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Promising antileishmanial effectiveness of doxorubicin and Doxil against *Leishmania major*: An *in vitro* assay

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ABSTRACT

Objective: To evaluate the effect of doxorubicin and its pegylated liposomal formulation (Doxil, Caelyx) on *in vitro* susceptibility of promastigote and amastigote stages of *Leishmania major*.

Methods: Throughout *in vitro* assays the IC_{50} was calculated in the promastigotes and amastigotes forms in J774 macrophage cell line. Also as cytotoxicity in J774 cell line macrophages.

Results: Doxorubicin and Doxil showed the same activity against promastigote form with IC_{50} values of 10.49 μg/mL and 9.63 μg/mL, respectively. Similarly, the amastigote stage was susceptible at concentration of at least 1 μg/mL when compared to positive control ($P < 0.0001$). Also, cytotoxicity assay against macrophage revealed no toxicity on the host cells at IC_{50} concentrations.

Conclusions: Our findings demonstrated the efficacy of both doxorubicin and its pegylated liposomal formulation on *L. major* at low concentrations. Further researches are needed for evaluating the safety of drugs in animal model particularly as topical formulation.

1. Introduction

Leishmaniasis is a disease ranging from mild self limiting skin lesions to severe fatal visceral forms [1]. Current treatment is based on chemotherapy. Pentavalent antimonials are considered as first line drugs with prolong period of treatment and high toxicity [2]. Second line drugs, including Amphotericin B and Pentamidine are used in antimonial failure. Furthermore, newly designed drug miltefosine and azoles are considered as therapeutic components in the treatment of leishmaniasis [3,4]. Considering adverse side effects of available drugs, the development of a safe, effective and affordable antileishmanial drug is a critical global public-health priority. According to our previous hypothesis about the effect of doxorubicin and Doxil on cutaneous leishmaniasis (CL), we attempt to evaluate their biological effects experimentally [5]. Doxorubicin (Ebedoxo) is an anti-cancer (anti-neoplastic or cytotoxic) drug classified as an anthracycline antibiotic. Several cancers including bladder, breast, head and neck, leukemia (some types), liver, lung, lymphomas,

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mesothelioma, multiple myeloma, neuroblastoma, ovary, pancreas, prostate, sarcomas, stomach, testis (germ cell), thyroid, uterus are treated with doxorubicin. Despite therapeutic effects of doxorubicin as anti-cancer agent, the drug has serious side effects commonly (occurring in greater than 30%) including: Early Side Effects: (within one week after treatment begins). Pain along the site where the medication was given, Nausea or vomiting, Later Side Effects: (within two weeks after treatment begins), Low blood counts. White and red blood cells and platelets may temporarily decrease. This can put patient at increased risk for infection, anemia and/or bleeding.

Pegylated liposomal doxorubicin (Doxil, Caelyx) is a formulation of doxorubicin in polyethylene glycol-coated (Stealth) liposomes with a prolonged circulation time and unique toxicity profile [6]. Liposomes increasing the microvascular permeability and leads to drug accumulation in tumoral tissues during circulation and maximum efficiency. The toxicity of Doxil is different from doxorubicin and can cause dose-dependent mucocutaneous toxicities, mild myelosupression, mild alopecia and vague toxicity for cardiac tissues. Despite the lower single maximum tolerated dose (MTD) of Doxil than doxorubicin, the cumulative MTD dose of Doxil is greater than free doxorubicin [7]. Doxil is used in Kaposi's sarcoma which is sarcoma in HIV-AIDS patients and also has a great effect in treatment of recurrent ovarian cancer. Although Doxil can be used in some types of cancers, but its therapeutic effect in other cancer types and also combination therapy with other drugs is under investigation. Little information is available concerning antileishmanial effects of Doxil and doxorubicin particularly on *Leishmania major* (*L. major*), as main causative agent of CL. So, for the first time, in the present study *in vitro* antileishmanial activities of both drugs are evaluated on *L. major*. This article outlines the effect of Doxil and doxorubicin on *Leishmania* parasite and identification of them as novel antileishmanial agents.

2. Materials and methods

2.1. Drug preparation

Meglumine antimoniate (MA, Glucantime Rhône–Poulenc, France), doxorubicin (Ebedoxo, Iran) and commercially available Caelyx® were obtained from Behestan Darou Company (Tehran, Iran). Also Doxil (Sina doxosome) was obtained from Iranian research company (Sina, Mashhad, Iran). All drug concentrations were prepared in culture medium. Prepared final concentrations for doxorubicin and Doxil were 20, 10, 4, 2, 1 μg/mL. Also MA diluted as a drug of choice [8].

2.2. Parasite culture

L. major promastigotes vaccine strain (MRHO/IR/75/ER) were grown in NNN medium and sub cultured in RPMI-1640 medium (Gibco, UK) supplemented with 20% heat-inactivated fetal calf serum (FCS), antibiotics, and HEPES(25 mM), pH 7.2 at 26 °C.

2.3. Promastigote assay

The susceptibility of promastigotes was carried out according to the method described by Carrio *et al* [8]. Serial dilutions of doxorubicin and Doxil in RPMI-1640 (PH, 7.2) were prepared in 96-well microtiter plate. Promastigotes (1×10^5) were harvested at log phase, and 100 μl of medium was added to each well and incubated at (25 ± 1) °C for 72 h. Promastigotes were cultured in medium with no drug and used as positive control, and medium with no organism was used as blank.

All experiments were performed in triplicate. Briefly serial dilutions of doxorubicin and Doxil were prepared. Final concentrations were 20, 10, 4, 2 and 1 μg/mL. Also MA was prepared in final concentrations of 75 μg/mL. All drugs were added to wells. MTT assay was performed by preparing MTT (Sigma Aldrich, USA) in sterile PBS and 10 μl of prepared solution was added in each well, incubated at (25 ± 1) °C for 3 h. The reaction was stopped by using isopropyl alcohol and the optical density was read by ELISA reader (Synergy H1, BioTeck) at 570 nm with filter 630 back ground. The IC_{50} values were calculated using CalcuSyn version 2 software (Biosoft, UK).

2.4. Amastigote assay (ex vivo assay)

Macrophage line J774A.1 was obtained from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Macrophages were kept in RPMI medium. Cells were diluted in medium then viability test was performed by adding 90 μl of trypan blue solution (0.2%) in saline containing 0.01% sodium aside to 10 μl of cell suspension (10^6 cells per Milliliter). After 2 min, cells were counted under light microscope, and viability was calculated as follows:

%Viability = (% of live cells/all counted cells) × 100

Briefly, 200 μl of the cells (10^6 cells/mL) was added into 8-chamber slide (SPL. Korea) and incubated at 37 °C with 5% CO_2 for 2 h. Promastigotes (10^7/mL) were added to macrophages and incubated at 37 °C with 5% CO_2 for 24 h. Then serial dilutions of doxorubicin and Doxil (10 μL) in medium was added to each wells of chamber slides and incubated at 37 °C for 72 h. Also, MA was used as a control drug.

Dried slides were fixed with ethanol, stained by Wright-Giemsa and studied under light microscope. Macrophages containing amastigotes with no drugs and macrophages alone were considered as positive and negative controls, respectively. Drug activity was evaluated by counting the number of amastigotes in the macrophages by examining 100 macrophages.

2.5. Cytotoxicity assay

In vitro toxicity against J774.A.1 macrophages was assessed with cells plated in 96-well plates at 2×10^5 cells/well. After cell adherence, the medium was removed and replaced by the media containing IC_{50} concentration of each compound. The plates were incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2. Control cells were incubated with culture medium plus DMSO. Cell viability was determined using MTT colorimetric assay [9].

2.6. Statistical analysis

SPSS was used to analyze the data. ANOVA test, multiple comparison test and *t-test* were used. The IC_{50} values of MA,

doxorubicin and Doxil for both promastigote and amastigote stages were compared using t-test, and $P < 0.05$ was considered as a significant difference.

3. Results

3.1. Promastigote assay

In promastigote viability assay, several concentrations of doxorubicin showed significant decrease ($P < 0.05$) in optical density (OD) as measured by MTT method. The overall growth rate of promastigotes treated with various concentrations of doxorubicin (IC_{50} = 10.49 μg/mL) was a little higher than Doxil (IC_{50} = 9.63 μg/mL). The IC_{50} value of MA was 197 μg/mL, being significantly higher than those of doxorubicin and Doxil ($P < 0.0001$).

3.2. Amastigote assay

The IC_{50} values against amastigote stage for doxorubicin, Doxil and MA were calculated. The results revealed that the activity of Doxil (IC_{50} = 1.68 μg/mL) was two times higher than that of doxorubicin (IC_{50} = 3.12 μg/mL). Its activity was about 20-fold greater than that of MA (IC_{50} = 33.11 μg/mL).

The effect of doxorubicin and Doxil on amastigote stage of parasite was evaluated by the mean infection rate (MIR) of macrophages and also by the mean number of amastigotes in each macrophage. Comparison of the MIR and amastigotes per macrophage showed that Both doxorubicin and Doxil significantly inhibited growth of macrophages and amastigotes by at least 1 μg/mL when compared to positive control ($P < 0.001$) (Tables 1 and 2). Doxorubicin at highest concentration of 20 μg/mL had no statistically significant difference with MA in reducing MIR, but MA reduced MIR more than doxorubicin in further concentrations (Table 1). The difference between doxorubicin in highest concentration (20 μg/mL) and MA in reducing amastigotes per macrophages was statistically significant and doxorubicin was more effective than MA. Otherwise in concentrations of 10, 4, 2 μg/mL no significant difference was observed, but in 1 μg/mL the efficacy of MA significantly was more than doxorubicin (Table 1).

Doxil in highest concentration (20 μg/mL) significantly was more effective than MA in reducing MIR, but in other concentrations, MA significantly was more effective. In reducing amastigotes per macrophages, no statistical significant between Doxil and MA observed, however in lowest concentration (1 μg/mL) the efficacy of MA was significantly more than Doxil (Table 2).

Table 1

Comparison between several concentrations of doxorubicin with control and MA in the effect on *L. major* amastigotes (Mean ± SD).

Concentration (μg)	Mean percent of infected macrophages (MIR)					Mean number of parasite (amastigotes) in macrophages				
	Doxorubicin	MA	P^*	Control (0) μg	$P^\#$	Doxorubicin	MA	P^*	Control (0) μg	$P^\#$
20	31.33 ± 1.54	30.33 ± 2.88	0.98	85.67 ± 2.08	0.00	1.05 ± 0.05	1.70 ± 0.10	0.03	3.66 ± 0.63	0.00
10	37.00 ± 1.73	30.33 ± 2.88	0.01	85.67 ± 2.08	0.00	1.28 ± 0.02	1.70 ± 0.10	0.21	3.66 ± 0.63	0.00
4	42.33 ± 1.52	30.33 ± 2.88	0.00	85.67 ± 2.08	0.00	1.42 ± 0.02	1.70 ± 0.10	0.59	3.66 ± 0.63	0.00
2	54.66 ± 1.15	30.33 ± 2.88	0.00	85.67 ± 2.08	0.00	1.80 ± 0.10	1.70 ± 0.10	0.99	3.66 ± 0.63	0.00
1	58.33 ± 4.04	30.33 ± 2.88	0.00	85.67 ± 2.08	0.00	2.41 ± 0.10	1.70 ± 0.10	0.01	3.66 ± 0.63	0.00

MA = Meglumine antimoniate (Glucantime®), Control = Infected macrophage without any drug, SD = Standard deviation. * P value = Comparison between doxorubicin and MA groups. # P value = Comparison between doxorubicin and control groups.

Table 2

Comparison between several concentrations of Doxil with control and MA in the effect on *L. major* amastigotes (Mean ± SD).

Concentration (μg)	Mean percent of infected macrophages (MIR)					Mean number of parasite (amastigotes) in macrophages				
	Doxil	MA	P^*	Control (0) μg	$P^\#$	Doxil	MA	P^*	Control (0) μg	$P^\#$
20	15.00 ± 3.00	30.33 ± 2.88	0.00	85.67 ± 2.08	0.00	1.19 ± 0.16	1.70 ± 0.10	0.22	3.66 ± 0.63	0.00
10	42.66 ± 2.51	30.33 ± 2.88	0.00	85.67 ± 2.08	0.00	1.47 ± 0.19	1.70 ± 0.10	0.86	3.66 ± 0.63	0.00
4	55.66 ± 2.08	30.33 ± 2.88	0.00	85.67 ± 2.08	0.00	1.91 ± 0.10	1.70 ± 0.10	0.90	3.66 ± 0.63	0.00
2	63.00 ± 2.64	30.33 ± 2.88	0.00	85.67 ± 2.08	0.00	1.56 ± 0.14	1.70 ± 0.10	0.08	3.66 ± 0.63	0.00
1	67.33 ± 4.04	30.33 ± 2.88	0.00	85.67 ± 2.08	0.00	2.45 ± 0.38	1.70 ± 0.10	0.04	3.66 ± 0.63	0.00

MA = Meglumine antimoniate (Glucantime®), Control = Infected macrophage without any drug, SD = Standard deviation.* P value = Comparison between Doxil and MA groups. # P value = comparison between Doxil and control groups.

Table 3

Comparison between several concentrations of doxorubicin with Doxil in the effect on *L. major* amastigotes (Mean ± SD).

Concentration (μg)	Mean percent of infected macrophages (MIR)			Mean number of parasite (amastigotes) in macrophages		
	Doxorubicin	Doxil	P	Doxorubicin	Doxil	P
20	31.33 ± 1.54	15.00 ± 3.00	0.00	1.05 ± 0.05	1.19 ± 0.16	0.18
10	37.00 ± 1.73	42.66 ± 2.51	0.04	1.28 ± 0.02	1.47 ± 0.19	0.18
4	42.33 ± 1.52	55.66 ± 2.08	0.04	1.42 ± 0.02	1.91 ± 0.10	0.06
2	54.66 ± 1.15	63.00 ± 2.64	0.03	1.80 ± 0.10	1.56 ± 0.14	0.06
1	58.33 ± 4.04	67.33 ± 4.04	0.07	2.41 ± 0.10	2.45 ± 0.38	0.06

Statistical difference among doxorubicin and Doxil observed in concentration of 20 μg/mL and Doxil decreased MIR significantly more than doxorubicin ($P < 0.0001$) (Table 3).

The difference in number of amastigotes in macrophages for doxorubicin and Doxil was not significant for all concentrations ($P > 0.05$). The inhibitory effect on MIR for MA and positive control was 30.33 ± 2.88 and was 85.66 ± 2.08 respectively and was statistically significant ($P < 0.0001$).

4. Discussion

In the present study, we evaluated the *in vitro* and *ex vivo* efficacy of anti-cancer drugs, doxorubicin and Doxil in decreasing the growth rate of amastigote and promastigote stages of *L. major*. Despite the vast use of pentavalent antimonials as first line drugs, the efficacy of them has decreased and resistance has occurred in some of endemic areas [10]. Also, prolonged treatment period, painful injections and expensive price in endemic areas were the main reasons of increasing usage of second line drugs like Amphotericin B and Miltefosine. However, the side effects, drug resistance, and relapse ultimately lead to treatment failure.

There is an urgent need for new and more effective drugs. Miltefosine, an alkylphosphocholine derivative, was originally designed as anti-cancer agent but showed antiamoebic, antifungal, and leishmanicidal activity. Miltefosine causes apoptosis by interfering with cell membrane phospholipids metabolism. Also, it can increase the production of gama interferone (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin 12 (IL-12) which play critical role in healing of leishmanial sores [11]. Similarly doxorubicin is an anti-cancer drug with effective use in soft tissue sarcomas of the adult. The effectiveness of monotherapy with doxorubicin is about 20 and its toxicity is increased by combination therapy. Despite its serious side effects in some cases, it is considered the choice in advanced soft tissue sarcomas [12].

Despite the advantages of doxorubicin in tumor cell destruction, serious side effects such as myelosuppression and high toxicity to myocard limits its efficacy. An alternative formulation for reversing toxicity of doxorubicin is pegylated liposomal doxorubicin (Doxil). Clinical and preclinical studies revealed that Doxil can enhance the doxorubicin efficacy along with decreasing the toxicity of free doxorubicin [12].

In the present study, we have evaluated the efficacy of doxorubicin and Doxil on decreasing the MIR and number of amastigotes in infected macrophages. The obtained results were statistically compared with positive control. In addition, the efficacy of MA as a choice drug for leishmaniasis was evaluated on promastigotes and amastigotes as well as doxorubicin and Doxil. Interestingly, both doxorubicin and Doxil showed significant effect on decreasing both MIR and the number of amastigotes per macrophage in compare with control ($P < 0.0001$). The obtained IC_{50} values against promastigotes for doxorubicin and Doxil were 10.49 and 9.63 μg/mL, respectively. As the IC_{50} value of MA was 197 μg/mL it is estimated that the activities of doxorubicin and Doxil are at least 18-fold superior than MA. This revealed that the therapeutic effect of both doxorubicin and Doxil were stronger than drug of choice MA. Comparing IC_{50} values among doxorubicin, Doxil and MA showed that both doxorubicin and Doxil inhibits the growth of promastigote and amastigote stages of *L. major* at lower concentrations respect to the traditional drug MA. As the IC_{50} value of doxorubicin (IC_{50} = 3.12 μg/mL) is 1.8 folds higher than that of Doxil (IC_{50} = 1.68 μg/mL) for amastigote stage, we conclude that Doxil is more effective than doxorubicin on amastigote stage. The calculated IC_{50} value of MA (IC_{50} = 33.11 μg/mL) against amastigote stage is significantly (≈10 times) higher than those of doxorubicin and Doxil.

Similar to amastigote stage, Doxil inhibit the growth of promastigote at slightly lower concentration (IC_{50} = 9.63 μg/mL) in comparison to doxorubicin (IC_{50} = 10.49 μg/mL). A significant difference was found among doxorubicin and Doxil in MIR at concentration of 20 μg/mL, and Doxil decreased MIR significantly more than doxorubicin ($P < 0.0001$) (Table 3).

Remarkably, the difference of amastigote's number inside the macrophages was not statistically significant between Doxil and doxorubicin. Our results are agreement with the reported data for *Leishmania donovani* (*L. donovani*). In a study carried out in 2013, the efficacy of core loaded nanocapsules with high payload of doxorubicin was almost 1.9 fold higher than that of free doxorubicin [13].

Otherwise, some studied on *L. donovani* revealed the efficacy of doxorubicin on promastigote and amastigote stages at lower concentrations compared to our study. As in a study carried out in India on *L. donovani*, the researchers indicated that promastigotes and amastigotes were destroyed by doxorubicin at 0.43 μM and 0.86 μM, respectively [14]. Their results were close to our findings as in our study the promastigotes were destroyed at 10 μg/mL (5.7 μM).

It is well known that liposomal formulation of doxorubicin increases the potential anti-tumor and leishmanicidal effect of drug in comparison to free doxorubicin [13]. The researchers believe that natural homing of liposomes in macrophages provokes the anti-tumor effect of component by stimulating the immunomodulators entrapped by liposomes. This leads to killing the resistant *Leishmania* spp. As mannose acts as receptor for taking *Leishmania* parasites, same function performed when manosated doxorubicin were added to parasitized macrophages. In a study carried out by Kole *et al* [15], the researchers revealed that Mandoxosome was the most effective than free doxorubicin and liposomal form (doxosome). The effective dosage (IC_{50}) for Mandoxosome, free doxorubicin and doxosome were 3.4, 480 and 9.6 ng/mL respectively. Empty liposome was tested for leishmanicidal effect and was not toxic for intracellular amastigotes. There was no toxic effect on macrophages *in vitro* by doxosome and mandoxosome at the highest concentration of doxorubicin (100 ng/mL) used as liposomal incorporated drug [15].

Similarly in other study, doxorubicin significantly inhibited the growth rate of *L. donovani* promastigotes at concentrations up to 250 ng/mL, but surprisingly the liposomal form of drug induced little effect even at 1 μg/mL. There are little studies about doxorubicin and Doxil on *Leishmania* parasites.

In general, our study revealed the relevant effect of both doxorubicin and Doxil against *L. major* promastigotes and amastigote stages. In particular, the activity of Doxil was about 20-fold greater than that of MA as drug of choice. These drugs were not toxic at IC_{50} concentrations for macrophages and that's good news for more affect of component on *Leishmania* spp selectively. Comparison between Doxil and doxorubicin revealed that except at highest concentration of 20 μg/mL, doxorubicin decreased MIR more than Doxil. As there was no statistical difference in decreasing amastigotes per macrophage between Doxil and doxorubicin, the

advantage of Doxil than doxorubicin seems to be more circulation time and better accumulation in the cells which decreases toxic side effects.

Further investigations are needed for better definition of drugs as antileishmanial and vast usage of them in human. Our results offer the potential use of Doxil and doxorubicin in topical formulation with MA for effective anti-leishmanial therapy.

Conflict of interest statement

Authors declare that have no competing interests.

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