HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2016.09.014

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

Azar Shokri[1,4], Javad Akhtari[2], Masoud Keighobadi[3], Mahdi Fakhar[4✉], Saeed Hosseini Teshnizi[5], Saeed Emami[6], Sajede Sadjjadian[1]

[1]*Students Research Committee, Department of Parasitology and Mycology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*

[2]*Immunogenetic Research Center, Department of Physiology and Pharmacology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*

[3]*Students Research Committee, Department of Medicinal Chemistry and Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran*

[4]*Molecular and Cell Biology Research Center, Department of Parasitology and Mycology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*

[5]*Clinical Research Development Center of Children Hospital, Hormozgan University of Medical Sciences, Bandar Abbas, Iran*

[6]*Department of Medicinal Chemistry and Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran*

ARTICLE INFO

Article history:
Received 15 May 2016
Received in revised form 7 Apr 2017
Accepted 20 May 2017
Available online xxx

Keywords:
Leishmania major
Doxorubicin
Doxil
In vitro

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,

First author: Azar Shokri, Students Research Committee, Molecular and Cell Biology Research Center, Department of Parasitology and Mycology, School of Medicine, Mazandaran University of Medical Sciences, Farah Abad, Sari 481751665, Iran.
Tel/Fax: +981133543248
E-mail: azar_sh1969@yahoo.com
✉Corresponding author: Mahdi Fakhar, Molecular and Cell Biology Research Center, Department of Parasitolgy, School of Medicine, Mazandaran University of Medical Sciences, Farah Abad, Sari 481751665, Iran.
Tel/Fax: +981133543248
E-mail: mahdif53@yahoo.com
Peer review under responsibility of Hainan Medical University.
Foundation project: The authors would like to thank of financially supported by Vice Chancellors for Research and Technology of Mazandaran University of Medical Sciences (project number: 1919).

Please cite this article in press as: Shokri A, et al., Promising antileishmanial effectiveness of doxorubicin and Doxil against *Leishmania major*: An *in vitro* assay, Asian Pacific Journal of Tropical Medicine (2017), http://dx.doi.org/10.1016/j.apjtm.2016.09.014

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,

Please cite this article in press as: Shokri A, et al., Promising antileishmanial effectiveness of doxorubicin and Doxil against *Leishmania major*: An *in vitro* assay, Asian Pacific Journal of Tropical Medicine (2017), http://dx.doi.org/10.1016/j.apjtm.2016.09.014