not mean that this mechanism is more important in vivo. For instance, irradiation of sensitizer localized in membranes will probably lead to membrane lysis, which can lead to cell and/or organelle lysis [34]. Conclusive evidence has shown that membranes are key targets for photomodification in experimental models including tumor cells [35], insect [36] and yeast cells [37].

There are some characteristics of biological membranes that make them specially important in cell-killing events by PDT: (i) their high surface area; (ii) the photosensitizers tend to localize on them; (iii) the large protein concentration present in the biological membranes; and (iv) damage to plasmatic or organelle membranes can easily cause their lysis and a great misbalance in the cell homeostasis [34].

MB has a long history with membrane related phenomena. It has been used for long time in the treatment of methemoglobinemia [38,39]. This pharmacological activity has been modeled in mimetic systems, and it is related to the fact that it has redox properties that allow it to be easily reduced and oxidized in biological media and also due to the fact that it easily crosses membranes [40]. The crossing of MB through membranes has also been studied in mimetic systems and it is due to the diffusion of MB monomers through bilayers [41].

The efficiency of a photosensitizer in generating ROS within membranes is dependent on the intrinsic characteristics of the sensitizer in aqueous solution and within the membranes as well as their partition in the membrane [42]. Singlet oxygen that is generated out of the membrane may partition into the membrane with efficiency varying from 0.1 to 0.9 [34], and the lifetime of singlet oxygen inside the hydrophobic environment of micelles and vesicles is around one order of magnitude larger than in aqueous media [43].

The initial attack of singlet oxygen in lipids is by the specific reaction with double bonds to form allylic hydroperoxides [44,45]. The efficiency of this reaction is dependent on the lowest ionization potential of the olefins and also on the availability of allylic hydrogens. In fact, the quenching constant of $^1O_2$ by fatty acids and cholesterol varies by two orders of magnitude from 10^3 to $10^5\,M^{-1}\,s^{-1}$ and increases with the number of double bonds present in the lipid molecule [46]. Lipid peroxidation has been linked to several effects, i.e., increased ion permeability, loss of fluidity, cross-linking and inactivation of membrane proteins [47].

Photoexcitation of MB is known to promote lipid peroxidation in egg lecithin vesicles. The mechanism involves the initial attack by $^1O_2$, which was determined to be 200 times more efficient in inducing liposome lysis than the radical initiated reactions. No lysis was observed in vesicles made with saturated lipids in agreement with the main reaction mechanism being related to the formation of hydroperoxides [48]. Grossweiner and Grossweiner [49] have shown that the lysis rate of vesicles treated with MB and light increases with the solution mixing during the course of photo-oxidation. These results were related with changes in the hydrodynamic characteristic of the membrane due to the formation of hydroperoxides.

Methylene blue: photobiology

The photodynamic action of any photosensitizer in biological systems is a puzzle, since it may be affected by several variables including the photochemical mechanism, photosensitizer localization, reactivity of biological targets and cell signaling network, and each one of these variables may affect the properties of the other. In other words,

the localization defines the biological targets that define the mechanisms and vice versa.

Targeting cell organelles instead of targeting tumor vascular system, which is the accepted mechanism of hematoporphyrin derivative drugs, is one strategy that has been thought to avoid tumor regrowth after PDT treatment [2,3,13]. In fact, targeting mitochondria is an important research subject in PDT, since it is known that damaging mitochondria may induce the apoptotic cascade [50,51]. Positively charged sensitizers with the correct octanol/water balance are known to concentrate in mitochondria, due to the attraction by the negatively charged potential of properly functioning mitochondria [52]. Finally, understanding the mechanisms of photoinactivation of prokaryotic and eukaryotic cells can form a basis to propose new and more efficient protocols to PDT.

Interaction with cell organelles

Drugs can be selectively targeted to mitochondria when they present sufficient lipophylicity to cross membranes and positive charges, which are attracted to the negative electrochemical environment of the mitochondrial matrix [51—53]. Typical values of plasma membrane and mitochondrial membrane potentials are 75 and 180 mV, respectively [54]. In these cells, the relative concentration of a membrane-permeable cation can be calculated using the Nernst equation to be 18 times more concentrated in the cytosol than extracellularly, and 1000 times more concentrated in the mitochondrial matrix than in the cytosol [54].

Once localized in the mitochondria the sensitizer can damage it [51—54]. Photoinduced depolarization of the mitochondria membrane was measured for a series of positively charged dyes and shown to be dependent on three parameters, i.e., the optical density of the dye at the excitation wavelength, the singlet oxygen quantum yield and the fraction of dyes bound to mitochondria [50]. However, only molecules with no binding tendencies to other biomolecules would accumulate only in mitochondria [54]. Most of the photosensitizers have a distribution in several cell compartments and organelles. As it will be clear from the discussion below, MB is probably one of these drugs that have a distribution to several cell compartments including lysosomes and mitochondria.

When incubated with a suspension of mitochondria, MB actively binds this organelle and enters the matrix in a manner stimulated by the mitochondrial proton potential [55]. The larger accumulation of MB in mitochondria with elevated proton potentials or treated with large concentrations of MB results in the formation of MB dimers, previously shown to be less effective generators of $^1O_2$. Accumulation of MB within mitochondria also results in MB reduction to the leuco-MB and in consequent oxidation of mitochondrial NAD(P)H. Indeed, irradiation of mitochondria with high proton potentials in the presence of MB results in the generation of approximately half the quantity of $^1O_2$ than mitochondria with low proton potentials; the other half being probably dislocated to the formation of radical species [55]. These results indicate that changes in MB binding and aggregation properties in mitochondrial suspensions can strongly influence $^1O_2$ generation efficiency, which are in agreement with studies made in membrane mimetic systems, that show that MB may induce both the formation of radical and $^1O_2$ species [20,21,55]. These differences in photochemical properties certainly influence the cytotoxic effects of PDT in the presence of MB.

The fact that NAD(P)H, which is localized in the mitochondrial matrix, is oxidized by the photoexcitation of MB is in favor of the MB designation as being a mitochondrial sensitizer [55,56]. The mentioned MB behavior is different from other PDT sensitizers such as photofrin, protoporphyrin IX, Al(III) phthalocyanine chloride tetrasulfonic acid, meso-tetra(4-sulfonatophenyl)porphine dihydrochloride and visudyne, which are not able to oxidize mitochondrial NAD(P)H [57].

Due to its hydrophilic/lypophilic balance and membrane affinity, MB is expected to bind other cell organelles [58]. However, the actual cytolocalization of MB in cell culture has been the subject of controversy in the literature. Lysosome is certainly also a site of localization and action of MB. Santus et al. [59] have shown that MB is able to liberate lysosomes enzymes of mouse L and human fribroblast cells under the course of irradiation with visible light. Mitochondria localization has also been observed [60]. Some authors have suggested that MB is localized in lysosome and migration to nucleus is observed only after light activation [61]. Others show evidence that cell nucleus is the main site of localization [62]. Gutter et al. [63] demonstrated that MB induces photo-oxidation of guanine residues in intracellular DNA of human KB cells. The probable reasons for this discrepancy are due to the fact that the microenvironment experienced by MB affects its properties (emission efficiency) and therefore perturb the exact definition of its cytolocalization by fluorescence microscopy. For example, even if MB were mainly localized in mitochondria of a specific cell type, it would not be easily characterized by the usual method of fluores-

cence co-localization, because it is easily reduced to the leuco-form by NAD(P)H inside the mitochondria [55]. We have experiments under way trying to resolve this question. They are based on the competition between fluorescence organelle markers and MB for the sites of localization.

Mitochondria cytolocalization of MB derivatives have been confirmed in some studies [64,65]. The increase in the methylation in the MB molecular structure has been related to the increased phototoxicity in cells. These results have been explained in terms of larger partition in the membranes and smaller tendency to be reduced inside the cell [64].

Photoactivity in cell culture

The photodynamic activity of MB in cell culture depends on variables in the cell line. MB photoaction is similar in transformed human keratinocytes, squamous cell carcinoma [66], kidney cells [67] and human brain tumor cells [68]. However, erythroleukemia cells are resistant to MB plus light excitation [67]. Understanding the mechanisms of photoinduction of mammalian cell killing is an important direction to the search for new and more efficient treatments for cancer diseases. Cell cultures studies have demonstrated that polychromatic light can be as effective in exciting of MB as 662-nm laser light, opening the opportunity to have PDT performed with more accessible light sources compared to lasers [69].

One of the major drawbacks of cancer chemotherapy is the development of multidrug-resistant (MDR) tumor cells, which are cross-resistant to a broad range of structurally and functionally unrelated agents, making it difficult to treat these tumors. Concerning MDR phenotype expressed in five cell lines, MB was able to revert it [67]. Therefore, MB could have the advantage of being used simultaneously as a MDR reverser and a photodynamic agent [70].

Using a series of MB analogs modified with different alkyl chain lengths Mellish et al. [71] have studied the in vitro photoactivity of MB and MB analogs with longer alkyl chains in RIF-1 murine fibrosarcoma cells. They show that the larger efficiency is observed in the propyl, pentyl and hexyl derivatives, which accumulate preferentially in mitochondria, in accordance with the studies published by Wainwright et al. [65,71].

PDT-induced cell death may occur in two different ways: apoptosis or necrosis. Apoptosis starts with an intra/extra cellular signal (intrinsic/extrinsic pathway) leading to caspase activation and DNA fragmentation. In apoptosis, the cell shrinks, the nuclear chromatin becomes pycnotic and condenses against the nuclear membrane, and eventually the cytoplasm and nucleus break up into apoptotic bodies. Necrosis typically damages the cellular ion homeostasis, a process that leads to water influx and a loss of membrane integrity [72,73]. Apoptosis is preferred over necrosis for several reasons: (i) it can occur in lower doses of irradiation than those required for necrosis; (ii) it shows an anti-inflammatory effect; and (iii) apototic cell death may stimulate the immune system [72,73].

The induction of apoptosis or necrosis may be determined by the photochemical mechanism (type I or II), by the types of cell and photosensitizer, and by the light dose [74,75]. To induce apoptosis, the damage must be severe enough to avoid cell repairing, but mild enough to produce energy for the apoptotic pathway, otherwise the cell will die by necrosis [72,76]. Mitochondria sensitizers are known to induce apoptosis in higher efficiency than sensitizers that accumulate in other cell compartments [51—53,67,71—77].

The actual role of mechanisms type I and type II after photoexcitation of MB inside the cell is still a matter of debate. Although MB is well known for producing $^1O_2$ in solution, its generation from intracellular MB is not certain. Due to the facility by which MB is reduced, Tuite and Kelly [19] believed that mechanism type I is the most important for MB. In order to check whether or not $^1O_2$ was generated from intracellular MB, we obtained the NIR emission spectra from Hela cells incorporated with MB. Controls were performed to make sure that under these conditions MB is totally internalized. Note that the observed spectrum is the characteristic emission spectra of $^1O_2$ with maximum around 1270 nm (Fig. 3). Therefore, this experiment proves that MB does generate $^1O_2$ within Hela cells.

The generation of ROS species in the mitochondria induce inner membrane permeabilization, depolarization and swelling, causing the release of apoptosis signaling molecules (cytochrome *c*, AIF, SMAC/DIABLO) to the cytosol leading to apoptotic cell death. In the cytosol, cytochrome *c* binds pro-apoptotic factors, leading to the formation of the apoptosome, which activates caspases [76,78].

Phenothiazine neuroleptics (PN) like chlorpromazine, levomepromazine, promethazine, trifluoperazine and thioridazine suppressed proliferation and induced apoptosis in cultured leukemic cells [79]. MB is known to bind mitochondria [55] to generate $^1O_2$ and radicals that induce damages in cytochrome *c* [80] and there are some reports in the literature showing that a methylene blue derivative (MBD)-induced apoptosis in several cell lines

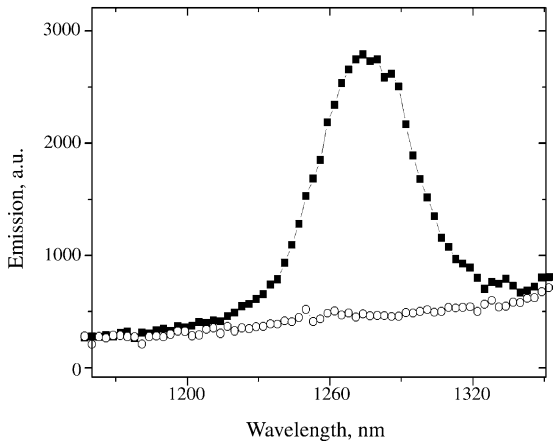


Figure 3 Emission spectra of singlet oxygen in Hela cells with (■) and without (◯) MB adsorbed. 10^6 Hela cells/mL suspension was treated with 50 μM MB in PBS buffer for 2 h. The cells were washed twice with cold PBS buffer and re-suspended in cold D_2O and measured instantaneously. The system for the measurement of singlet oxygen emission is the same as that used in Refs. [20,21,55]. It is basically a NIR fluorimeter from Edinburgh and the light excitation is provided by a Nd:YAG laser from *Continuum* (Surelite III).

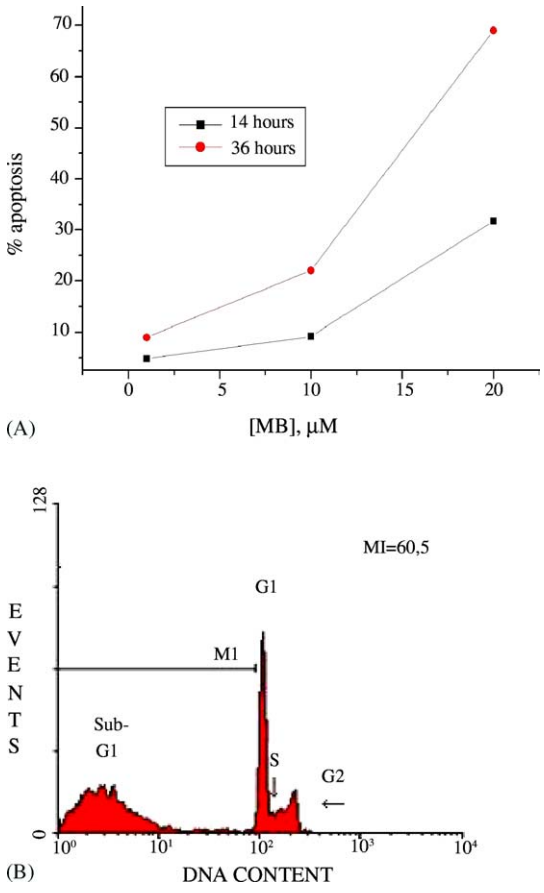


Figure 4 (A) Percentage of apoptose response as a function of MB concentration at different times after illumination. (B) Fluorescence of Propidium Iodide bound to DNA in He-La cells. The peak of sub-diploid DNA characteristic of apoptose, M1, marker one = sub G1. MI is the percentage of events in the M1 regions, i.e., sub-diploid DNA. The regions above M1 shows the characteristic peaks of the cell cycles G1, S, G2. Cells were treated with 20 mM MB and irraditated with 532 nm laser light 1.5 J/cm^2.

[67,71,81]. However, the structures of both MBD and PN are very different from the MB structure. In fact, the mechanism of cell death induced by MB itself is not yet well established. In order to test whether or not MB and proper illumination induced apoptosis, Hela cells were incubated with MB and illuminated with 634 nm diode laser. The presence of sub-apoptotic DNA fragments were detected by iodide propidium fluorescence in a flow cytometry equipment, as it can be clearly observed in Fig. 4B. MI reflects the percentage of sub-diploid nucleus, which is related with apoptosis [82]. 60.5% of cell death occurred by apoptosis. The percentage of apoptosis is proportional to MB concentration and to the incubation time after irradiation, as shown in Fig. 4A. Testing the experimental conditions that favor apopotic cell death by MB plus light excitation is under way in our laboratory.

Virus, bacterial and fungi

Given the rise of bacterial and fungal resistance, developing new antimicrobial therapies to treat infection is of great necessity to the medical community. Controlling microbial growth in living organisms as well as in blood components are interesting applications of PDT, and will certainly become increasingly important as the costs of PDT treatments decrease [83,84]. As early as in 1930s, it was shown that phages and viruses stained with phenothiazinium dyes were killed by light illumination, and in the early 1960s quantitative studies of photodynamic inactivation of bacteria and viruses were reported [2,85—87].

Lee et al. [88] have studied the photoinactivation of Qβ bacteriophage as a function of MB and oxygen concentrations. The dependence of bacteriophage killing efficiency on MB concentration follows a hyperbolic saturation isotherm suggesting that MB binds the viruses before photoinactivating them. The efficiency of MB and MB derivatives to inactivate bacteriophage virus R17 in vitro in the presence of red blood cells has been shown to correlate mainly with the differential binding of the dye between the erythrocyte membrane and virus DNA. The most effective derivative being 1,9 dimethylmethylene blue [89]. MB and light is effective in the killing of herpes viruses (HSV 1) giving

rise to DNA damage and blocking DNA replication [90,91].

Another application of PDT that has received attention lately is its blood sterilizing potential [92]. MB and toluidine blue at low concentrations were active for photoinactivating HIV-1 viruses in fresh plasma [93]. Sensitive essays using polymerase chain reaction show that HIV-1 inactivation is due to destruction of its RNA [94].

The literature dealing of photoinactivation of bacteria with phenothiazinium dyes is extensive [95,83]. Wainwright et al. [96,97] have shown photobactericidal activity of MB against vancomycin-resistant *Enterococcus* ssp and against methicillin-resistant strains of *Staphylococcus aureus*.

Several studies of photodynamic action of MB on *Escherichia coli* have been performed [98]. Binding, photoinactivation efficiency, mutation induction and mechanism of action have been determined [99,100]. Small amounts of mutations have been observed after MB photoaction [101], which were smaller in strains altered to produce carotenoid pigments [102].

The mechanism of inactivation of bacteria by MB also seems to be a mixture of type I and type II processes, and the relative efficiency of each of them depends on the cell type and experimental conditions [97]. An example of type I process was reported by Martin and co-authors. They observed that the photodynamic action was initiated by the reduction of the photoexcited dye with NADH. Superoxide dismutase expression, which was induced during photodynamic treatment, caused a protection effect [103]. Ito and Kobayashi [37] reported the involvement of $^1O_2$ in the photoinactivation by using azide suppression of $^1O_2$ as an indicator. However, the azide effect may be related, at least partly, with the release of MB that was previously bound inside *Salmonella typhimurium* and *Saccharomyces cerevisiae* [104]. Consistent experimental evidence of the role of $^1O_2$ in PDT of bacterial photokilling was obtained by analyzing the damage induced in DNA of *S. typhimurium* by MB plus light. The damage profile in the bacterial DNA closes resembled those obtained by MB plus light in isolated DNA [105].

Fungi, including yeast, have also been subjected to phenothiazinium dye photodynamic treatment [106]. Toluidine blue photosensitized the inactivation of *S. cerevisae* cells. MB was able to sensitize *Candida albicans* to killing by low-power laser light [107]. MB was also able to kill dermatophytes fungi *Trichophyton mentagrophytes* and *Microsporum gypseum* [108]. Using seven different dermatophytes in vitro, Ouf et al. [109] tested the survival rate in the presence of MB, toluidine Blue-O and hematoporphyrin derivative using light excitation that mimic the sun. Excellent recovery rates were also observed using guinea pig infected with three different dermatophytes. MB in concentrations of 450 and 500 μg/mL plus light totally eradicated *C. albicans* from the oral cavity [110]. Hamblin and Hasan [111] reviewed recently the area of photoinactivation of microorganisms by PDT emphasizing the double selectivity (light and drug localization) and the fact that it works in multi-resistant strains and does not induce resistance.

Methylene blue in clinical practice

There are several studies considering the application of MB for the treatment of tumor tissues [9,12,17,18,112,113]. The roles of type I and type II photosensization processes in vivo is still not yet clear [19]. MB is selectively accumulated in certain tumors [114,115]. Although the mechanism of accumulation is not certain, it may be related with the larger affinity to negatively charged interfaces and to melanin. However, the main fault that hinders the widespread use of MB is that it does not stain as well as HPD derivatives most of the tumor tissues when it is delivered in intra-venous injections, probably because it is easily reduced in biological environments and it is also excreted far more rapidly [116].

A new therapeutic concept with locally applied MB into the tumor and subsequent radiation after 1 h without skin photosensitivity was proposed by Orth et al. [12,17,18]. Due to its photochemical and photobiological characteristics, widespread availability and ability to be excited with polychromatic light, we decided to extend this concept and test it in several types of cancerous and non-cancerous diseases. We developed an inexpensive light source called RL50® that emits light that covers the red spectrum region from 600 to 750 nm. The RL50® spectral radiance also overlaps with the absorbance spectrum of the MB solutions [9].

Based upon these considerations, after ethical approval of the FM-ABC Human Research Committee, we obtained informed consent from 10 patients with 17 superficial tumors that could not be treated systemically, and from several other individuals with dermatological infections. In all cases we employed PDT with MB and RL50®. PDT was applied in multiple sessions every week, every other week or once a month. No toxicity was observed with MB. Both itching and pain occurred according to patient's sensibility. Edema and redness could be observed just after irradiation. Some patients complained of burning when light was on, and this

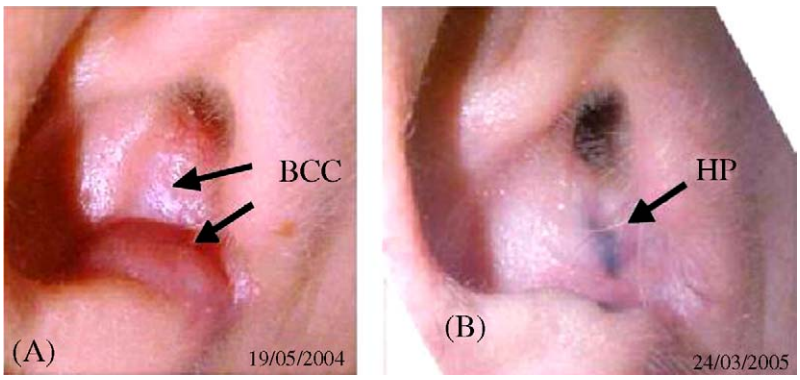


Figure 5 P.H.G.C., male, 37-year-old, with a basal cell carcinoma (BCC) in the external ear, (A) before and (B) after three MB PDT treatments with one month interval between each treatment. HP is hyperpigmentation.

symptom began typically after ~12 s of irradiation. Therefore, light dose was fractionated in 12 s intervals of light and dark. Curiously, Rueck et al. [117] concluded that fractionated light application with 15 s interruption intervals enhanced phototoxic response. We have always used light doses varying from 18 to 36 J/cm^2 and observed that 30 s is enough as the delay time between MB administration in the target tissue and light irradiation. There follows a small description of the cases treated with PDT using MB and RL50®.

Basal cell carcinoma

Basal cell carcinoma in difficult surgery locations is a clear indication for PDT treatment with MB and RL50®. The patient's complete response after three PDT sections can be observed in Fig. 5. Surgery should remove an extended area, which would probably be mutilating, needing posterior plastic surgery. If surgery were associated with radiotherapy there would be the risk of neurological damage in the internal ear. PDT avoids mutilation and there is no risk of neurological damage. The most frequent cosmetic problem is residual hyperpigmentation [2] due to MB accumulation after treatment (Fig. 5B).

Kaposi's sarcoma

Kaposi's sarcoma is caused by Herpes virus VIII, and it is the most prevalent malignancy in AIDS patients [118]. Although there are several clinical options, effective treatments are still the objects of conjecture [119—121]. Bernstein et al. [122] used PDT with Photofrin® and dye laser as treatment against Kaposi's sarcoma, with very good results (32.5% of complete results and 63% of partial response). Our patient J.A. had several lesions of Kaposi's sarcoma (Fig. 6A) and after five sections of chemotherapy presented no improvement. After several sections of PDT, complete response (negative biopsies were obtained) and excellent cosmetic result were obtained (Fig. 6B) [123].

Melanoma

Due to the difficulty in achieving good light excitation of porphyrin derivatives and due to ethical considerations concerning the aggressiveness of the disease, the application of PDT for treating melanoma lesions is still not developed [3]. Tests with ALA and HPD have shown relative low efficiency [3]. Some representatives of the new generation sensitizers, for example, a pheophorbide derivative as well as MB, show improved photodynamic action against melanoma in cell cultures [124,125]. Due to the characteristic of MB accumulating in melanoma and due to its favorable photochemical characteristics, it presents potential to be used to treat malignant melanoma. A patient with multiple skin melanoma lesions not deemed as a good surgical candidate received intratumoral injection of an aqueous solution of 2% MB with 2% lidocaine. Typically, 0.5 mL was injected. Thirty seconds after the injection, RL50® was applied at fluence of 18 J/cm^2 for 3 min for five sessions every other week. After the treatment, CR (disappearance of tumor) was observed in five out of six sites of tumors, and PR (at least 50% reduction in tumor volume) was observed at one site. A typical result is shown in Fig. 7. Few months after starting the treatment, clinical tumor remission was observed, and little or no scars remained. Therefore, by using the association of RL50® with MB injected locally, it is possible to efficiently treat relatively large melanoma lesions [9].

The conclusions obtained in our experience in treating cancer patients with PDT using MB and RL50® is shown in Table 1. Eighty-two per-

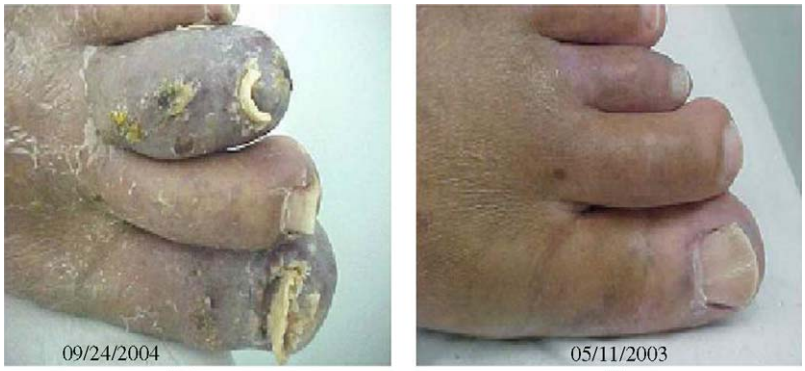


Figure 6 J.A., male, 40-year-old, with diagnosis of Kaposi's sarcoma, (A) before and (B) after 15 MB PDT treatments applied every other week. The photosensitizer solution contained 2% MB and 2% Toluidine Blue.

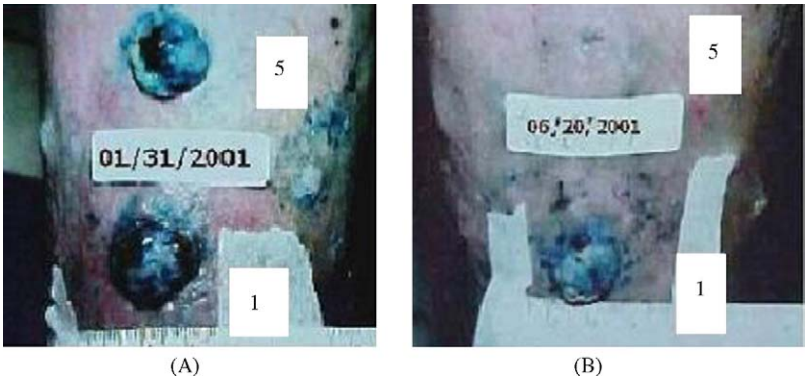


Figure 7 General view of two of the six treated lesions of a 92-year-old patient from ABC Foundations School of Medicine Clinics (FM-ABC) (A) before and (B) after five PDT sections, applied every other week. The response of lesion 5 was classified as CR and the response of lesion 1 was classified as PR. The volume of the lesions was measured using the semi-sphere volume ($\pi d^3/3$, where d is the tumor diameter). It was taken from Ref. [9].

cent response rate was observed (64.70% complete response and 17.64% partial response) in six metastatic melanoma, six basal cell carcinoma, one squamous cell carcinoma, three breast carcinoma and one Kaposi's sarcoma (Table 1). The exact protocol used for treating cancer tissues is: (i) obtain pictures before treatment; (ii) intra-tumor injection of a mixture of 2% MB and 2% Lidocaine until tumor becomes dark blue (usually few milliliters, less than 5 mL); (iii) 30 s after injection RL50® is turned on and light irradiation is applied with final dose of 18 J/cm^2; (iv) seven days after the treatment, new pictures are taken and protocol is repeated at the same site if necessary or another site or both.

Table 1 Clinical results of tumors treated with PDT using MB and RL50®.

Type	Number of patients or sites	Response		
		CR	PR	NR
Metastatic melanoma	6	5	1	
Basal cell carcinoma	6	5	1	
Squamous cell	1			1
Breast cancer	3		1	2
Kaposi's sarcoma	1	1		
Total	17	11	3	3

PDT with methylene blue in dermatology of non-cancerous diseases

The expanding use of this relatively new therapeutic modality in dermatology at many centers around the world has revealed the PDT efficacy for the treatment of cutaneous pre-cancer and cancer, as well as selected benign skin disorders [2]. In their article, Kalka et al. [2] discuss the application of PDT in dermatology of cancerous

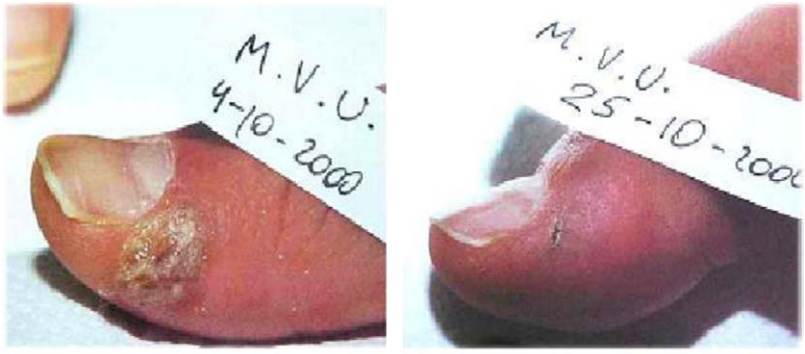


Figure 8 M.V.U., male, 31-year-old, with verrucae vulgares on the finger, (A) before and (B) 21 days after one MB PDT session.

and non-cancerous diseases. The treatment of psoriasis, viral diseases like HSV 1 and 2, human papillomavirus-induced lesions: laryngeal papillomatosis, verrucae vulgares and condylomata acuminate were reviewed and discussed [2]. Although there are several alternative treatments, i.e., acid, local surgery removal and CO_2 laser, the frequency of recurrence is smaller in the case of PDT [2]. Here, we show similar results observed when treating recalcitrant common warts with MB solution injected intradermal under the lesion and illumination with RL50® just after dye injection. Usually the warts vanish after three weeks (Fig. 8). Around 20 lesions were treated without any recurrence. We also have had the opportunity to treat HSV 1 using topical MB solution and irradiation with RL50® (data not shown). The results are excellent: no adverse effects and no toxicity, fast pain relief, shorter healing time and decreased recurrence rates. The clinical protocol used to treat papillomatosis is the same as the protocol used for treating cancer tissues. In the case of treating Herpes simplex, instead of injecting the MB solution, blisters are ruptured with a sterile needle (26 G (1/2)) and 2% MB solution is applied topically with a swab.

Onychomycosis

Onychomycosis are becoming more prevalent especially in immune-suppressed patients and are of difficult treatment Fig. 9 [126]. Topical and oral medications are possible. The treatments are long and the oral medications are hepatotoxic with less than 80% complete response [126]. PDT has the potential to become new treatment approach with smaller toxicity [2,127]. The data in Fig. 7 show a typical result obtained with MB PDT and excitation with RL50®. We have so far treated more than 60 patients using this protocol with complete response. An ongoing trial of PDT using MB and RL50® in onychomycosis with fungus culture at ABC Medicine School aims to compare different responses of PDT among different species of der-

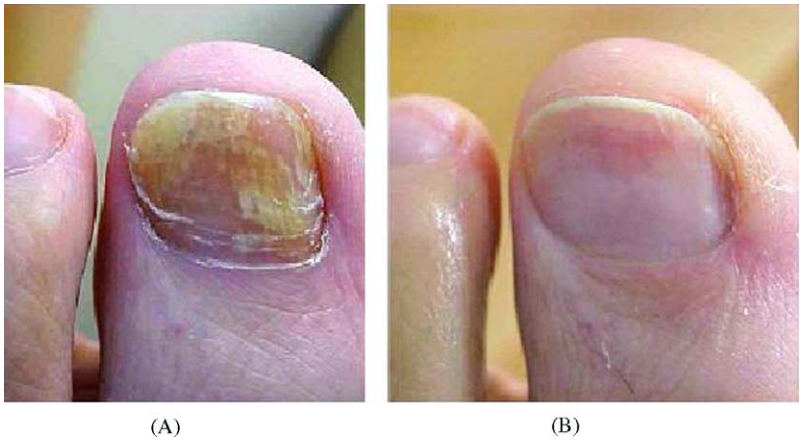


Figure 9 G.F.E., male, 59-year-old, with onychomycosis, (A) before and (B) after four (once a month) topical PDT treatments with MB and RL50®.

matophytes. The exact protocol used to treat onychomycosis is: (i) take pictures before treatment; (ii) clean the nail bed after scraping the nail surface with a sharp curette; (iii) send material for diagnosis; (iv) drip 2% MB dissolved in acetone between nail plate and nail bed; (v) apply light irradiation with RL 50 with final dose of 18 J/cm^2; and (vi) take pictures and repeat the protocol after 30 days if necessary.

Conclusion

MB has interesting characteristics conferring to this molecule a great potential for application in PDT. It absorbs light intensively in the therapeutic window; it has a well characterized and effective photochemistry that triggers both photosensitization mechanisms type I and type II; it damages biomolecules and efficiently induces death in several target cells, tissues and organisms. Therefore, upon irradiation it can be used to treat a variety of cancerous and non-cancerous diseases. We show that when used together with the RL-50® it treats efficiently several types of cancer, virus and fungi infections.

During the last 30 years most of the PDT protocols were based on the use of hematoporphyrin and its derivatives, irradiated with lasers or very sophisticated non-coherent light sources, turning the method restricted to some reference centers. The combination of MB and RL50® provides a new PDT protocol that is inexpensive, safe and efficient. Therefore, we can conclude that there is a great potential for the widespread use of PDT.

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