Chapter 17

Treatment and Control of Leishmaniasis Using Photodynamic Therapy

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Additional information is available at the end of the chapter

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1. Introduction

Leishmaniasis is a chronic disease affecting the skin, mucosal and/or internal organs, caused by flagellate protozoa *Leishmania* of the *Trypanosomatidae* family. [1] It is among the six most important disease in terms of its impact in public health. The world incidence of leishmaniasis is very large with about half a million new cases per year. About 12 million people are infected with *Leishmania ssp* parasites worldwide. New treatment alternatives are highly needed. Our goal here is to critically revise the literature in order to show the potential of Photodynamic Therapy in the treatment and comprehensive control of this disease. We have separated this chapter in nine sections, besides this brief introduction, which are: Leishmaniasis: Background and treatment strategies; Mechanisms in Photodynamic Therapy; Treatment of animals infected with leishmaniasis using PDT; Vector control using PDT; PDT alternatives for Blood purification; PDT on the treatment of Old World Tegumentary Leishmaniasis; PDT - *In vitro* tests in species that cause Tegumentary Leishmaniasis; Conclusions; References.

2. Leishmaniasis — Background and treatment strategies

There are two main forms of leishmaniasis, visceral (VL) and tegumentary (TL) leishmaniasis, which are also respectively called Kala Azar and Bauru ulcer. The later, received its name because of the original high prevalence in Bauru, a city in the countryside of the State of São

Paulo, in Brazil. The tegumentary leishmaniasis is characterized by skin lesions (cutaneous-CL) and mucocutaneous lesions (such as, nasal and mouth regions) [2].

Leishmaniasis is a common zoonosis, with domestic (dogs and cats) and wild (rodents, marsupials, edentulous and wild canids) reservoirs. It is transmitted to humans by sand flies, which comprise the genus Lutzomyia and Phlebotomus. Details of the etiology and pathophysiology of the disease are out of the scope of this chapter and we suggest that the reader consult reviews that focus on these subjects [3].

The current scenario of leishmaniasis treatment is not promising. Therapeutic approaches include systemic administrations of anti-parasitic medications, which often present serious side effects. Few drugs are available in the clinic, mainly antimonials and amphotericin, and the frequency of resistance development is rising. Therefore, there is an urgent need to establish new and more effective treatments for both VL and TL. The treatment of TL (the focus of this chapter) urges new drugs and new therapeutic forms, that allows for more effective and conveniently administered treatments [4].

One of the promising approaches, and the one discussed in here, is photodynamic therapy (PDT). The main expectation of this approach is that it treats lesions in a localized manner, without damaging healthy tissues [5]. The few reports that are available in the literature have validated this hypothesis. In addition, no sign of systemic toxicity is reported in PDT, eliminating one of the major health issues related to existing TL treatments.[6] These points will be further discussed in this chapter.

The use of light as a therapeutic modality has gained strong impulse recently due to the development of efficient and affordable light sources. Consequently, photo-activated drugs (PhotoSensitizers-PS) play key roles in the present clinical portfolio, and more importantly, are the major lead in the development of new drugs to treat a variety of diseases such as cancer, microbial infections and tropical diseases. However, increasing the efficiency of PDT photosensitizers remains challenging [7-9].

The use of PDT in veterinary is much less common even considering the benefits that such strategies could bring in the treatment of high-value reproducing animals, as well as, in the treatment of animals that are reservoirs of human diseases [10].

In terms of developing effective treatments against leishmaniasis in endemic areas, it is important to think of comprehensive strategies that could cause a quick decrease in the pool of infected patients (Figure 1). It is also important to emphasize that leishmaniasis is a neglected tropical disease and, therefore, it is highly relevant to consider low-cost strategies that would serve as an alternative for public medicine in poor countries [9]. Developing efficient clinical protocols that would cure/control the disease would not only favor the patient itself, but also, would decrease the chance of this infection being transmitted to others by the vectors or by blood transfusion. In the next sections, we will explain how PDT can be helpful in the treatment of patients, as well as, of all the possible reservoirs and transmitting vectors that would favor the parasite infection cycle (Figure 1). Some of this potential has been attained and some are still in the step of hypothesis testing.

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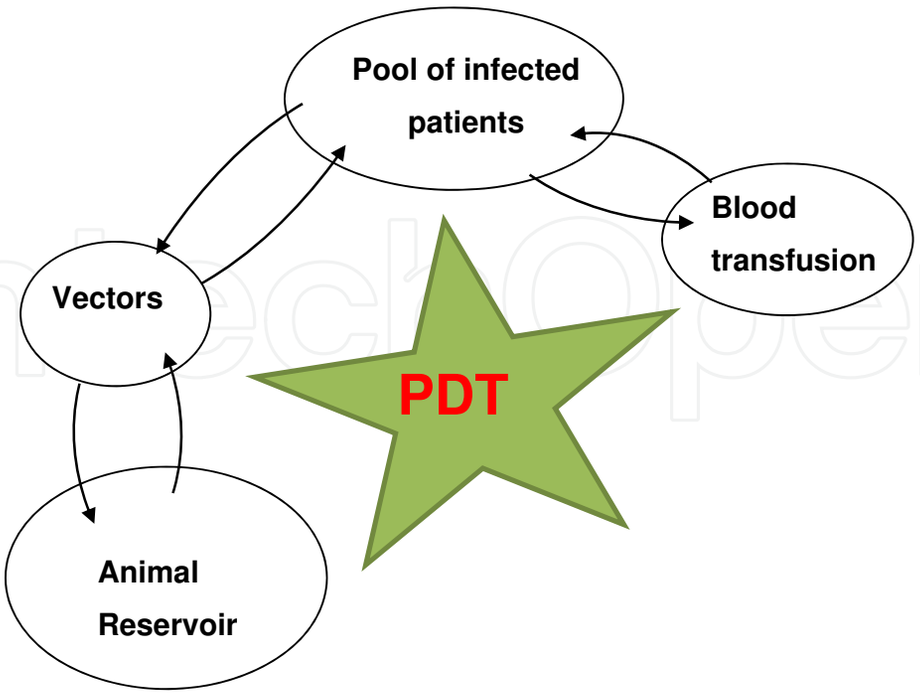

Figure 1. Schematic representation of a comprehensive strategy to control leishmania disease in endemic areas by using PDT. Besides treating patients and animals; killing vectors and disinfecting blood, should be considered in a PDT strategy to control leishmaniasis. The star represents the multi-target characteristic of the PDT strategy.

3. Mechanisms in photodynamic therapy

PDT is a clinical modality based on the damage caused in biological tissues or in infecting microorganisms by light-induced reactions, generically called photosensitization reactions. Photosensitization occurs when PS absorb light and transfer its energy to neighboring molecules, such that light converts into chemical reactivity [11-13]. After the end of a photocycle, PS returns to the ground state and may absorb another photon. The photophysical step that allows the formation of an efficient PS is the intersystem crossing (ICS), that converts singlet into triplet species, which are long lived and highly reactive (Figure 2) [13].

The photooxidation of biomolecules is responsible for changes in their structure and function. It can occur by two main mechanisms: electron transfer reaction (excited states are stronger oxidizing and reducing species than their respective ground states) catalyzing the formation of various radical species, including the highly reactive hydroxyl radical. These reactions are classified as type I. The photooxidation can also occur through energy transfer with molecular oxygen, catalyzing the formation of singlet oxygen, a mechanism called type II (Figure 2) [14].

It is considered that type II mechanism is the most relevant effector of photooxidation, because type I reactions usually lead to PS degradation [15]. However, in biological systems, there usually is shifts between these two mechanisms (type I versus type II), for several reasons,

including local concentrations of oxygen and of reducing species, interaction of PS with other biomolecules and PS aggregation [17-21].

Free radicals and singlet oxygen have different reactivity towards biological targets, but both can react with them [14,22]. Singlet oxygen mainly reacts by addition to double bonds (Figure 2). The efficiency of photo-induced cell killing seems to depend more on the amount of PS that is located in the intracellular environment and on the specific intracellular location than on the *in-vitro* photophysical efficiency of the PS [23-28].

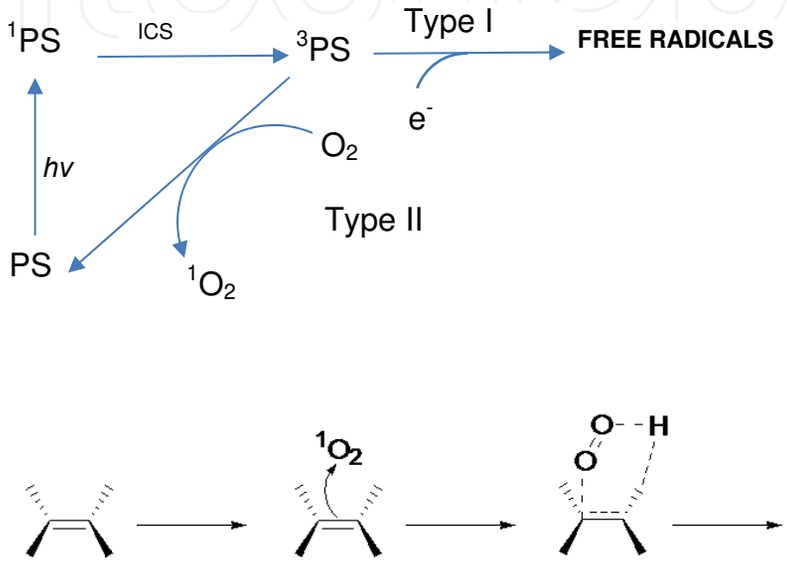

Figure 2. Top scheme. Main mechanisms of photooxidation. PS, ^{1}PS, ^{3}PS: photosensitizer ground state, singlet and triplet species, respectively. O_2 and $^1O_2$ correspond to oxygen in the ground state and the singlet excited state, respectively. *hv* represents light absorption at a specific wavelength and ICS is intersystem crossing between the singlet and the triplet states. Bottom scheme: Reaction of singlet oxygen with a double bond forming a hydroperoxide, which is the main reaction of singlet oxygen with lipid double bonds.

PDT combines three components to kill cells (eukaryotic and prokaryotic) and non-cellular organisms such as virus: PS, light and oxygen. PS is applied either topically or systemically and it must incorporate in the biological tissue to be treated, which is exposed to light in the presence of oxygen. The PS needs to absorb efficiently the incident light and form triplet species [14]. There are hundreds of PS molecules that have been synthesized and tested. In Figure 3 we present the chemical structures of few that are worth commenting in this chapter, because they either have been involved on treatments of leishmania or have the potential to be. Methylene Blue (MB) and Crystal Violet (CV) are positively charged and low-cost photosensitizers that enter cells and react mainly by type II and type I mechanisms, respectively. MB has been used to treat several diseases including leishmania [27], while CV should be tested since it has a great potential as a positively dye that mainly accumulates in mitochondria [28]. Riboflavin (RF, vitamin B2), is a natural PS that absorbs in the 400-500 nm region and has been used for blood disinfection as well as in test-tube leishmania killing assays [29]. Hypericin is another natural PS that is extract from St. John's wort and has been used in several PDT studies [30]. ALA is the first compound in the porphyrin synthesis pathway. Protoporphyrin IX is

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formed intracellularly after the treatment with ALA and/or methyl ALA and is the most used PS in leishmaniasis treatment [31-35]. Chlorophyll is the main pigment of photosynthesis and their derivatives hold promising potential as low-cost PS [36].

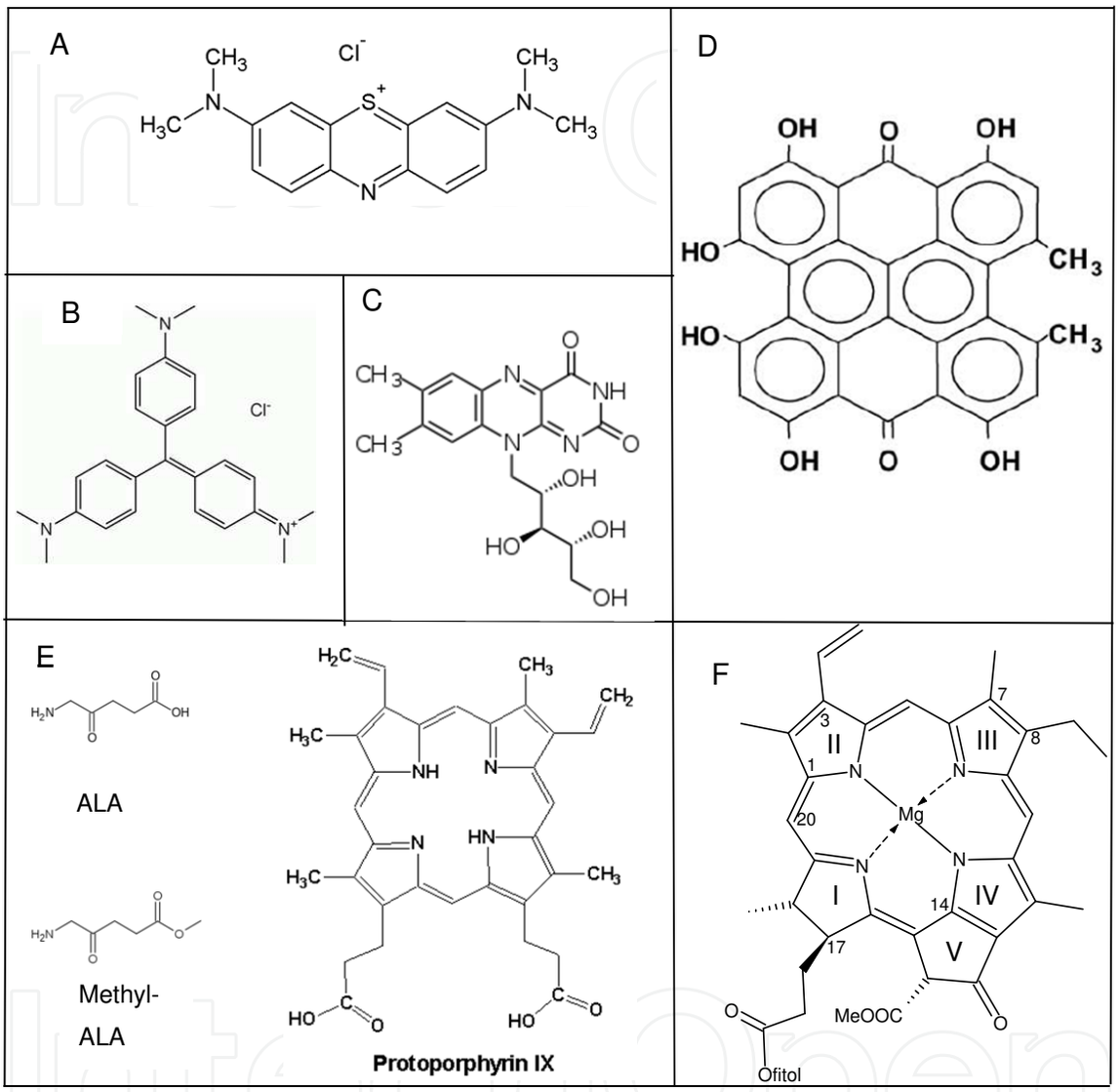

Figure 3. Molecular structure of relevant photosensitizers in PDT: (A) methylene blue; (B) crystal violet; (C) Riboflavin, (D) Hypericin; (E) ALA, Methl ALA and Protoporphyrin IX; (F) chlorophyll.

The ability of PDT to act as an anti-microbial treatment, i.e., to treat fungi, bacteria and virus infections, is well described in the scientific literature [37-39]. Many research groups have developed experiments that prove the effectiveness of this therapy for a large number of diseases, including certain parasitic diseases [40]. *In vitro* studies of photoinduced inactivation of parasites have been used to unravel important aspects of the therapy including, the action mechanisms, light dosimetry, structural-activity relationships, PS uptake and localization. PDT has been used in the treatment of human and experimental murine leishmaniasis of the Old and New Word. Despite the small number of cases related, literature highlights the ability

of PDT to deliver better results compared to traditional treatments, emphasizing its better effectiveness in leading to amastigote-free lesions in a shorter time periods, in addition to its excellent esthetic results.

4. Treatment of animals infected with leishmaniasis using PDT

PDT has emerged in the treatment of cutaneous diseases among human and different animal species [41]. Researchers have shown that PDT offers an effective alternative in the treatment of CL indicating that it also has a great clinical potential in the treatment of this disease within Veterinary Medicine [27]. The initial studies using PDT to treat leishmaniasis were performed in humans and are further described on section 7 [31-35]. Although some animals, especially mammals, constitute important reservoirs of the parasites, leishmaniasis also has clinical importance because some species can develop injuries, become sick and die due to the disease and its complications. Therefore, from this point of view, Veterinary Medicine has special interest, not only to control the disease epidemiology, but also to treat infected and sick animals.

The main vertebrate hosts (domestic and wild) described and classified as hosts of these protozoan through natural and/or experimental infections, are: foxes, opossums, armadillos, anteaters, sloths, rodents, cat, dog, goat, sheep, buffaloes, horses and primates [42-47]. While the treatment of infected animals provides possibilities for partial or total removal of cutaneous lesions, it is still not possible to guarantee the elimination of the infectious agents from the carrier animal, remaining the possibility that it remains as a host reservoir. Therefore, there is a great need to further investigate the treatment of domestic and wild animals with leishmaniasis, by using PDT.

Among all involved animals, the domestic dog and some rodents are the main sources of human infection in America and in the Middle East, respectively; therefore, being the majors urban reservoir hosts of leishmaniasis [44,48]. The proximity of this animal to humans complicates the disease control. The lack of identification of infected animals becomes a challenge, mainly due to the numerous generic clinical manifestations, and sometimes the absence of pathognomonic lesions in the dogs [49]. The skin disorders are quite common in animals, and include opaque hair coat, alopecia, depigmentation, hyperkeratosis of nasal plan and digital cushions, mucocutaneous ulcers, intradermal nodules, onychogryphosis and excessive flaking [50,51] but the most common presentation of the cutaneous disease is a symmetrical alopecia accompanied by intense flaking with silvery appearance that often starts on the head and spreads to other parts of the body [52]. However, these symptoms are sometimes not correlated with leishmaniasis. Regarding the condition of the dogs as reservoir hosts in the epidemiology of the disease, clinical treatment is not recommended so far [51], making euthanasia of the infected animals mandatory in many countries [50] and keeping the controversial discussion among public health authorities, animal protectors and veterinarians [53,54]. Despite the importance of dogs in the epidemiology of the disease, the most used animal model and the one that has shown success in the treatment of the cutaneous disease are rodents, mainly mice and hamsters.

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Several studies demonstrated the possibility of using PDT in animal models, especially on murines. In 2007 *Akilov et al.* reported an evaluation of the use of ALA (precursor of PpIX) in TL caused by Old World species in ears of Balb/c mice [55]. *Akilov et al*. also highlighted the action of ALA-PDT in murine with leishmaniasis compared to a control group treated with ALA [56]. The results showed a significant reduction of 24.5 folds in the parasite load compared with the control group. Nevertheless, they observed vascular damage in ears of the PDT-ALA group probably caused by PDT. According to the authors, a wide inflammatory and immunologic response was noted in Balb/c ears of ALA-PDT group, which correlated with the expressive decrease of parasite load and with the healing of the tissue.

Despite ALA, other classes of photosensitizers already widely used in PDT began to be tested. The phenothiazine 3,7-bis(di-n-butylamino)phenothiazin-5-ium bromide (PPA904) was tested by *Akilov et al.* in mice [57]. Ears of female Balb/c were infected with metacyclic parasites of *Leishmania* sp. Following infection, mice were treated with PPA904 cream and irradiated with a broad band light source. They tested the PS concentration, time of uptake and absorption site in the ear. The results showed that PPA904 applied during at least 90 min in consecutive sessions of PDT decreased parasite load around 5.2 log compared to the controls groups. However, PPA904 application also lead to skin irritation. Another study was carried out with female Balb/c infected with *L. major* parasites expressing green fluorescent protein (GFP) to monitor the parasitic load and the efficacy of PDT [58]. PPA904 was applied in the ears of the mice and the parasitic load was compared with control group (only infected). The fluorescence of GFP parasite in the ear of mice after the PPA-PDT decrease significantly, about 80%, compared to control group. The authors emphasized that this result was obtained after more than one PDT session.

*Peloi et al.*chosen a different murine, which is also considered an appropriate model to develop leishmaniasis caused by some New World *Leishmania* spp. Hamsters were used to investigate the effectiveness of PDT with methylene blue (MB) photosensitizer [59]. A light-emitting diode (LED) was chosen as light source. The footpads of hamsters were infected with *Leishmania* sp. The control presented an increase in thickness throughout the treatment. An opposite reaction occurred in the group A and B treated with oil/water lotion MB+LED and aqueous solution MB+LED, respectively. Statistically significant reductions on the thickness of the footpad and parasitic load were observed.

The scientific reports in PDT-treated animal models mentioned in this chapter show similar results to those reported in humans. In other words, PDT is capable to treat infected wounds reducing the parasitic load. In some cases, the complete disappearance of the parasite from tissue is achieved. Other aspect to highlight is its ability to inactivate both Old and New world *Leishmania* spp. Details of parameters from scientific studies using PDT on Old World and New World TL in murine models are described in table 1. However, treatment conditions of infected animals out of experimental controlled environment have not been described. Therefore, PDT has to become a more common procedure to be used in the clinical practice of Veterinary Medicine. It certainly has the unfulfilled potential to become a therapeutic alternative in veterinary medicine, and to help controlling the parasitic cycle in humans.

Author/year	Parasite	Animal	Photosensitizer	Uptake	Light source	Exposure time	wavelength	Irradiance	Fluence	Results
Akilov *et al*. 2007	*L. major*	BALB/c mice (age: 6–8 weeks) - ear	ALA (20%)	4 hours	Diode laser	#	635 nm	66 mW/cm2	50 J/cm2	1) Significant reduction in the parasitic load 2) Large tissue damage 3) Decrease of macrophages, increased levels of interleukin 6 in tissue
Akilov *et al*. 2009	*L. major*	BALB/c mice (age: 6–8 weeks) - ear	PPA 904 - 3,7-bis(N,N-dibutylamino) phenothiazinium bromide	30, 60 ,90 e 120 minutes	LC-122 non-coherent light source + broadband optical filte + fibra otica (665 ±15 nm)	#	665 ±15 nm at	50 mW/cm2	50 J/cm2 (50mW/cm2).	1) Best result of the treatment and cure in 90 min 2) Penetration of the PPA is dependent on time 3) Decreased parasite load and ulcer with 4 days of treatment
Latorre - Esteves *et al*. 2010	*L. major GFP*	BALB/c mice (age: 6–8 weeks) - ear	PPA 904 - 3,7-bis(N,N-dibutylamino) phenothiazinium bromide	60 minutes	LC-122A,non-coherent light source +fiber optic (665 ±15 nm)	11,6 minutes	665 ± 15 nm	30 mW/cm2	21 J/cm2	1) Significant reduction in parasite load in the ear of the animal compared to controls 2) After 4 days of PDT treatment appeared more effective 3) Decrease of parasites in limiting dilution
Peloi *et al*.2011	*L. amazonensis*	Hamsters - footpad	CG - lotion MB and water+MB GA -oil/water (O/W) lotion 10 nM AM GB - aqueous solution 10nM AM	10 minutes	LED light system using 6 units (in series) that emit red light (663 nm)	1 hour	665 nm	5 mW/cm2	12 J/cm2	1) Significant reduction in parasite load in groups A and B 2) After 6 weeks of treatment absence of lesions in the GA and presence in 13.3% in the animals in GB 3) Decrease the size of the footpat in the group A and B compared to control

CG: control group / GA: group A / GB: group B

Table 1. Parameters used in PDT to the treatment of Old World tegumentary leishmaniasis and New World tegumentary leishmaniasis in murine models

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5. Vector control using PDT

The field of insect photo-killing by administration of photosensitizer molecules and light exposition (usually sun light) is one of the areas of possible PDT application that has received small attention of the scientific community [60-63]. The few studies, which were mainly reported by Jori and co-authors, sustain that there is indeed great potential on this area. There are reports showing that the PS activity is a function of its log P_{OW} value and of its amphiphilic character [62,63]. PDT was also shown to be efficient for Larva control of dengue vector *Aedes aegypti* [60]. However, there is no scientific report on the use of PDT to control the vector (*Phlebotomus* sand flies) and its larva, which are responsible for the transmission the leishmania parasites. It is also important to emphasize that the amount of information available concerning larva development of phlebotomine sand flies is much less than what is known for the mosquitoes whose control have been studied by PDT. Nevertheless, for the matter of bringing new ideas to the field of *Leishmania* treatment, the concentration of photosensitizers that are needed to neutralize larva and to kill those mosquitoes is several orders of magnitude smaller than the concentrations of chemical insecticides, which are currently used for vectors control, causing great disturbance in the whole ecosystem. Therefore, it is up to our community to develop and test strategies to control vectors of *Leishmania* parasites using PDT.

6. Blood purification

The purification of blood products is critical to avoid disease transmittance through blood transfusion. Although this is not the main route of transmission of leishmaniasis, it is a possible one, and cases have been reported in the literature [64]. The focus of the disinfection strategy is to kill microorganisms without harming the cellular and plasma components. PDT offers great potential to be successful in blood disinfection, because it is a multi–target strategy, i.e, the reactive species that are formed (after light absorption and photosensitization reaction) are effective against viruses, bacteria, fungi, and parasites [37-40]. This strategy has even been proved effective to promote pathogen inactivation in the presence of fragile blood components, such as stem cells from blood of embryo's cord [65-68]. It is better than UV treatments, because it does not cause direct damage to blood components. Several PS have been used for blood disinfection including MB, CV and RF (Figure 2). Molecules that have intracellular targets such as MB and CV can be used to treat plasma derivatives but not whole cell blood, because they will cause extensive hemolysis. RF, however, is an aqueous based photosensitiser, which do not enter cells and can be used to disinfect whole blood derivatives. RF reacts either by type I or by type II mechanisms and is already in use. Several companies commercialize kits for blood and plasma decontamination, like Macopharma, whose technology for plasma decontamination is based on MB photosensitization (http://www.macopharmausa.com/). In the case of leishmaniasis, parasites remain mostly in the intracellular environment, except when they are in transit from a lysed cell to infect a macrophage or other phagocytic cell. We could think of using PDT to remove parasites in the plasma or to develop strategies to target PSs to destroy only infected cells of contaminated blood.

7. Photodynamic therapy on the treatment of old world tegumentary Leishmaniasis

There are several reports on the literature dealing with the treatment of leishmaniasis by PDT [5,6,33,34]. The first report was conducted by Enk's group in 2003 [5,6]. Both studies reported the use ALA and MAL, combined with red light. These authors performed the treatment of 32 TL lesions from 11 Israeli patients. The diagnostic was accomplished by verifying the amastigote presence in direct smear from the lesions [5]. This work showed that about 96% of the lesions healed, leaving some mild scars and pigment in place of the old lesions. Just one lesion presented amastigotes forms after PDT. Gardlo *et al.* published the case of a patient, aged 34, with CL confirmed by histology. According to the authors, the patient developed resistance to the treatment with sodium stibogluconate and presented 10 lesions, which were treated five times with PDT and five times with paromomycin sulfate ointment [6]. The result obtained is similar to the previous work and showed that the five ulcers treated with PDT healed without signs of amastigotes, while two ulcers treated with paromomycin partially responded to the drug, one of them did not respond and two lesions were shown to have no amastigotes. The ulcers that did not responded to paromomycin ointment were subsequently treated with PDT successfully.

Asilian and Davami developed a placebo-controlled, randomized clinical trial that provided definitive evidence of the efficacy of PDT in the treatment of CL [34]. 60 patients with confirmed CL by clinical and parasitological diagnosis were separated in 3 groups with different treatments. Group 1 was treated with PDT once a week, group 2 received twice daily paramomycin plus methylbenzethonium chloride ointment and in group 3 was used a paraffin-based ointment without active ingredients with same application time of the group 2. During four weeks, the groups received the treatments described above. At the end of the study healing was present in 93.5% of the patients of group 1, 41.2% of group 2 and 13.3% of group 3. At the same time, 100%, 64.7% and 20% of the lesions had parasitological cure in group 1, 2 and 3, respectively.

Other studies accomplished in Iran and German corroborated with the results described above. According to the authors, PDT showed to have the capacity to treat wounds caused by Old Word *Leishmania* species. We emphasize that most of the reports claim that this therapeutical modality can achieve results above 90% healing of wounds, however, a caveat must be held since some of these studies indicate that not all healed wounds become free of parasite [35,56]. The mechanism of ALA PDT in the case of leishmaniasis was shown to be due to the killing of infected host-cell killing (macrophages) instead of direct parasite killing (see further discussion about this issue on section 8).

One CL case of the New World leishmaniasis is described in the literature. Song *et al.* reported the case of a Brazilian patient presenting cutaneous leishmaniasis confirmed by smear stained by Giemsa. PDT was carried out using MB. In this specific case because of ethical concerns of possible development of evolution to mucocutaneou disease, the patient received at the same time a low dose of pentavalent antimony and PDT. The patient had two ulcers. One receive PDT and the other was only being treated with the low-dose pentavalent [27]. The treatment

showed 100% of cure in both lesions, but the lesion treated with PDT presented a faster wound recovery compared to the antimony alone (Table 2).

Author/year	Parasite	Photosensitizer	Uptake	Light source	wavelength	Irradiance	Fluence	Treatment sessions	Frequency	Patientes	Results
Enk *et al.* 2003	*L. major*	ALA*	4h	Curelight, Photocure	570-670 nm	150 mW/cm²	100 J/cm²	once weekly	until parasite was not detectable in the direct smear	11	96% cure 1 patiente with 1 lesion presented parasite
Gardlo *et al.* 2003	*Suspeita L. donovani*	MAL	5h	Curelight, Photocure	570-670 nm	150 mW/cm²	75 J/cm²	1º Twice weekly (12 weeks) + 2º once weekly (4 weeks)	1º 12 weeks / 2º 4 weeks	1	100% cure
Asilian et al. 2006	*L. major*	ALA	4h	Omnilux(visible red light)	633 nm	#	100 J/cm²	once weekly	4 weeks	20	93.5% cure
Ghaffarifar *et al.* 2006	*L. major*	ALA	4h	Red light	570-670 nm	150 mW/cm²	100 J/cm²	once weekly	4 weeks	5	100% cure
Sohl *et al.* 2007	*L. tropica*	MAL	3h	Waldman PDT 1200L	580-700 nm	#	100 J/cm²	once weekly	1 to 4 weeks	1	100% cure
Song *et al.* 2011	*L. amazonensis*	0.5 % Methilene Blue	#	RL 50 - LED	570-750 nm	35 mW/cm²	20 J/cm²	once weekly	4 weeks	1	100% cure

* First compound in the porphyrin synthesis pathway, precursor of Protoporphyrin IX (PpIX)

Table 2. Parameters used in PDT on the treatment of Old World and New World tegumentary leishmaniasis in humans

This brief account of the use of PDT for the treatment of CL demonstrates the ability of this therapeutic modality and encourages its use. It also stimulates research in the pursuit of new protocols with new PS, which could ensure not only healing but also clinical and parasitological cure of these patients.

Details of parameters from scientific studies using PDT on the treatment of Old World and New World tegumentary leishmaniasis in humans are described in Table 2.

8. Photodynamic therapy — *In vitro* tests in species which cause Tegumentary Leishmaniasis

The effectiveness of PDT on CL treatment was first conducted in humans and in animal models. *In vitro* tests began less than ten years ago to allow testing of PDT parameters like the efficiency of different types of photosensitizers, their respective uptakes and concentrations and accumulation sites.

Sujoy Dutta *et al*. began *in vitro* studies with the New World specie, *L. amazonensis* in 2005 [69]. The first part of that work evaluated *Leishmania* transfectants expressing GFPs. The PS tested was aluminum phthalocyanine chloride (AlPhCl) in different concentrations. The principal factor tested was the light-mediated cytolysis when cells were in the presence or pre-incubated with the AlPhCl. In the dark there was no phototoxicity for both promastigote and amastigote forms of the parasite. The opposite effect occurred when the photosensitizer received red light illumination, showing that promastigotes appear to be more sensible than amastigote forms. In addition, the loss of fluorescence of the GFP parasites indicated cell death. On the second part of the study, J774 cells (cell line immortalized murine Balb⁄ c monocyte ⁄ macrophage) were tested at the same conditions reported above. The authors observed that they were 10-20 fold more resistant than promatigotes. According to the authors, the photosensitized *Leishmania* cells are susceptible to cytolysis, probably due to the generation of reactive oxidative species after illumination, an indicative of inefficiency of their antioxidant mechanisms. ALA did not induce protoporphyrin IX (PpIX) production in the *Leishmania* cells, because of a deficiency in the heme biosynthetic pathway in this parasite [57, 70].

Tests with other phthalocyanines were developed by Pinto *et al.* using species of Old and New world *Leishmanias*, *L. major* and *L. braziliensis*. The parasites were incubated with aluminum phthalocyanine tetrasulfonate (AlPcS4) at different concentrations and irradiated with a GaAlAs diode laser (λ= 659 nm, 40 mW). The experiments indicated a significant reduction of viable parasites in both species compared to controls, however *L. braziliensis* demonstrated higher mortality than *L. major* [71].

In Brazil, Song *et al*. performed tests to understand mechanism of action of PDT using MB in a case report. Promastigotes of *L. amazonensis* were incubated with different concentrations of MB, washed with PBS and illuminated using a home-built LED light source with a wavelength of maximum emission at λ= 650 nm. After the irradiation, cell survival was determined with MTT assay that detected cell toxicity after irradiation of light in the presence MB. There was

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an increase in the phototoxicity with the increase in the MB concentration indicating a concentration-dependent response [27]. Differently from PPIX induced by ALA and MAL, MB has parasite intracellular target. In fact, PS seems to be localized in mitochondria (Figure 4).

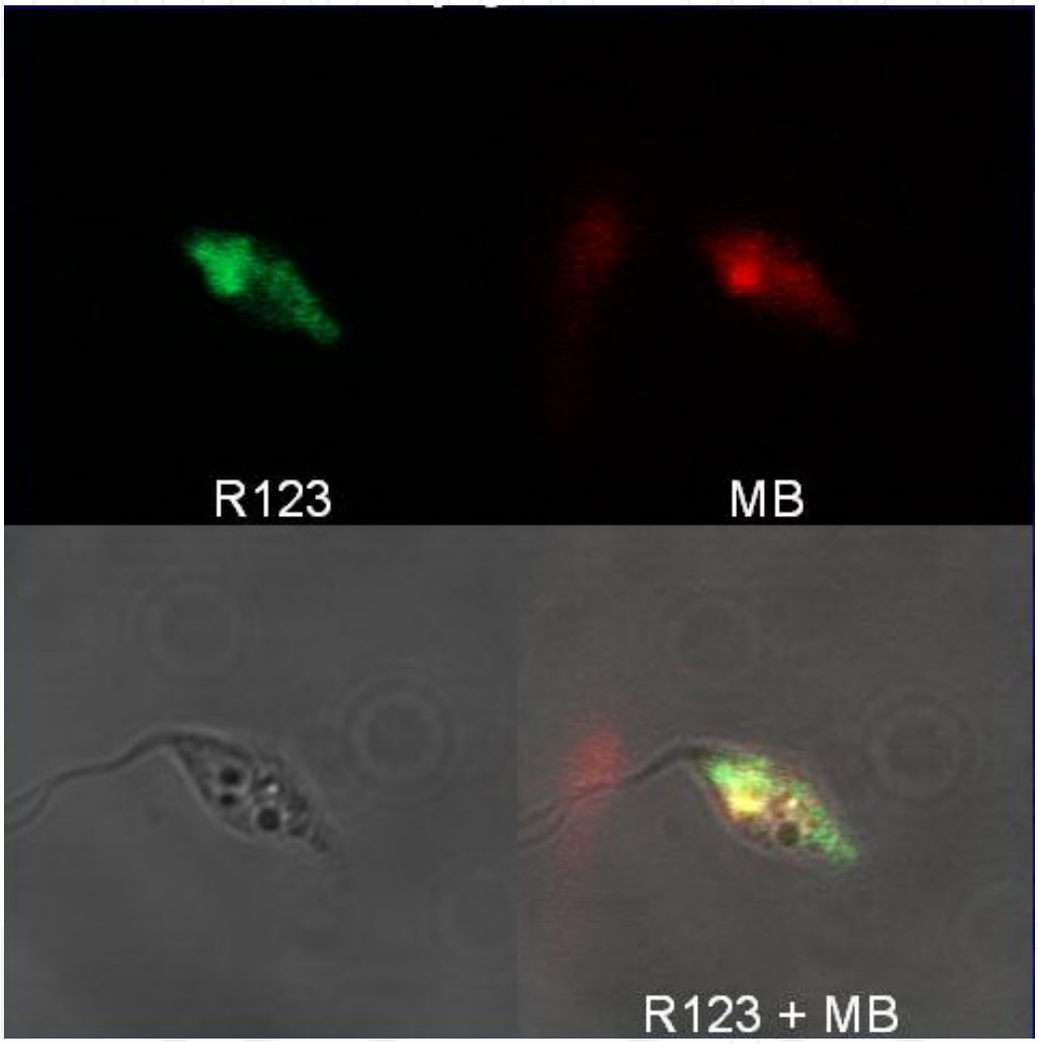

Figure 4. Top: Rhodamine 123 and Methylene blue fluorescences in promastigote parasites of *L. amazonensis.* Bottom: transmission image and colocalization of R123, MB and transmission images.

Other researchers have investigated the susceptibility of *L. amazonensis* regarding PDT. In order to verify the lethality of phenothiazine's derivatives on the promastigote forms, Barbosa *et al* [72] tested TBO (toluidine blue O), MB and a TBO/MB solutions. Irradiation was performed with a diode laser (λ= 660 nm, P= 40 mW). They tested different PS incubation time (5 and 60 min) and two energy densities (4.2 and 8.4 J/cm^2). The results showed a representative decrease

on the viability of *L.amazonensis* promastigotes for all treated groups in comparison to their controls. The authors did not find statistical differences between the dyes, but reported that the best result was observed with TBO.

Dutta *et al*. published an article that described the use of a combination of photosensitizers. Uroporphyrin (URO1) and aluminum phthalocyanine chloride (AlPhCl) were used in uroporphyrinogenic mutants of *L. amazonensis* (RAT/ BA/ 74 /LV78) 12-1 clone, transfected with pX-alad and p6,5-PBGD [73]. This transfected *Leishmania* is able to absorb ALA and turns it into URO 1. The authors evaluated the combination of both drugs into promastigotes with and without irradiation of red light. Results showed photolysis of the irradiated parasites with both photosensitizers whereas non-irradiated parasites showed no damage.

Hernández *et al*. published another study that compared encapsulated chloroaluminum phthalocyanine (CLAlPc) in liposomes (UDL-CLAlPc) and free in solution. The experiments were conducted with two species of New World *Leishmania* in promastigote and amastigote forms and in THP1 cells. The experiments tried to verify the ability of the photosensitizer in reaching the *Leishmania* inside THP1 host cell. According to the authors, the UDL-ClAlPc photosensitizer was almost 10 times more photoactive than free ClAlPc on THP-1 cells as well as on promastigotes and with intracellular amastigotes of *L. chagasi* and *L. panamensis* [74].

9. Conclusions

- First reports of cutaneous leishmaniasis using PDT were performed in humans;
- Treatments using porphyrin precursors, ALA and MAL, showed positive results on the cure of patients with CL;
- The low-cost phenothiazine methylene blue and red light can be used to treat patients with CL;
- More than one PDT session is necessary to achieve wound healing.
- Both New and Old World *Leishmania* can be treated with PDT.
- Murine models of infection such as Balb/c and hamster show to be appropriate for PDT studies of CL treatment.
- *In vitro* tests demonstrate that Old and New world *Leishmania* species can be used to test new photosensitizers and to establish structure/activity relationships.
- PDT also has the potential to control leishmaniasis transmission by the treatment of vectors and infected animal reservoirs, although the development of these potentials will need further investigation.

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