Phytomedicine 61 (2019) 152894

Contents lists available at ScienceDirect

Phytomedicine

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

Original Article

Natural anthraquinones as novel photosentizers for antiparasitic photodynamic inactivation

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ARTICLE INFO

Keywords:
Leishmania amazonensis
Chlorinated anthraquinones
Dimeric anthraquinones
LED
Cytotoxicity

ABSTRACT

Background: Cutaneous leishmaniasis (CL) is a vector-borne disease caused by obligate protist parasites from the genus *Leishmania*. The potential toxicity as well as the increased resistance of standard treatments has encouraged the development of new therapeutical strategies. Photodynamic inactivation (PDI) combines the use of a photosensitizer and light to generate reactive oxygen species and kill cells, including microorganisms. Vegetal kingdom constitutes an important source of bioactive compounds that deserve to be investigated in the search of naturally occurring drugs with leishmanicidal activity.

Purpose: The purpose of this study was to test the antiparasitic activity of PDI (ApPDI) of five natural anthraquinones (AQs) obtained from *Heterophyllaea lycioides* (Rusby) Sandwith (Rubiacae). To support our results, effect of AQ mediated-PDI on parasite´s morphology and AQ uptake were studied. Cytotoxicity on fibroblasts was also evaluated.

Study design/Methods: Two monomers, soranjidiol (Sor) and 5-chlorosoranjidiol (5-ClSor) plus three bi-anthraquinones (bi-AQs), bisoranjidiol (Bisor), 7-chlorobisoranjidiol (7-ClBisor) and Lycionine (Lyc) were selected for this study. Recombinant *L. amazonensis* promastigote strain expressing luciferase was subjected to AQs and LED treatment. Following irradiation with variable light parameters, cell viability was quantified by bioluminescence. Alteration on parasite's morphology was analyzed by scanning electron microscopy (SEM). In addition, we verified the AQ uptake in *Leishmania* cells by fluorescence and their toxicity on fibroblasts by using MTT assay.

Results: Bisor, Sor and 5-ClSor exhibited photodynamic effect on *L. amazonensis*. SEM showed that promastigotes treated with Bisor-mediated PDI exhibited a significant alteration in shape and size. Sor and 5-ClSor presented higher uptake levels than bi-AQs (Bisor, Lyc and 7-ClBisor). Finally, Sor and Bisor presented the lowest toxic activity against fibroblasts.

Conclusion: Taking together, our results indicate that Sor presents the highest specificity towards *Leishmania* cells with no toxicity on fibroblasts.

Introduction

Leishmaniasis is a vector-borne disease caused by parasites from the genus *Leishmania* transmitted through the bite of infected female sandflies. This disease is endemic in large areas of the tropics, subtropics and Mediterranean basin, globally spanning 97 countries (WHO, 2018) and is considered a neglected tropical disease with a strong linkage to poverty (Seifert, 2011).

Abbreviations: AQs, anthraquinones; bi-AQs, bi-anthraquinones; ApPDI, antiparasitic PDI; Bisor, bisoranjidiol; 7-ClBisor, 7-chlorobisoranjidiol; 5-ClSor, 5-chlorosoranjidiol; CV, cell viability; CL, cutaneous leishmaniosis; *La*-LUC, Recombinant *L. amazonensis*; Lyc, lycionine; NC, negative control; PC, positive control; PDI, photodynamic inactivation; PDT, photodynamic therapy; Sor, soranjidiol; $O_2^{\cdot -}$, superoxide anion radical; PS, photosensitizer; PIT, pre-irradiation time; ROS, reactive oxygen species; SEM, scanning electron microscopy

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https://doi.org/10.1016/j.phymed.2019.152894
Received 19 December 2018; Received in revised form 23 February 2019; Accepted 12 March 2019

Cutaneous leishmaniasis (CL) is a clinical form characterized by the appearance of skin lesions with raised borders (volcano sign), eventually ulcerated, and limited to a specific area (Stebut, 2015). In some patients, lesions may disseminate and manifest as multiple raised non-ulcerated skin lesions (diffuse and disseminated CL).

The potential toxicity as well as the increased resistance of standard treatments has led to development of alternative medication for CL (Seifert, 2011). Photodynamic inactivation (PDI) has been suggested as a new approach to treat CL (de Oliveira et al., 2017). This treatment uses photosensitive drugs combined with light to kill microbial cells. Light excites the photosensitizer (PS) that in presence of oxygen generates cytotoxic reactive oxygen species (ROS). Due to its action mechanism, PDI would not produce resistance on microorganisms (Cieplik et al., 2018). Besides, as a topical treatment, PDI combines the advantages of both physical therapy and chemotherapeutic agents, avoiding side effects of systemic therapy (Akilov et al., 2007).

Literature is rich on PDI studies on *Leishmania* spp. with synthetic PSs but natural PSs have been poorly explored. In fact, vegetal kingdom constitutes an important source of bioactive compounds that deserve to be investigated in the search of naturally occurring drugs with leishmanicidal activity (Taylor et al., 2013; Da Silva et al., 2018).

It is known that the genus *Heterophyllaea* Hook f. (Rubiaceae) is toxic to cattle that feed on it and is exposed to sun light (Bacigalupo, 1993). This toxicity is attributed to the presence of 9,10-anthraquinones (AQs) that demonstrated to have photosensitizing properties (Núñez-Montoya et al., 2005; Comini et al., 2007). Recently, chemical studies on *H. lycioides* (Rusby) Sandwith species allowed the isolation of several AQs (Dimmer et al., 2017) that had already been obtained from *H. pustulata* Hook. f (Núñez-Montoya et al., 2006). Among them, three new structures were identified: 5-chlorosoranjidiol (5-ClSor), 7-chlorobisoranjidiol (7-ClBisor) and lycionine (Lyc) (Fig. 1), which promoted an increase in the production of superoxide anion radical ($O_2^{\cdot -}$) under irradiation (Dimmer et al., 2017). This feature makes them potential PSs to be used in photodynamic therapy (PDT).

The present work was designed to explore the antiparasitic photodynamic inactivation (ApPDI) of soranjidiol (Sor) and other four derivatives of this AQ: 5-ClSor, bisoranjidiol (Bisor), 7-ClBisor and Lyc. We verified the effect of AQ-mediated ApPDI on cell viability of promastigotes of *L. amazonensis*. Alterations on parasite's morphology following ApPDI were analyzed by scanning electron microscopy (SEM). AQ uptake was measured to clarify our findings. Finally, as they could be used in a topical application, we also investigated the in vitro toxicity of PDI against fibroblasts.

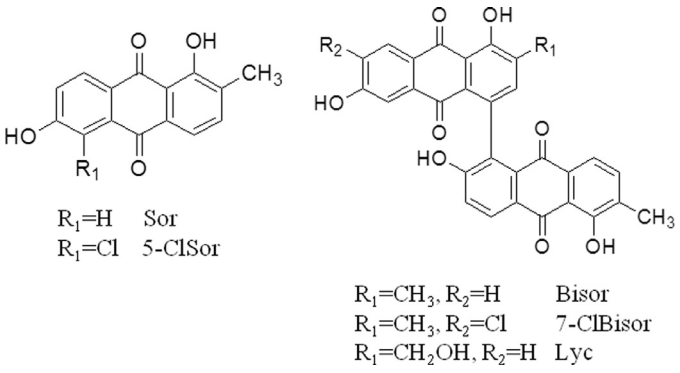

Fig. 1. Anthraquinones isolated from *Heterophyllaea lycioides,*
Sor: soranjidiol; 5-ClSor: 5-chlorosoranjidiol; Bisor: bisoranjidiol; Lyc: lycionine; 7-ClBisor: 7-chlorobisoranjidiol.

Materials and methods

Natural photosensitizers

AQs were obtained as described by Dimmer et al., 2017. Purity was determined by HPLC analysis: Sor (93.7%), 5-ClSor (99.3%), Bisor (87.4%), 7-ClBisor (87.4%) and Lyc (82.3%). A Varian Pro Star chromatography apparatus (model 210, Agilent, Santa Clara, CA, USA), equipped with an UV-Vis detector and a Microsorb-MV column 100-5 C-18 (250 × 4.6 mm i.d.), was used at 25 °C. The mobile phase was a gradient elution of formic acid 0.16 m dissolved in ultrapure water (solvent A) and MeOH-formic acid 0.16 m (solvent B). The following gradient elution program was used: 1–3 min, 50%; 3–5 min, 50–80% B; 5–46 min, 80%; 46–47 min, 80–100% B; 47–61 min, 100%, 61–63 min, 50% B. Detector was set at 269 nm. Samples were dissolved in MeOH (HPLC grade, Sintorgan, Bs. As., Argentina), filtered through cellulose (Merck Millipore, São Paulo, SP, Brazil) and manually injected (20 µl). Data analysis was performed using the Varian software (Star Chromatography Workstation 6.41).

For each assay, AQs were dissolved in phosphate buffer solution (PBS) and dymethyl sulfoxide (DMSO) as co-solvent in a concentration below 1%. Solutions were filtered through a sterile 0.22 µM filter membrane.

Parasite culture

Recombinant *L. amazonensis* species expressing the luciferase gene (*La*-LUC) were obtained by transfection of *L. amazonensis* wild type strain (MHOM/BR/73/M2269). *La*-LUC lines were a kind gift from Prof. Silvia Uliana from Institute of Biomedical Sciences, University of São Paulo (ICB-USP). The enzyme luciferase (LUC), which is over expressed by recombinant *Leishmania*, catalyzes the reaction between luciferin substrate and ATP to generate photons (bioluminescence). Therefore, ATP from viable cells can be quantified by bioluminescence detection (Reimao et al., 2013). It is important to point out that it was demonstrated that *La*-LUC and the wild type parental line produce the same clinical pattern of lesion development as well as they have the same susceptibility to antileishmanials (Reimão et al., 2013).

Metacyclic promastigotes of *La*-LUC were grown in 25 cm^3 tissue culture flasks containing M199 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; Gibco™ Invitrogen Corporation, New York, NY, USA), 40 mM HEPES at pH: 7.4 (Sigma-Aldrich), hemin (0.005%) (Sigma-Aldrich) and penicillin/streptomycin 100 µg/ml (Sigma-Aldrich). They were incubated at 25 °C for 7 days.

Antiparasitic photodynamic assay

To determine the AQ concentration to be tested on *La*-LUC, Sor was used as it has the basic structure of the five compounds. Therefore, Sor was tested in a concentration range from 2.5 to 40 µM. Each concentration ($n = 3$) was incubated with a suspension of 5×10^6 promastigotes/ml during 30 min without irradiation, with the aim to choose a concentration that is non-toxic in the dark. Another sample set with the same variable concentrations of Sor was pre-incubated for 10 min under darkness [pre-irradiation time (PIT)] and immediately irradiated during 12 min with a violet-blue LED system ($\lambda = 410 \pm 10$ nm, 50 mW/cm^2, 36 J/cm^2), which matches to the characteristic absorbance of the studied AQs (Fig. 2). Cell viability (CV) of both set of samples (dark and irradiated) was quantified by means of bioluminescence detection, using a proper spectrophotometer (Spectramax M4, Molecular Devices, Sunnyvale, CA, USA). Measures were taken after 30 min of luciferin substrate addition, prepared following all manufacturer´s instructions (Promega Corporation, Fitchburg, WI, USA). The luminescence was then normalized in relation to non-treated control [negative control (NC)], so that results were expressed as a

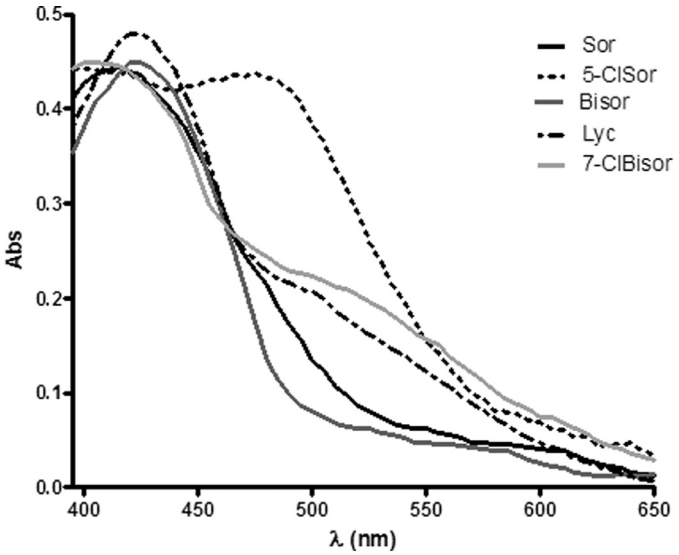

Fig. 2. AQ spectra in PBS.
Sor: soranjidiol; 5-ClSor: 5-chlorosoranjidiol; Bisor: bisoranjidiol; Lyc: lycionine; 7-ClBisor: 7-chlorobisoranjidiol.

percentage of CV (%CV) respect to NC (100%CV).

The compounds were then tested at one concentration but varying radiant exposures. For this procedure we used a low AQ concentration (2.5 μM) to prevent any effect of dark toxicity. The same irradiation system abovementioned was used to deliver the following radiant exposures: 9, 18, 27 and 36 J/cm^2 (Irradiance = 50 mW/cm^2) corresponding to exposure times of 3, 6, 9 and 12 min, respectively. Thereafter, the CV was measured.

A dose-response curve between%CV and radiant exposure was plotted to estimate the lethal light dose for killing 50% (LD_{50}) and 90% (LD_{90}) of parasites.

The curve is expressed by:

$$CV(\%) = CV_{min} + \frac{CV_{max} - CV_{min}}{\left(\frac{D}{LD_{50}}\right)^{k}}$$

where CV_{max} and CV_{min} are the maximum and minimum cell viability, respectively, D is the radiant exposure (dose at J/cm^2) and k is the hill slope.

To determine how different wavelengths affect cellular viability following ApPDI, two more wavelengths (450 and 520 nm) were selected to irradiate AQs, considering the absorbance band of each AQ (Fig. 2). Experiments were performed with a radiant exposure of 36 J/cm^2.

Each experiment included four control groups: non-treated group (NC), cells treated with sodium dodecyl sulfate (SDS) [positive control (PC)], LED group (irradiated parasites with the highest radiant exposure = 36 J/cm^2, LEDG) and dark control (each AQ in dark condition).

Morphological analysis

Promastigote morphology of NC and Bisor-mediated ApPDI (λ = 410 nm) were analyzed under a SEM (TM 3000 Tabletop, Hitachi, Tokyo, Japan) at high vacuum mode. Each sample was centrifuged at 1200 g for 10 min and then, a solution of glutaraldehyde 2% in sodium cacodylate buffer (0.1 M) was added for fixation. After overnight incubation, samples were washed twice and then stained in a solution 10 mM of Ru(bipy)3 (Sigma-Aldrich) for 30 min. Finally, cells were washed twice again, the supernatant was removed and the pellets were added to coverslips prior to analysis. Flagellum length and parasite size were measured using ImageJ 1.52i software.

AQ uptake

AQs (2.5 μM) and La-LUC (5×10^6) were incubated in a 96-well microplate at 10 min (PIT) and 22 min (PIT + 12 min of irradiation). Thereafter, the plates were centrifuged for 20 min at 1200 g and 15 °C (model 5430R, Eppendorf AG, Hamburg, Germany). The supernatant (SN1) was collected and transferred to wells and the pellet was resuspended in 50 μl of PBS. After a second centrifugation, SN2 was removed and added to SN1 to determine AQ extracellular fluorescence (ECF). Then, one hundred-μl of 2% SDS was added to the pellets to dissolve promastigote cells. Samples were incubated overnight. After 24 h, samples were mechanically homogenized and centrifuged (1200 g for 20 min). Supernatant (SN3) was transferred to wells and 50 μl of PBS were added to pellet to perform a second centrifugation (SN4). Supernatants obtained (SN3 and SN4) were pooled to measure intracellular fluorescence (ICF). ICF and ECF were measured at 665 nm (λ_{exc} = 420 nm) using a microplate reader (Spectramax M4, Molecular Devices, Sunnyvale, CA, USA).

Toxicity of AQ-mediated PDI on fibroblasts

Experiments were performed in FN1 human fibroblast cells kindly provided by Prof. Durvanei Augusto Maria from Butantã Institute, São Paulo, Brazil. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEN, Gibco™ Invitrogen Corporation, New York, NY, USA), supplemented with 10% FBS (Sigma-Aldrich), 100 μg/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) at 37 °C with 5% CO_2 humidified air. A fibroblast monolayer (5×10^6 cells/wells), in a 24-well plate, were treated with each AQ (2.5 μM) under dark and light conditions. After a PIT of 10 min, they were photoactivated with blue LED (λ = 410 ± 10 nm; irradiance = 50 mW/cm^2) and exposed to a radiant exposure of 36 J/cm^2. Negative control (NC, non-treated group), PC (cells treated with SDS 2%) and LED group (LEDG, cells irradiated with 36 J/cm^2) were included. Once finished, each solution was removed, the cells were washed with 400 μl of PBS solution and fresh medium was added to each well. After 24 h of incubation, cell viability was evaluated with MTT [3-(4,5-dimethylthiazol-2-yl) − 2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) assay. In each experimental condition, medium was removed from the well, and 40 μl of MTT solution (5 mg/ml) plus 360 μl of PBS was added. After 4 h of incubation at 37 °C, the solution was removed and 100 μl of DMSO was added. Thirty min later, each well was homogenized and the optical density (OD) was measured in a spectrometer (Spectramax M4, Molecular Devices) at 570 nm. Results were expressed as the mean percentage of CV compared with NC group.

Statistical analysis

All experiments were performed in triplicate. Data are presented as mean values ± standard deviation (SD). Data obtained were submitted to One Way ANOVA test for ApPDI and toxicity of AQ on fibroblasts. Uptake data were submitted to Two Way ANOVA test. Mean comparisons between groups were carried out with Bonferroni post-test. SEM measures were analyzed by unpaired *t*-test. Statistical analyses were performed by using GraphPad Prism 5.03 software and differences were considered significant as $p < 0.05$(*), $p < 0.01$ (**) and $p < 0.001$ (***).

Results

In darkness conditions (Fig. 3), the lowest concentration tested of Sor (2.5 μM) showed the same CV as NC, while the other concentrations promoted a significant reduction. Nevertheless, under LED irradiation, all concentrations showed CV similar to PC. Thus, we proceeded with the other assays using 2.5 μM.

Fig. 4 displays the ApPDI of tested AQs on *La*-LUC promastigotes.

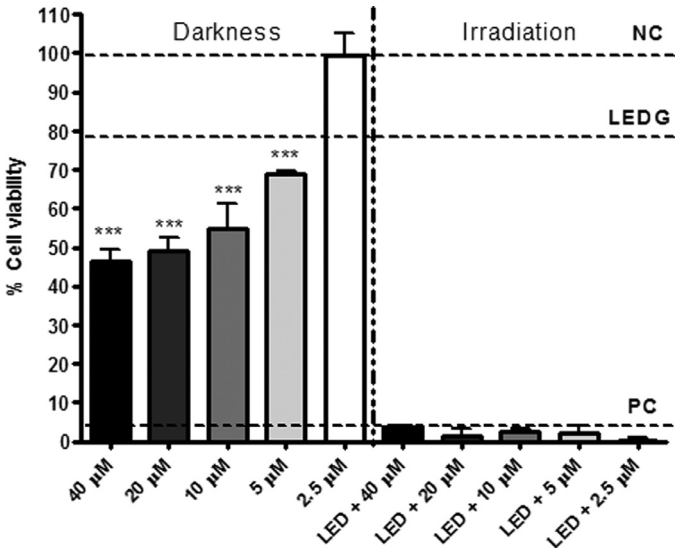

Fig. 3. Percentage of cell viability of Soranjidiol-sensitized *La*-LUC under darkness and light conditions. Radiant exposure was 36 J/cm^2 delivered by a LED emitting at λ = 410 ± 10 nm with irradiance of 50 mW/cm^2 (n = 3). ***p < 0,001 *vs.* negative control.
NC: negative control. PC: positive control. LEDG: treated group with LED.—:% cell viability reached by NC, PC and LEDG.

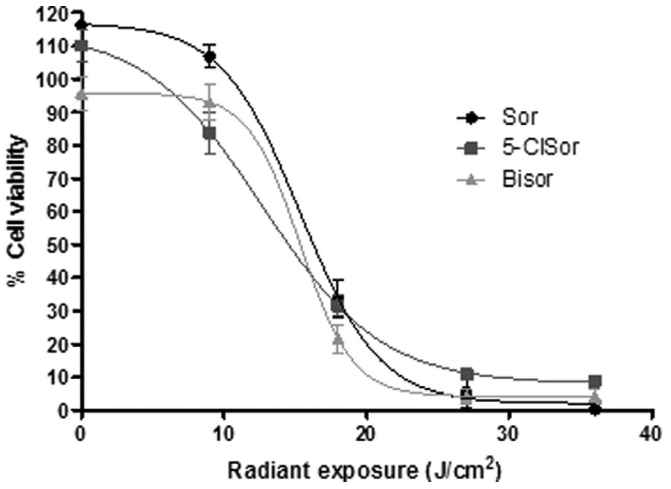

Fig. 5. Dose-response curve between percentages of cell viability *vs.* radiant exposure (n = 3).
Sor: soranjidiol; 5-ClSor: 5-chlorosoranjidiol; Bisor: bisoranjidiol.

Table 1
Lethal light dose for killing 50% and 90% of parasites of active AQs (LD_{50} and LD_{90}, respectively).

AQs	LD_{50} (J/cm^2)	LD_{90} (J/cm^2)
Sor	16.3	22.1
5-ClSor	13.8	22.2
Bisor	15.2	19.3

AQs in darkness had no significant effect on CV of *La*-LUC promastigotes (0 J/cm^2). Treatment with violet-blue LED alone produced a CV reduction of 20% due to endogenous chromophores of *Leishmania* cells. The combination of violet-blue light and Sor, 5-ClSor or Bisor promoted a significant decrease in parasite viability, in a light exposure-dependent manner, showing similar effect as PC (reduction above 90%) with a radiant exposure of 27 J/cm^2 (9 min). In comparison, 7-ClBisor and Lyc exhibited no reduction in CV, showing a similar effect as LED group with a radiant exposure of 36 J/cm^2 (12 min).

To compare the AQs that promoted more pronounced ApPDI, we adjusted a dose-response curve between cell viability and radiant exposure (Fig. 5) to identify LD_{50} and LD_{90} (Table 1).

It is possible to notice that the photoinactivation kinetics depends on AQ structure. In fact, LD_{50} is lower for 5-ClSor but Bisor exhibited the lowest LD_{90}.

We tested other two wavelengths (450 and 520 nm) that are also absorbed by AQs (see Fig. 2). Interestingly, Bisor and 7-ClBisor, that have no ApPDI at 520 nm, reduced cell viability in about 40% under 450 nm (Fig. 6A). Also, our results demonstrated that 5-ClSor was able to decrease the CV in more than 90% when combined with 450 or 520 nm (Fig. 6A and B). For Sor and Lyc, no significant inactivation was observed by using either 450 or 520 nm.

Morphological changes promoted by ApPDI were observed by SEM. Untreated promastigotes showed their typical morphology with an elongated shape and flagellum (Fig. 7A). After Bisor-mediated ApPDI (lowest LD_{90}), we noticed a drastic alteration in shape and size. The cells appeared rounded with a shortened cell body (about 35%) indicating changes in the cell volume. The shortening of the flagella (about 43%) was also perceived (Table 2).

To clarify the results obtained, we measured the AQ uptake by promastigotes (Fig. 8). The assay showed that following 10 min of incubation, higher ICF was observed for all AQs, meaning that all of them

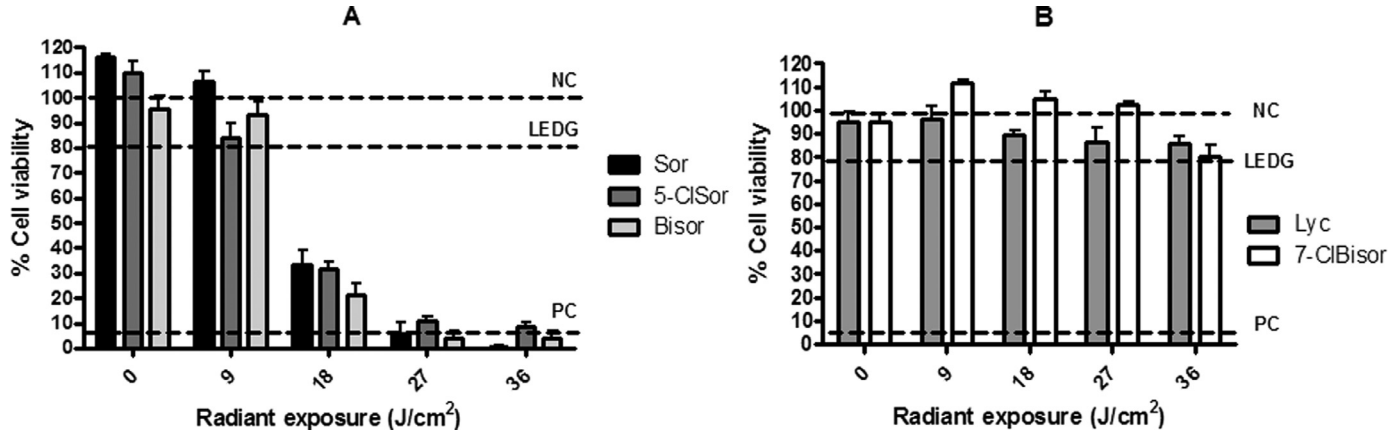

Fig. 4. Percentage of cell viability of *La*-LUC promastigotes following AQ-mediated ApPDI (n = 3). (A) AQs with significative decrease on CV. (B) Inactive AQs. AQ concentration = 2.5 µM.
NC: negative control. PC: positive control. LEDG: treated group with LED.—:% cell viability reached by NC, PC and LEDG. Sor: soranjidiol; 5-ClSor: 5-chlorosoranjidiol; Bisor: bisoranjidiol; Lyc: lycionine; 7-ClBisor: 7-chlorobisoranjidiol.

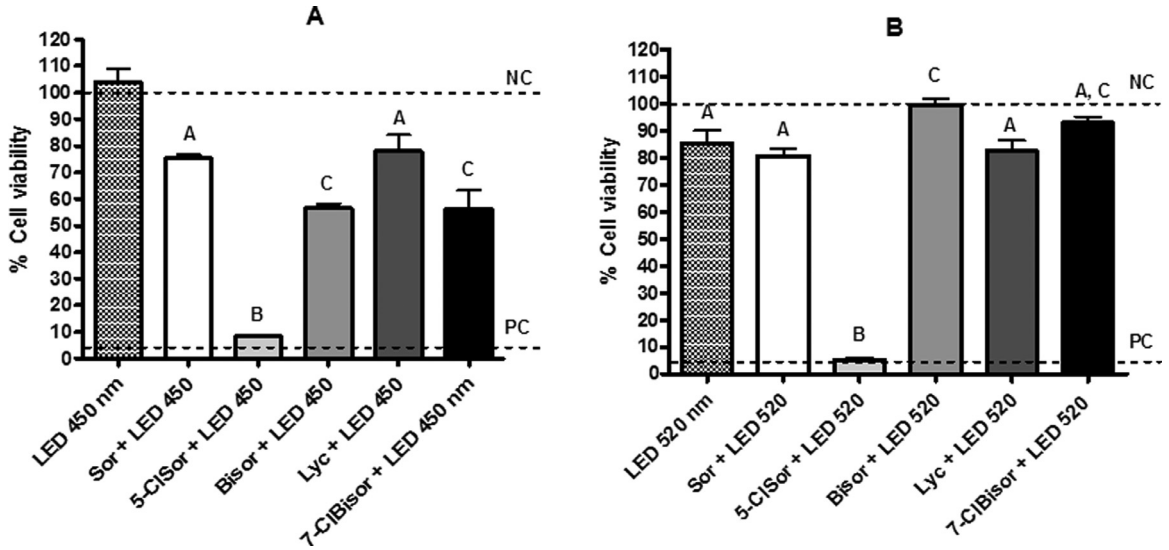

Fig. 6. Percentage of cell viability of *La*-LUC promastigotes following AQ-mediated ApPDI using different LEDs: (A) 450 nm (B) 520 nm. The radiant exposure was 36 J/cm^2 and irradiance 50 mW/cm^2 ($n = 3$). Different letters denote statistically significant differences ($p < 0.05$). NC: negative control. PC: positive control. —:% cell viability reached by NC and PC. Sor: soranjidiol; 5-ClSor: 5-chlorosoranjidiol; Bisor: bisoranjidiol; Lyc: lycionine; 7-ClBisor: 7-chlorobisoranjidiol.

enter inside the parasite. AQ monomers (Sor and 5-ClSor) showed the highest uptakes in comparison to bi-anthraquinones (Bisor, Lyc and 7-ClBisor). 7-ClBisor showed the lowest fluorescence, while Bisor and Lyc exhibited similar fluorescence ($p > 0.05$). After 22 min of incubation, ICF decreases in all cases. At the same time, 7-ClBisor, Sor and 5-ClSor increases the ECF while Bisor maintained it and Lyc showed a decrease.

Results in Fig. 9 indicate that Sor and 7-ClBisor are the compounds with the lowest cytotoxicity, because they exhibited no toxicity under irradiation and dark conditions, with CV similar to NC and LEDG. Bisor and Lyc promoted a decrease in CV of 30% under LED irradiation, although they have showed no toxicity in darkness. Finally, 5-ClSor showed a CV of 75% in darkness and a reduction of more than 90% under irradiation (same as PC), hence it was the highest toxic compound on fibroblast cell line.

Discussion

Synthetic PSs combined to different light sources have been reported for ApPDI of *L. amazonensis* promastigotes. Methylene blue at 50 μM associated to red light at 106.2 J/cm^2 reduced parasite viability at 80% (Aureliano et al., 2018). In contrast, rose bengal derivatives combined to green LED showed more than 90% of parasite inactivation at 3 J/cm^2 and PS concentrations higher than 4 μM (Navasconi et al., 2017). In this work, we explored the photodynamic activity of five natural AQs with photosensitizing properties: Sor and their derivatives with little skeletal differences: 5-ClSor, Bisor, Lyc and 7-ClBisor (Dimmer et al., 2017). Sor, 5-ClSor and Bisor at 2.5 μM associated to violet-blue LED at 20 J/cm^2 were able to inactivate more than 90% of parasites indicating their potential as antileishmanials.

Table 2
Measures of flagella length and parasite size ($n = 7$). Statistical differences between treated and non-treated group. *$p > 0.01$/**$p > 0.001$ *vs.* negative control.

	Negative control	Treated group
Flagella length (μM)	15.2 ± 2.3*	9.9 ± 0.7*
Size of parasites (μM^2)	12.3 ± 1.3**	6.5 ± 0.5**

Sor showed photodynamic effects at concentrations below 40 μM, a non-cytotoxic concentration in Vero cells (Konegheim et al., 2012). It is emphasized that the lowest tested concentration (2.5 μM) resulted the most active in presence of light, being at the same time not toxic in dark conditions. The remaining AQs also were not cytotoxic at 2.5 μM in the dark; nevertheless, at this concentration only 5-ClSor and Bisor decreased the CV of *La*-LUC, when they were irradiated with violet-blue.

The three most photodynamic active AQs have a structure derived from Sor. Thus, 5-ClSor has a moiety of -Cl, whereas Bisor is a homo bi-anthraquinone formed by two monomers of Sor. Probably these differences in chemical structure could explain the different

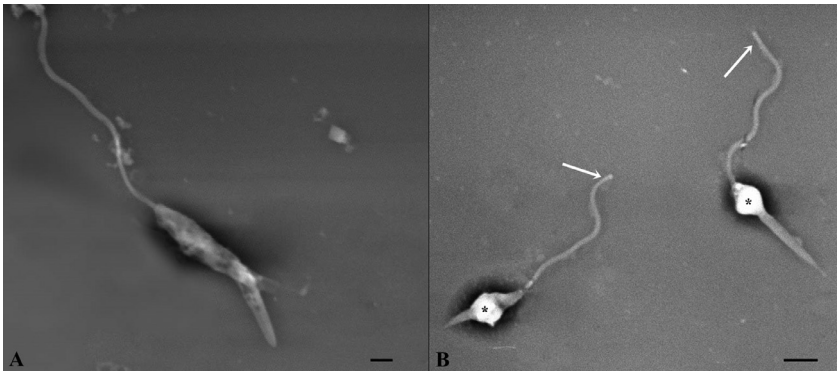

Fig. 7. Electron-micrographs of *L. amazonensis* promastigotes before (A) and after bisoranjidiol-mediated ApPDI (B). Observe the rounding of cell body (*) and shortening of flagella (arrows) in B. Bars represent 5 μM in (A) and 2 μM in (B).

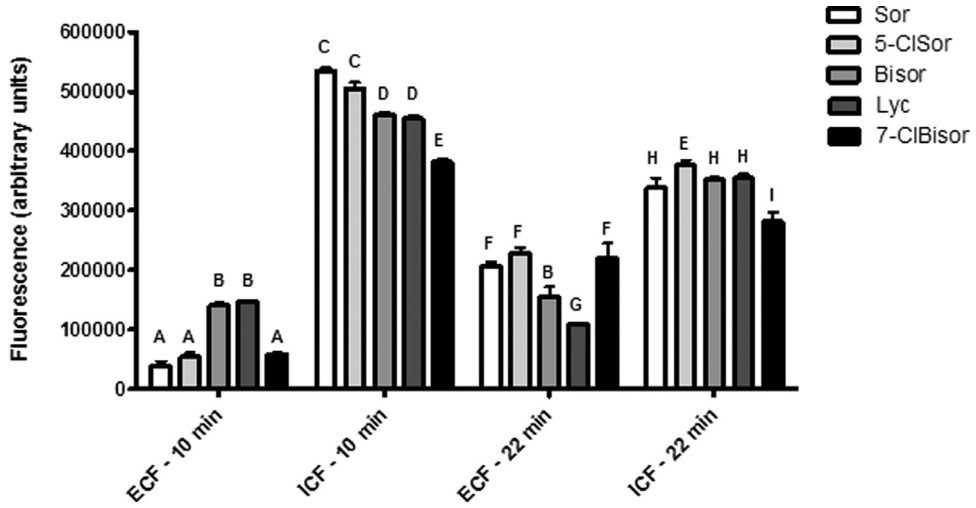

Fig. 8. Relative intracellular (IC) and extracellular (EC) fluorescence of AQs in *La*-LUC at 10 and 22 min of incubation ($n = 3$). Different letters denote statistically significant differences ($p < 0.05$).
Sor: soranjidiol; 5-ClSor: 5-chlorosoranjidiol; Bisor: bisoranjidiol; Lyc: lycionine; 7-ClBisor: 7-chlorobisoranjidiol.

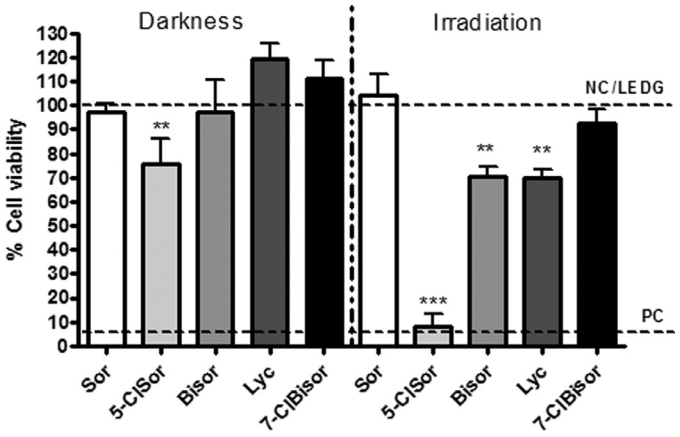

Fig. 9. Percentage of cell viability of fibroblasts treated with natural AQs and violet-blue LED (410 ± 10 nm, irradiance: 50 mW/cm^2) ($n = 3$). $^{**}p < 0.01$/ $^{***}p < 0.001$ *vs.* negative control.
NC: negative control. PC: positive control. LEDG: treated group with LED. —:% cell viability reached by NC, PC and LEDG. Sor: soranjidiol; 5-ClSor: 5-chlorosoranjidiol; Bisor: bisoranjidiol; Lyc: lycionine; 7-ClBisor: 7-chlorobisoranjidiol.

photoinactivation kinetics observed for AQs in this work. In addition, it was demonstrated that monomers of AQ (Sor and 5-ClSor) are relatively efficient singlet oxygen ($^1O_2$) producers compared to phenalenones (Núñez-Montoya et al., 2005), whereas Bisor stands out because it increases the $O_2^{\cdot -}$ production (Comini et al., 2007).

The other two bi-AQs, Lyc and 7-ClBisor, did not show a significant reduction in parasite viability. In fact, they only exhibited a slight decrease at 450 nm that could be related to the presence of absorption peaks at higher wavelengths (see Fig. 2). Both structures are hetero-bi-AQs formed by two monomers; one of them is Sor, and the other, is a monomer different of Sor linked in position 5-5´. It is known that bi-AQs are less efficient in the production of $^1O_2$ because they present a deactivation mechanism given by the rotation of the 5,5′ bridge (Turro, 2001). In addition, Lyc is not a good ROS producer and 7-ClBisor generates less $O_2^{\cdot -}$ than Bisor (Dimmer et al., 2017).

Our group have demonstrated that PDI of *Staphyloccocus aureus* by Sor and Bisor is related to photodynamic generation of singlet oxygen and radicals (Comini et al., 2011), whereas *Candida tropicalis* biofilm reduction is mainly mediated by radical formation (Marioni et al., 2017). Therefore, both photodynamic reaction types could be contributing to the mechanisms of our findings.

Morphological changes following ApPDI were expected. In fact, some authors have shown that PDI can lead *L. amazonensis* to apoptotic death due to oxidative stress (Aureliano et al., 2018). Apoptosis in protozoa is characterized by structural changes, which encompass cell retraction and reduction of cell size (Jimenez-Ruiz et al., 2010). Thus, our data suggest that apoptosis could be involved in AQ-mediated ApPDI.

We hypothesized that the uptake of the AQs by the parasite could clarify our results. Previous studies showed that AQs have LogP between 1.29 and 2.83, indicating that those derivatives have passive diffusion through cellular layer (Comini et al., 2006). Thus, we could assume that bi-AQs (Bisor, Lyc and 7-ClBisor), especially those one with voluminous substituents (Cl and CH_2OH moieties, Fig 1), probably limits passive diffusion through the parasite's cell membrane. In contrast, monoAQs can enter into the parasite in a higher ratio compared to bi-AQs, supporting the significant antiparasitic activity showed especially by Sor and 5-ClSor. Nevertheless, Bisor and Lyc have similar ICF at 10 min but opposite photodynamic activity. This could be due to low ROS production presented by Lyc with respect to Bisor or subcellular localization of bi-AQs (Oliveira et al., 2011; Comini et al., 2011). In comparison, the low uptake of 7-ClBisor by parasites suggests that the minimum active concentration is not reached inside the parasite.

According to measures of ECF, we can hypothesize that Sor, 5-ClSor and 7-ClSor are pumped out by ABC transporters in *Leishmania* (Leandro and Campino, 2003). However, following 10 min of PIT there is enough AQ to trigger photodynamic reactions. In contrast, Bisor and Lyc probably aggregate to lipophosphoglycan (LPG), the dominant molecule in *Leishmania* surface (Naderer et al., 2004).

One of the requisites for effective antimicrobial PDT treatment is selective targeting of the microbial cells with minimal collateral damage to the surrounding normal cells (Cieplik et al., 2018). Thinking in a topical treatment, we assessed the in vitro cytotoxicity of AQs on human fibroblasts, a mammalian cell line, to evaluate toxic effects of AQ-mediated ApPDI. Results showed that monomeric AQs have opposite effects. Sor exhibited similar viability as NC, while 5-ClSor showed a higher cytotoxicity. This result could be due to the presence of Cl substituent. It is known that this substituent produces an increase in $^1O_2$ production, leading to a higher intracellular damage (Valeur, 2001).

Due to the pronounced action of 5-ClSor in reducing fibroblast viability, we suggest that this AQ could be a new photosensitizer to mediate the photodynamic treatment (PDT) of cutaneous fibrosis, since

PDT has also been reported as an attractive therapeutic strategy for abnormal skin scarring (Mendoza-Garcia et al., 2015; Ud-Din et al., 2013).

Regarding the bi-AQs, Bisor and Lyc reduced fibroblast viability by approximately 30% that is acceptable according to the ISO 10993-5 (International Organization for Standardization, 2009). Finally, 7-Clbisor has no effect on fibroblasts as well as on parasites under the experimental conditions tested. It is a bi-anthraquinone with a voluminous substituent like Cl, which limits penetration as it was demonstrated in uptake assay. Thus, the lack of activity could be associated with a poor penetration into the cells.

Conclusion

This is a first attempt to identify potential AQs to be used in cutaneous leishmaniasis. Taking together, our results indicate that from five tested AQs, Sor and Bisor seem to be the most promising for CL due to its significant selectivity towards parasites when compared to fibroblasts.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by Red de macrouniversidades de América Latina y el Caribe, SeCyT-UNC [Res. n 366/16 and 113/17], CONICET [PIP 2010, Res. n 918/10], FONCYT [PICT 2014 n 2204] and Photonics Institute from Conselho Nacional de Pesquisa e Desenvolvimento [CNPq, grant #465763/2014-6].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2019.152894.

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