IN VITRO EVALUATION OF CHLOROQUINE AND CLARITHROMYCIN AGAINST LEISHMANIA TROPICA

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ABSTRACT

Objective: To evaluate the efficacy of chloroquine and clarithromycin against Leishmania tropica KWH23.

Methodology: This experimental study was conducted at the Institute of Basic Medical Sciences, Khyber Medical University Peshawar, Pakistan. Pre-formed cultures of Leishmania tropica KWH23 were inoculated in Roswell Park Memorial Institute (RPMI) 1640 medium and incubated at 24ºC for 7 days. Stock solution of chloroquine and clarithromycin of 1000 µg/ml were prepared and further diluted serially. Approximately, 180 µl of RPMI was added in different wells of 96 well microtiter plates. For the test compound, 20 µl was added in the first well and then serially diluted to keep the final volume up to 180 µl. Subsequently 20 µl was discarded from the last well. About 100 µl of parasites (2×106 cells/ml) were added in each well and 2 rows were left for positive and negative control. Dimethyl sulfoxide (DMSO) was taken as negative control and was serially diluted in RPMI 1640 medium. Amphotericin B was taken as positive control. Microtiter plates were incubated at 24° C for 72 hours. Assay was performed in triplicate. After the incubation period, 20 µl was taken from each dilution, put on improved Neubaur counting chamber and live parasites were counted under microscope. IC_{50} values of the compounds possessing anti leshmanial activity were calculated by GraphPad Prism 5 software.

Results: The IC_{50} values of chloroquine dosage and pure forms (without excipients) against the promastigote of Leishmania tropica KWH23 were 0.023µg/ml and 0.019 µg/ml respectively while IC_{50} values of clarithromycin dosage and pure form were 4.548 µg/ml and 27.13 µg/ml respectively. Clarithromycin inhibited promastigotes growth but its IC_{50} was higher than chloroquine. The IC_{50} values of combination of chloroquine and clarithromycin dosage (500 mg) and pure form were recorded as 0.049 µg/ml and 0.0023 µg/ml respectively.

Conclusion: Both chloroquine and clarithromycin are potential drugs for the treatment of cutaneous leishmaniasis.

Key Words: Chloroquine, Clarithromycin, Cutaneous leishmaniasis, Leishmania tropica.

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INTRODUCTION

Leishmaniasis is caused by a genus of Leishmania parasite, and is transmitted to mammals by the bite of female sand flies[1]. It is an obligatory intracellular parasite of reticuloendothelial cells in the macrophages of liver, spleen and bone marrow. The three clinical forms of leishmaniasis are cutaneous, mucocutaneous and visceral, caused by different types of the parasite species [2]. Leishmaniasis is prevalent in 88 countries affecting 12 million people worldwide, out of which 1.5-2.0 million cases of cutaneous leishmaniasis and 500,000 visceral leishmaniasis occurs annually[3]. The number of deaths is 70,000 annually mainly due to visceral leishmaniasis[2].

Cutaneous leishmaniasis is a major problem in Pakistan, including Khyber Pakhtunkhwa because of the emigration of displaced people in various cities from the hilly areas of Pakistan and neighbour country Afghanistan[4]. In Pakistan two different Leishmania species are involved in causing cutaneous leishmaniasis. L. tropica gives rise to anthroponotic cutaneous leishmaniasis (CL) and L. major results in zoonotic CL[5]. Considering the treatment preferences, pentavalent antimonials are the prominent drugs of treating the disease, but as far as the drug availability is concerned, there is shortage

of these drugs in Pakistan. Currently, glucantime used in treatment of Leishmaniasis, is neither available nor registered in Pakistan. The situation needs investigation for a safe drug that is easily available to the community everywhere.

So far, the intracellular active antibiotics and antimalarial agents have been investigated against the Leishmania amastigotes and promastigotes[6,7]. Chloroquine is an antimalarial drug that raises the lysosomal pH and precludes the fusion of endosome with lysosome. A preliminary study of 10 patients, on intra lesional chloroquine showed 100% response[8]. A comparative study of cutaneous Leishmaniasis using intra-lesional chloroquine vs meglumine antimoniate showed 100% satisfactory response[9]. Mefloquine and chloroquine also showed good results against cutaneous Leishmaniasis [10].

Clarithromycin is macrolide derivative antibiotic which blocks protein synthesis of susceptible organisms. It concentrates in tissue macrophages where amastigote reside[11]. Azithromycin and clarithromycin concentrations were observed 100 to 200 times higher in macrophage tissues as compared to blood serum levels. High tissue concentrations pose high antibiotic activity of azithromycin and clarithromycin[12]. Various studies have reported the efficacy of azithromycin and clarithromycin against different parasites including Pneumocyctis carnii, Toxoplasma gondi, Cryptosporidium parvum and Plasmodium species[13]. This study was aimed at the anti leshmanial activity of the above two drugs against Leishmania tropica, the commonly reported species of cutaneous leishmaniasis in Khyber Pakhtunkhwa, Pakistan.

METHODOLOGY

Drugs and control

The dosage and pure forms of clarithromycin and chloroquine dosage were evaluated against Leishmania tropica KHW23. Chloroquine and clarithromycin dosage were purchased from local market and the pure forms were obtained from local manufacturing pharmaceutical in Peshawar, Pakistan. The efficacy was determined through micro-dilution method. Chloroquine 10 mg was dissolved in 1 ml saline and clarithromycin 10 mg was first dissolved in 0.5 % DMSO (0.05ml DMSO plus 9.95 ml distilled water). Amphotericin B was used as standard anti leishmanial drug and 1% DMSO was used as a negative control.

Parasites culture

The stock culture of Leishmania tropica KWH 23 was obtained from Infectious Disease Laboratory, Quaid -I-Azam University, Islamabad. Promastigotes were cultured in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (Biowest) and incubated at 24°C. Parasites were cultivated until log phase was reached.

Treatment of promastigotes with drugs

Microtiter culture plates of 96 wells were used for assay. Two different series of clarithromycin (dosage and pure form) concentrations were prepared. The first concentration series of the drugs was 2000µg, 400µg, 80µg, 16µg, 3.2µg and 0.64µg which were five folds dilution. The 50 µl of drug was used in the first well of 96 well culture plates and serial dilutions were performed up to the last well and 50 µl was discarded from the last well. Other concentrations of clarithromycin (dosage and pure) were 1000µg, 100µg, 10µg, 1µg, 0.1µg and 0.01µg which were ten folds dilution. In the pure chloroquine and dosage as well as combination of both drugs the concentrations were 1000µg, 100µg, 10µg, 1µg, 0.1µg, 0.01µg, 0.001µg and 0.0001µg respectively. The 25 µl of drug used in these concentrations after serial dilution up to the last well and discardeding 25 µl from the last well. The promastigotes that were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum were counted with haemocytometer and adjusted as 1×106 promastigotes /ml in each well having a final volume maintained to 250 µl. The assays were performed in triplicate and mean values were calculated. The culture plates were labelled and incubated at 24°C for 72 hours. After 72 hours, the cultured parasites were diluted 1/100 with phosphate buffer saline, and live and dead parasites were counted using Neubaur chamber.

RESULTS

The anti leishmanial activity of chloroquine dosage and pure form was determined against L. tropica KHW23 promastigotes at a concentration of 1000-0.0001 µg. The IC_{50} values were calculated by GraphPad Prism software version 6. The IC_{50} values of chloroquine dosage and pure form against the promastigotes of Leishmania tropica KWH23 were recorded as 0.023µg/ml and 0.019µg/ml respectively. The chloroquine both in pure and dosage form showed anti leishmanial activity against promastigotes of Leishmania tropica KWH 23. (Table1 and Fig.1)

Clarithromycin pure and dosage form were used at a concentration ranging from 0.01µg/ml to 2000-µg/ml and their IC_{50} were calculated as 27.13 µg/ml and 4.548 µg/ml. The IC_{50} of dosage drugs was found lower than pure form. The dosage clarithromycin was found effective in inhibition of promastigotes growth (Table 2 and Fig 2).

DISCUSSION

Treatment preferences for cutaneous leishmaniasis in Pakistan are a major health problem. It is extremely important to select the best treatment choice which

Table 1: The IC_{50} values of Chloroquine (dosage and pure) and Amphotericin B

Drugs	IC_{50} ug/ml
Chloroquine dosage (250 mg)	0.023
Chloroquine pure	0.019
Amphotericin B	0.02
DMSO (1%)	Nil

Table 2: The IC_{50} values of clarithromycin (dosage and pure) and amphotericin B

Drugs	IC_{50} ug/ml
Clarithromycin dosage (500mg)	4.548
Clarithromycin pure	27.13
Amphotericin B	0.02
DMSO (1%)	Nil

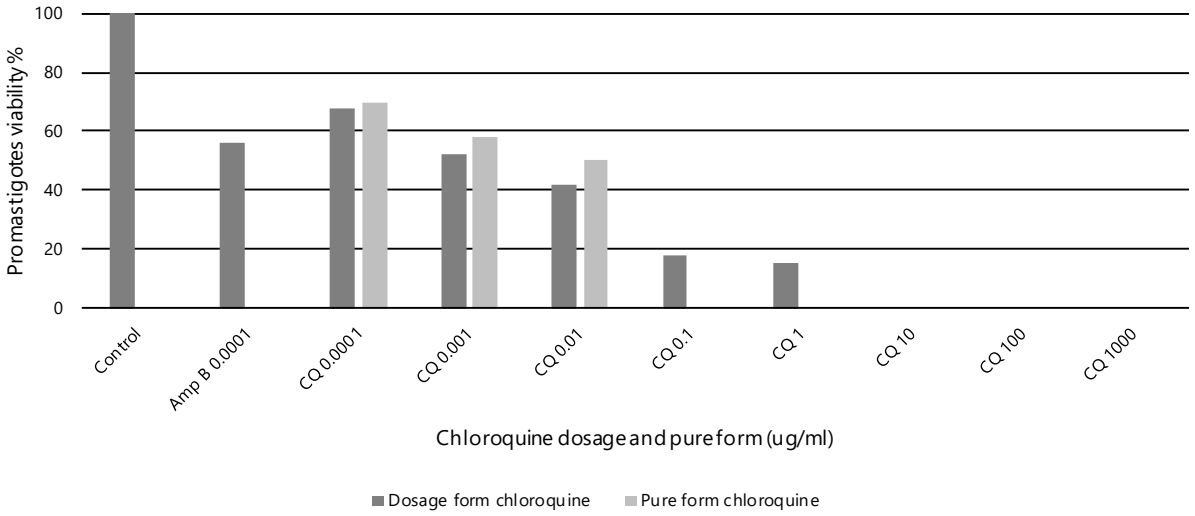

Figure 1: The efficacy of chloroquine dosage and pure form against Leishmania tropica KHW23 promastigotes

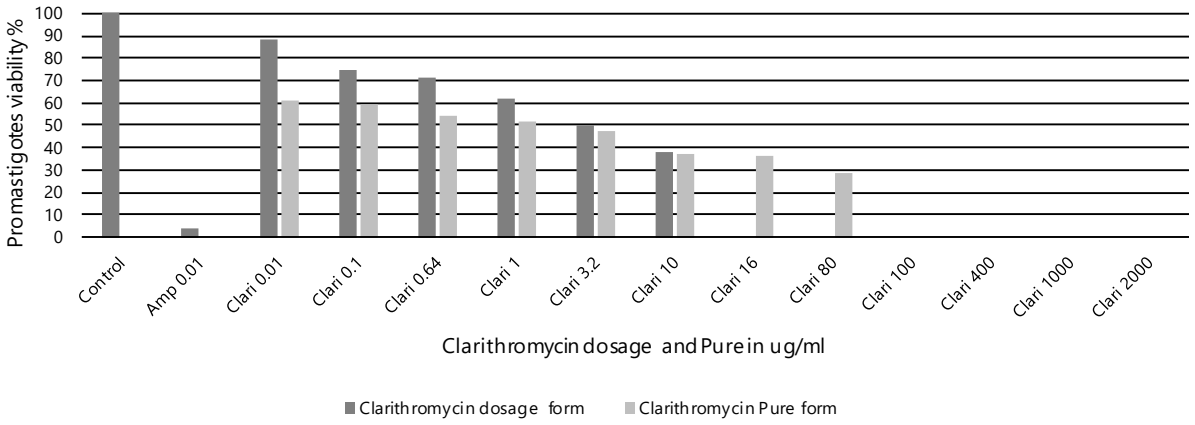

Figure 2: Efficacy of clarithromycin dosage and pure at different concentration against Leishmania tropica KHW23 promastigotes

is safe, less expensive and easily available for the patients. Chloroquine is an antimalarial drug that raises the lysosomal pH and precludes the fusion of endosome with lysosome. The efficacy of chloroquine was determined by using intra lesional injection and was found very effective in curing skin lesion[15]. In vitro efficacy of artesunate, chloroquine, hydroxychloroquine, mefloquine and primaquine has also been evaluated against Leishmania amazonensis promastigotes at a concentration 0.6-50 µM for 72 hours. Chloroquine and primaquine did not effectively inhibit promastigote growth at this concentration while the artesunate and primaquine showed effective inhibition less than 50%. Interestingly chloroquine and its derivatives were sig-

nificantly effective against amastigotes than promastigotes because of high accumulation of the drug in the acidic compartment of cells[16]. The result of chloroquine against Leishmania amazonensis showed deviation from our results. It may be due to the varying genetic constitution of different Leishmania species' sensitivity to the same drug. The IC_{50} value of pure and dosage chloroquine are 0.019 µg/ml and 0.023 µg/ml respectively. The IC_{50} of pure and dosage chloroquine was found approximately equal. The pure form chloroquine gave us an encouraging result that is equivalent to the standard anti leishmanial drugs.

Clarithromycin is an antibiotic that inhibits protein synthesis intracellularly concentrated in the tissues of phagocytic cells, where amastigotes exist. The macrolides have been found effective in eliminating intracellular microorganisms' growth[17]. The efficacy of azithromycin was evaluated against L. major in vitro and in vivo. The ED_{50} was found 12 µg/ml against L, major promastigotes 12. Anti leishmanial activity of azithromycin was evaluated on promastigote and amastigote of L. amazonensis, L. (Viannia) braziliensis and L. chagasi. The result of azithromycin was dose dependent which shows agreement with previous study. Clarithromycin activity was also found dose dependent that compensates the parasite resistance[18]. We used a concentration of 0.01 -2000 µg/ml of clarithromycin dosage and pure form and IC_{50} were calculated as 4.548 µg/ml and 27.13 µg/ml respectively. The IC_{50} determined for dosage form was found lower than pure form. The result showed that clarithromycin was efficient in both dosage and pure form against Leishmania tropica KWH 23. Ameneh et al. have shown that in in vitro, promastigotes were affected by clarithromycin in both liposomal and non-liposomal forms. The ED_{50} values were 169 and 253.6 µg/ml for liposomal and non-liposomal forms. The liposomal form showed oromising result than non-liposomal form. Clarithromycin showed in vitro activity against both liposomal and non-liposomal forms of L. major promastigote[19]. In comparison to this study, IC_{50} value found in this report was also found effective against Leishmania tropica KWH 23.

CONCLUSION

Chloroquine and clarithromycin exhibited the best anti leishmanial activity against Leishmania tropica KWH23 and are potential candidate drugs for the treatment of cutaneous leishmaniasis.

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CONTRIBUTORS

MK conceived the idea, wrote initial manuscript carried out laboratory work and collected data. JF and IK assisted in laboratory work, manuscript writing, data collection and analysis. MZ and IU searched literature, wrote introduction and discussion sections and finalized the manuscript. All authors contributed significantly to the submitted manuscript.