Bioorganic & Medicinal Chemistry 16 (2008) 7033–7038

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Carbaporphyrin ketals as potential agents for a new photodynamic therapy treatment of leishmaniasis

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ARTICLE INFO

Article history:
Received 19 December 2007
Revised 9 May 2008
Accepted 14 May 2008
Available online 20 May 2008

Keywords:
Leishmania
EPR
Photodynamic therapy
Carbaporphyrin ketal
Reactive oxygen species

ABSTRACT

Dimethyl and diethyl carbaporphyrin ketals inhibit the growth of *Leishmania tarentolae* promastigotes in vitro. The concentration dependency of the inhibitory effect was tested using the MTT assay. The presence of reactive oxygen species, such as singlet oxygen and superoxide, was detected using electron paramagnetic resonance spectroscopy with selected spin traps and confocal microscopy in cultures exposed to these carbaporphyrin ketals. These unique porphyrinoids show promise as potent inhibitors of *Leishmania*.

1. Introduction

Leishmania spp. are protozoan parasites that infect a variety of hosts ranging from reptiles to humans leading to leishmaniasis, a disease that affects the skin, mucosa, and/or internal organs.[1,2] These parasites are found in areas where there are female phlebotomine of sandflies, which serve as the vector. Leishmaniasis appears to be a disease with a long association with humans. For instance, PCR analysis of bone marrow samples of Egyptian and Nubian mummies indicates that humans have been exposed to visceral leishmaniasis infections for at least 4000 years.[3]

Current treatments for leishmaniasis involve pentavalent antimonial drugs such as sodium stibogluconate. However, these drugs have a high incidence of side effects such as joint pain, fatigue, gastrointestinal upset, and anemia.[4,5] Newer drugs such as miltefosine and amphotericin B have been shown to have antileishmanial activity. Amphotericin B is very effective as an antileishmanial compound, but it has to be administered as mixed micelles in sodium deoxycholate while miltefosine is the first orally active antileishmanial treatment.[5] Approximately, 1.5 million new cases of leishmaniasis are documented each year and there is a critical need for better therapies to be developed.[6,7]

Porphyrin derivatives have been commonly used for photodynamic therapy (PDT) because of their ability to generate reactive oxygen species (ROS) when excited by a light source.[8,9] A thorough discussion on the cellular generation of singlet oxygen and superoxide and the oxidative stress produced from each of these reactive species has been published.[10] *Leishmania* cannot synthesize their own heme, thus they need to obtain this important molecule from their hosts or other exogenous sources[11,12] making porphyrins and derivatives (porphyrinoids) attractive candidates for medicinal therapies. It has been previously shown that photodynamic therapy, using δ-aminolevulinic acid and benzophenoxazine derivatives, can be valuable therapeutics to treat cutaneous leishmaniasis.[13–16] Dutta et al.[17] reported that aluminum phthalocyanine chloride negatively affected *Leishmania amazonensis* growth in vitro, but only after treatment with light.

In this study, carbaporphyrin derivatives were investigated as potential photosensitizing agents. Dimethyl and diethyl carbaporphyrin ketals[18,19] were tested using promastigotes of the reptile strain, *Leishmania tarentolae*. This species was selected since the promastigotes are easy to grow in the laboratory and are very safe to culture since their growth temperature optimum is 26 °C, and they have essentially no ability to infect humans. These unique porphyrinoid species (Fig. 1) have strong absorptions in the far red that are a desirable feature of photosensitizers in PDT.

This study tested the effect of the carbaporphyrin ketals on the growth and viability of axenic promastigotes of *L. tarentolae* in

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doi:10.1016/j.bmc.2008.05.037

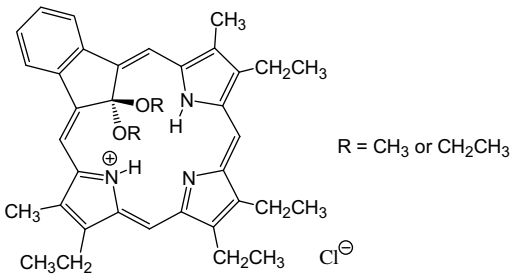
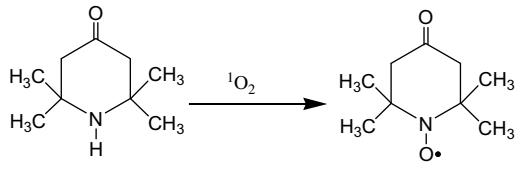
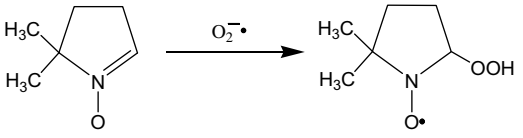


Figure 1. Structure of carbaporphyrin ketals.

vitro. In addition, it focused on the mechanism of action, emphasizing the detection of ROS generated within *L. tarentolae* induced by the presence of the carbaporphyrin ketals. The indirect detection of ROS was carried out via chemical traps such as the singlet oxygen trap 2,2,6,6-tetramethyl-4-piperidone (TEMP) and the superoxide trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO).[20] Once TEMP or DMPO reacts with singlet oxygen or superoxide, respectively, the stable free radicals TEMPO and DEMPO-OOH are generated (see Reactions 1 and 2, respectively) which can then be detected via electron paramagnetic resonance (EPR) spectroscopy. Additionally, confocal microscopy utilizing fluorescent probes for the mitochondria and mitochondrial superoxide was employed to observe the location of the carbaporphyrin ketals inside the parasites and the induced formation of superoxide in their mitochondria.



TEMP TEMPO (1)



DMPO DMPO-OOH (2)

2. Results

Growth curves of *L. tarentolae* exposed to several different concentrations (1.1–4.2 μM) of the carbaporphyrin ketals (R = CH_3 or CH_2CH_3) indicated that the ketal effectively inhibited *L. tarentolae* growth (Fig. 2) relative to the ethanol diluent control. It is clear that above 1.1 μM, the *Leishmania tarentolae* parasites are adversely affected in a dose-dependent manner by this dimethyl carbaporphyrin ketal compound especially after day 2 in culture. Concentrations up to 40 μM did not completely inhibit cell growth under our experimental conditions (data not shown). The mechanism of inhibition is unknown, but we hypothesized that the carbaporphyrin ketals may generate reactive oxygen species (ROS), such as singlet oxygen or superoxide that led to the observed growth inhibition. Excess ROS thus leads to protein/DNA damage, lipid peroxidation, and eventual cell death.[10,21]

The effect of light on the growth of the parasites was also investigated.

Figure 3 shows results obtained when *L. tarentolae* promastigotes were grown in the presence of 1.1 μM CKOMe and directly

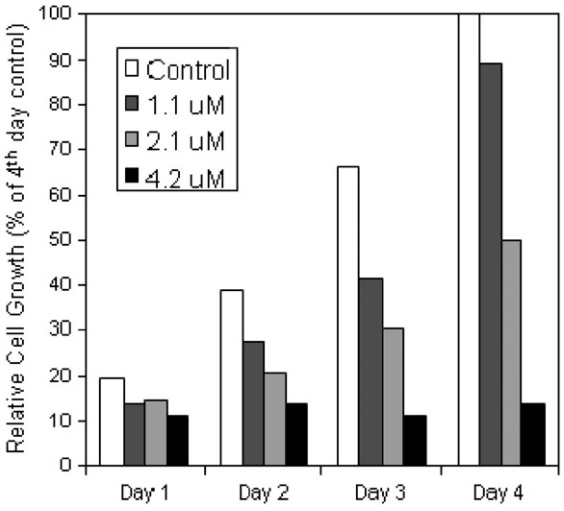


Figure 2. Inhibition of *L. tarentolae* growth by carbaporphyrin dimethylketal (CKOMe).

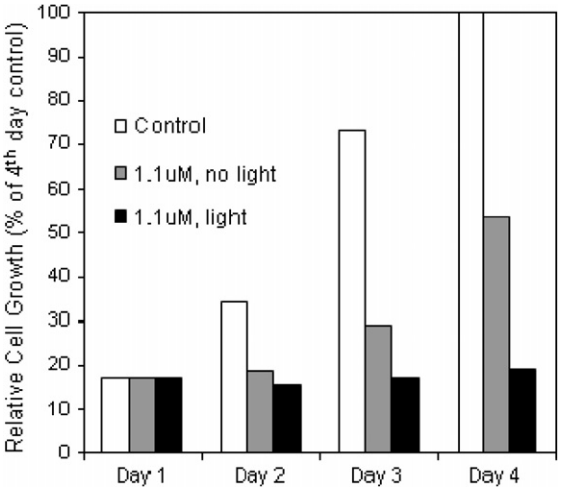


Figure 3. Photodynamic effect of CKOMe on the growth of *L. tarentolae*; control contains ethanol only.

exposed to visible light provided by a 20 W fluorescent lamp for 60 min per day. The cultures were kept in the dark following the exposure to direct visible light.

2.1. Viability of promastigotes using the MTT assay

Cell viability after exposure to different doses of the carbaporphyrin ketals and the unrelated 5,10,15,20-tetraphenylazuliporphyrin,[18,19,22] which was previously shown to not affect *L. tarentolae* promastigotes growth in culture, was tested using the MTT assay. Figure 4 shows viability curves of the compounds evaluated in terms of the absorbance at 595 nm of the MTT metabolite, formazan.

2.2. Singlet oxygen production within *Leishmania tarentolae*

Using the standard singlet oxygen generator, Rose Bengal,[23] we determined that the BHI medium alone was either interacting with TEMP or quenching produced singlet oxygen since the expected ^{14}N triplet splitting from the free radical TEMPO was not observed. However, when the same procedure was carried out in isotonic saline solution instead of the BHI medium, the characteristic signal

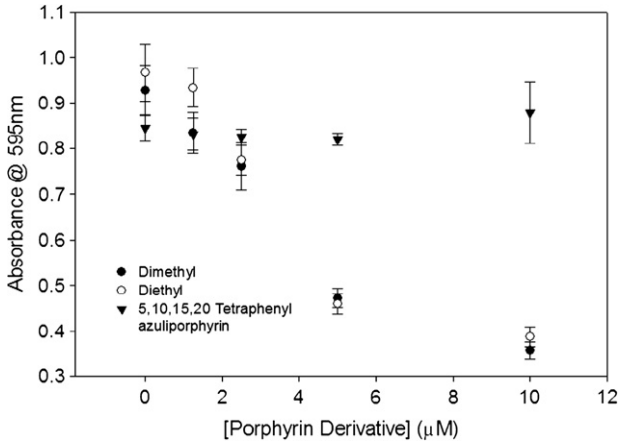


Figure 4. Formation of the formazan as a function of the concentration of dimethyl (●), diethyl (○) carbaporphyrin ketal, or 5,10,15,20-tetraphenylazuliporphyrin (▼). *L. tarentolae* culture (density at 1.5–1.8 AU at 600 nm) was diluted with BHI medium, and incubated for 30 min in ambient light with 0, 1.25, 2.5, 5, or 10 μM of selected porphyrinoid compound freshly prepared before incubation with the MTT reagent. (Incubation time = 3 h; each point is mean ± standard deviation for eight replicates.)

from TEMPO was observed in the EPR spectrum. Thus, all subsequent experiments involving promastigotes were performed in isotonic saline rather than in BHI medium. Figure 5 shows the EPR spectra collected.

Mitochondrial cytochromes, heme-containing proteins, are obligatory in the electron transport chain of aerobes. Thus, it was of interest to evaluate the ability of the carbaporphyrin ketal to associate with this organelle. Using a mitochondrial probe (MitoTracker® Deep Red dye at 0.5 mM incubated for 45 min) and confocal microscopy, it is observed that the carbaporphyrin ketal enters the parasite and appears to accumulate in the mitochondria of promastigotes (Fig. 6).

To assess whether functional electron transport is necessary for generation of singlet oxygen by the carbaporphyrin ketal, promastigotes were incubated with potassium cyanide, a potent inhibitor of cytochrome *c* oxidase.[24] Cultures that contained 2.3 mM potassium cyanide, 10 μM dimethyl carbaporphyrin ketal, and 40 mM of the singlet oxygen trap TEMP yielded no EPR signal after 30 min of incubation (data not shown).

We also attempted to evaluate the production of superoxide using the superoxide spin trap, DMPO (see Reaction 2) used by Turnbull et al.,[25] and EPR spectroscopy. Promastigotes were grown, isolated, and lysed using the same procedure as for the singlet oxygen experiments. *Leishmania* samples were incubated with DMPO, with and without dimethyl carbaporphyrin ketal, and analyzed using EPR spectroscopy. However, no EPR signal was detected using cells under any conditions tested suggesting that superoxide concentration is too low for detection by this methodology.

2.3. Confocal florescence microscopy detection of superoxide within *L. tarentolae*

In order to establish if the presence of the carbaporphyrin ketal induces the production of superoxide inside the parasites,cultures were incubated with a selective superoxide mitochondrial fluorogenic probe and evaluated by confocal microscopy. Figure 7 shows a field view of the confocal fluorescence images of *L. tarentolae* promastigotes incubated with different concentrations of the dimethyl carbaporphyrin ketal in the presence of the mitochondrial-specific superoxide probe MitoSox Red®.[26,27] The images clearly show a correlation between the amount of carbaporphyrin and the amount of parasites showing fluorescence. Fluorescence was not observed in the absence of the carbaporphyrin ketal.

It is clear that the presence of the carbaporphyrin ketal induces the formation of superoxide as evidenced from the increase in the amount of fluorescence detected (associated with oxidation of the probe by superoxide in the mitochondria) with an increase in the concentration of the compound. The results indicate that the carbaporphyrin ketals are able to accumulate inside the parasites and disrupt mitochondrial function, probably at the level of electron transport, which induces the formation of superoxide. The formation of superoxide may also be enhanced by a photodynamic mechanism via light absorption by the porphyrinoid, which may be excited to a state that induces the formation of superoxide via electron transfer to molecular oxygen.

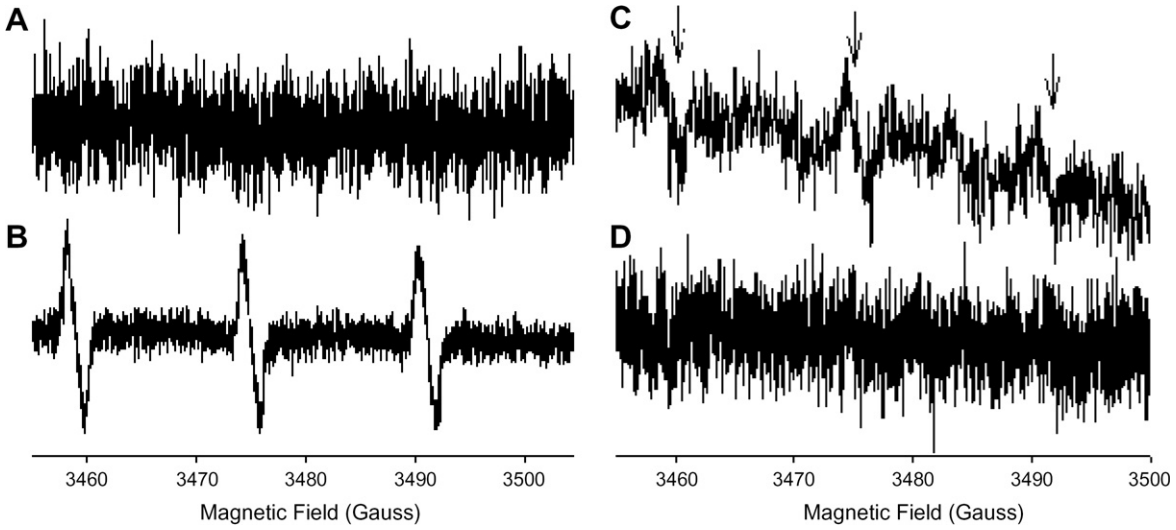


Figure 5. X-band EPR spectra recorded at 298 K. (A) A solution containing BHI medium, 10 μM of singlet oxygen generator Rose Bengal and 40 mM TEMP. (B) An isotonic solution containing 10 μM of Rose Bengal and 40 mM of TEMP. (C) A culture of *L. tarentolae* promastigotes resuspended in saline after exposure to a 20 W fluorescent lamp for 3 h in the presence of 10 μM of dimethyl carbaporphyrin ketal and 40 mM TEMP. The arrows mark the ^{14}N triplet from the TEMPO. (D) A *L. tarentolae* culture incubated with 40 mM TEMP but no carbaporphyrin ketal after exposure to light. Experimental EPR conditions: microwave frequency, 9.76 GHz; microwave power, 0.21 mW; modulation field, 2 G at 60 kHz; scan time, 30 min.

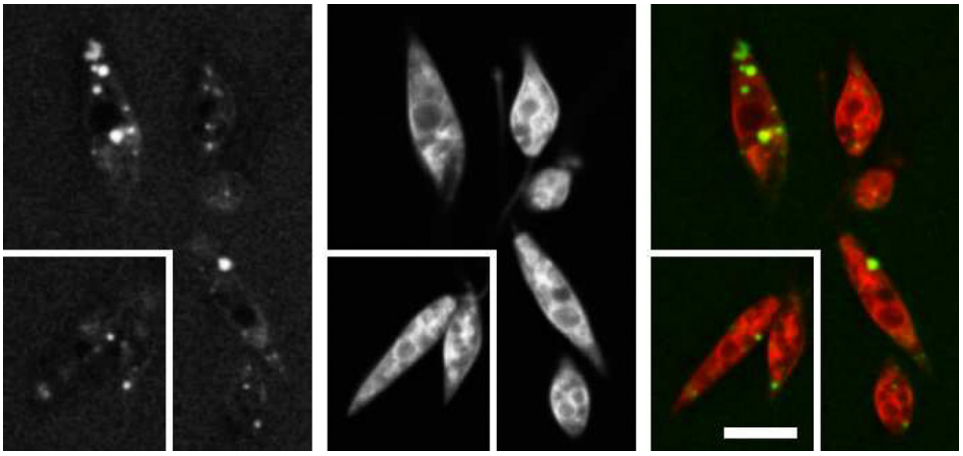


Figure 6. Confocal microscopy of a culture of *L. tarentolae* promastigotes incubated with both the MitoTracker® Deep Red mitochondrial probe, and 2 μM dimethyl carbaporphyrin ketal. Left: the dimethyl carbaporphyrin ketal was excited at 458/476 nm and the light emission was detected at 468–541 nm. Center: the Mito Tracker® was excited at 633 nm and the light emission was detected at 650–750 nm. Right: an overlay of the two images showing the location of the compound within the parasites. Scale bar is 5 μm.

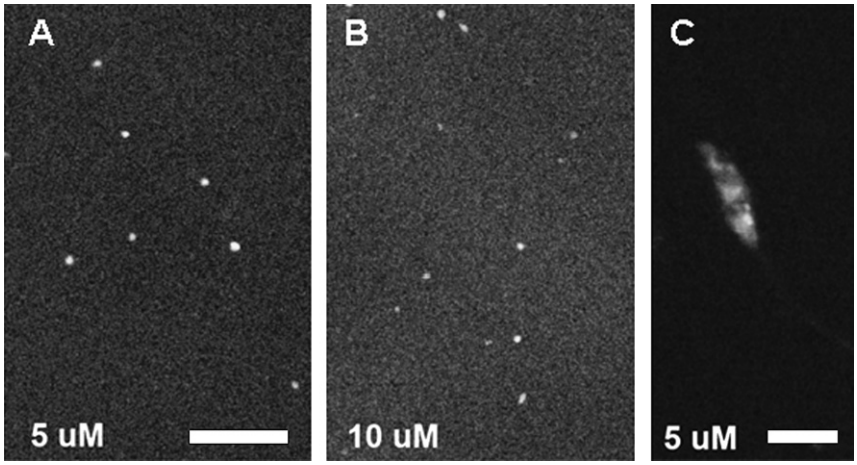


Figure 7. Confocal microscopy images showing fluorescence (white spots) generated by the oxidation of the superoxide probe MitoSOX™ Red as a function of dimethyl carbaporphyrin ketal: (A) 5 μM, (B) 10 μM, scale bar for (A and B) is 50 μm. Frame (C) shows an enlarged view of a promastigote at the same conditions as in frame (A), the scale bar is 5 μm.

3. Discussion

Both the growth curve (Fig. 2) and the cell viability assay (Fig. 4) indicate that the dialkyl carbaporphyrin ketals tested affect the in vitro growth and viability of axenic promastigotes of *L. tarentolae* in a concentration-dependent manner. Additionally, the disruption of growth is enhanced by direct illumination of the cultures with visible light demonstrating a photodynamic effect. It is interesting to note that at dialkyl carbaporphyrin ketal concentrations of 5 and 10 μM an apparent inhibitory asymptote is reached which correlates with the apparent ability of the organisms to withstand oxidative stress by their quenching processes. The effects due to the dimethyl and diethyl carbaporphyrin ketals were not significantly different from each other and both of these porphyrinoids were considerably more inhibitory than others tested, such as the azuliporphyrin.

A major goal of this study was to investigate the mechanism by which these compounds are effective. We hypothesized that they may induce the production of ROS such as singlet oxygen or superoxide in *L. tarentolae*. EPR spectroscopy in combination with the probe compound TEMP allows the detection of singlet oxygen. Experiments utilizing a known singlet oxygen generator (Rose Bengal) indicated that it was necessary to separate the promastigotes from the BHI media. Cultures of promastigotes of *L. tarentolae* that were incubated with 10 μM dimethyl carbaporphyrin ketal and 40 mM TEMP and further isolated from the media by centrifugation and exposed to light (from either a 20 W fluorescent lamp for 3 h, 75 W xenon lamp for 30 min, or ambient light during the time it took to add the selected reagents) gave EPR spectra that were indicative of the production of singlet oxygen. A weak ^{14}N triplet was observed in the EPR spectrum indicating that a small amount of singlet oxygen produced via photosensitization reacted with the TEMP. As expected, the EPR of cultures that were kept completely in the dark did not show the ^{14}N triplet EPR signal. Singlet oxygen was only detected from the promastigotes incubated in the presence of the dimethyl carbaporphyrin ketal and not those incubated with only the TEMP spin trap or with only the carbaporphyrin ketal. Even though the EPR detection limit is approximately 10^{-9} M,[28] signals are very weak. Detectable EPR signals were only evident using the post-centrifugation pellet fraction containing cells. The supernatant fraction containing medium, carbaporphyrin ketal and TEMP were also analyzed by EPR, but no EPR signal was detected. We conclude then that the spin trap and carbaporphyrin ketal were able to transverse the cell membrane and

that singlet oxygen is being generated in situ at concentrations of at least nanomolar amounts. However, if singlet oxygen is also generated in the medium, it is rapidly quenched by some unknown mechanism.

Leishmania parasites reside in the macrophages of their hosts and macrophages use oxidative stress to rid the cell of foreign objects. To test the ability of *Leishmania* to quench free radicals, the stable free radical TEMPOL was incubated with the cells prior to EPR evaluation. The EPR spectrum of this free radical also exhibits the ^{14}N coupling and shows the characteristic triplet as seen with the TEMPO. After the EPR spectra were collected, the intensity of the signal was compared at times 0, 30, and 60 min. It was found that between 0 and 30 min the intensity of the TEMPOL signal decreased, indicating that the levels of free radical were being diminished. However, between times 30 and 60 min the intensity of the signal no longer changed implying that a steady state was established (data not shown). From these data, we conclude that the *Leishmania* are very active in quenching free radicals as anticipated.

We also attempted to determine, using EPR spectroscopy and the superoxide trap DMPO, if the carbaporphyrin ketal induces the formation of superoxide. EPR spectra of the positive control (ofloxacin) were compared to those obtained when a sample of *Leishmania* was incubated with a carbaporphyrin ketal. The EPR spectrum of the cultures incubated with ofloxacin shows the typical EPR signal for DMPO-OOH, but no EPR signal was detected in the sample in which *Leishmania* was incubated with the carbaporphyrin ketal. Since similar experimental conditions were used for both the superoxide and the singlet oxygen experiments, it appears that the amount of superoxide produced intracellularly was below our EPR detection limits. Using the selective fluorogenic MitoSOX™ Red mitochondrial superoxide indicator and a confocal fluorescence microscope we were able to detect superoxide only when the *L. tarentolae* were exposed to the carbaporphyrin ketals. The results indicate that the amount of superoxide produced inside the parasites, more specifically in the mitochondria, is proportional to the amount of carbaporphyrin ketal utilized.

We have thus shown that the dialkyl carbaporphyrin ketals tested inhibit the growth of axenic promastigotes of *L. tarentolae* in culture. The detrimental effects of each carbaporphyrin ketal were dose-dependent and these were the only porphyrin derivatives tested that resulted in inhibition of the viability of *L. tarentolae* promastigotes. There is also a photodynamic effect on the growth of promastigotes. We also determined through the use of EPR spectroscopy and confocal fluorescence microscopy in combination with reactive oxygen probes that the carbaporphyrin ketals induce the generation of detectable amounts of singlet oxygen and superoxide inside promastigotes of *L. tarentolae*. The major effect on cell viability appears to involve these reactive oxygen species, which may be mediated by mitochondrial electron transport chain functioning. Since the effects appear to be increased by light exposure, some photosensitivity is involved and clearly several mechanisms are operating. Overall, the dimethyl and diethyl carbaporphyrin ketals show great promise as potent inhibitors of *Leishmania* and should be investigated further.

4. Materials and methods

4.1. Chemicals

The initial promastigote culture of *L. tarentolae* was obtained from the American Tissue Culture Collection (ATCC #30143, Manassas, VA). Hemin, penicillin–streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin; pen–strep), 2,2,6,6-tetramethyl-4-piperidone (TEMP), Rose Bengal, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), potassium cyanide, *n*-octyl β-D-glucopyranoside, Ofloxacin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) were purchased from Sigma Chemical Company (St. Louis, MO). 4-Hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL) was purchased from Acros Organics (Morris Plains, NJ). Fluorescent probes for confocal microscopy (MitoTracker® Deep Red and MitoSOX™ Red) were purchased from Invitrogen (Carlsbad, CA). Buffers, reagents, and cell media were prepared using nanopure water from a nanopure Ultrapure Water System obtained from BarnStead/Thermolyte (Dubuque, IA). Carbaporphyrin ketals (dimethyl and diethyl) were prepared as previously reported by Lash et al.[18,19] The ketals were prepared by reacting a benzocarbaporphyrin with 500 equiv of ferric chloride in chloroform–alcohol mixtures using the literature procedures.

4.2. Promastigotes culture

Promastigotes of *L. tarentolae* were cultured (at 26 °C) in the dark in heat-sterilized brain heart infusion medium (BHI; Becton–Dickinson and Company). After sterilization, 4 ml of a 2.5 mM hemin sterile solution and 5 ml of penicillin–streptomycin (pen–strep; 10,000 U of penicillin and 10 mg of streptomycin per milliliter in sterile 0.9% (w/v) sodium chloride; Sigma Chemical Company) per 500 ml of BHI were added. Stock cultures were grown in 250 ml Corning® flasks (Fisher Scientific, Fairlawn, NJ) containing 20 ml of medium and transferred every 4 days to fresh medium.

4.3. Promastigotes viability using the MTT assay

Leishmania tarentolae viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[29] following the procedure provided by Sigma–Aldrich.[30] Briefly, cultures to be evaluated were incubated with the MTT reagent for 3 h. Viable cells are able to metabolize the MTT reagent into purple-colored formazan. An increase in the absorbance at 595 nm, due to the formation of the formazan, indicates functional mitochondria and therefore viable cells. Assays were carried out in non-sterile 96-well polypropylene flat-bottomed immuno-module plates purchased from Fisher Scientific (Pittsburg, PA) and absorbance data were obtained at 595 nm using a microplate reader (Bio-Rad® Microplate Reader Benchmark).

4.4. Effect of carbaporphyrin ketals on promastigotes growth

A 100 μM stock solution of dimethyl carbaporphyrin ketal (MW 596 g/mol) in absolute ethanol was added to the promastigotes in culture to obtain different dilutions of the compound. In all cases the final ethanol concentration was less than 1% (v/v). *L. tarentolae* promastigotes were exposed for 4 days to several different concentrations (0.25–40 μM) of the dimethyl carbaporphyrin ketal and compared to control cultures in which promastigotes are exposed only to the ethanol diluent. Cell growth was evaluated by spectroscopy at 600 nm.

4.5. Detection of reactive oxygen species in *L. tarentolae* cultures incubated with carbaporphyrin ketal

Experiments were performed by incubating the promastigotes (day 3 after transfer) with the selected ROS trap and the carbaporphyrin ketal. The final concentration for the selected ROS traps in the assay was 40 mM and the carbaporphyrin ketal was 10 μM. On day four of growth, the culture flasks were exposed to either ambient light, a 20 W fluorescent lamp (visible range) for 3 h, or a 75 W xenon lamp equipped with an IR filter for 30 min to induce excitation of the carbaporphyrin ketal in the visible range. After exposure, the cells were collected by centrifugation, resuspended in isotonic saline, and then evaluated by EPR spec-

troscopy directly or lysed by addition of the detergent *n*-octyl β-D-glucopyranoside at a final concentration of 35 mM.[31] Samples with and without detergent and isotonic saline containing the ROS trap (TEMP or DMPO) were used as negative controls.

4.6. Mitochondrial superoxide detection using confocal fluorescence microscopy

Four-day-old cultures of *L. tarentolae* promastigotes were incubated with ethanol solutions of carbaporphyrin ketal to obtain a final concentration of 0, 1, 5, or 10 μM. Cultures were then incubated for 3 h before addition of the superoxide probe and confocal fluorescence microscopy analysis. Cultures were prepared for confocal fluorescence microscopy by incubating them with a 5 μM solution of the MitoSOX™ Red probe according to the manufacturer's specifications. Confocal microscopy images were collected using a Leica SP2 system from a slide containing an aliquot of sample using a 63× oil immersion objective. The probe was excited with an argon ion laser at 514 nm, while its fluorescence was collected with a photomultiplier in the 550–600 nm range.

Acknowledgments

M.A.J. acknowledges the support of the Eppley Foundation for Research, Inc., and a College of Arts and Sciences Research Enhancement Award; D.L.C. acknowledges the support of the Illinois State University College of Arts and Sciences Program of Excellence Grant (D.L.C. as Director) and of Research Corporation via a Cottrell College Science Award (CC6382); S.J.P. acknowledges the support of the Camille and Henry Dreyfus Foundation via a Supplemental Award through the Scholar/Fellow Program; T.D.L. acknowledges support From the National Science Foundation, Grant No. CHE-0616555.

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