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Improved treatment of visceral leishmaniasis (kala-azar) by using combination of ketoconazole, miltefosine with an immunomodulator—Picroliv

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ABSTRACT

Visceral leishmaniasis (VL) caused by the parasite *Leishmania donovani*, is a potentially fatal disease. It is characterized by prolonged fever, enlarged spleen and liver, substantial weight loss and progressive anemia. Available drugs are toxic, costly and require prolonged treatment duration *viz*; 28 days of oral treatment with miltefosine, 30 days infusion with Amphotericin B and 21 days intramascular with paromomycin sulfate. Drug combination for VL clinically proved to shorten the duration of treatment. The efficacy of drugs is also compromised due to suppression of immune function during the course of infection. To combat this situation leishmanicidal efficacy of already marketed standard antifungal drug, ketoconazole under the approach of 'therapeutic switching' in combination with standard antileishmanial drug, miltefosine and a potent immunomodulator agent, picroliv were evaluated in *L. donovani*/hamsters model. Animals treated with combination of ketoconazole (50 mg/kg, 5 days, po) + miltefosine (5 mg/kg, 5 days, po) showed augmentation in efficacy against *leishmania* parasite (72%) in comparison to those treated with ketoconazole (54.67%) and miltefosine (54.77%) separately. Co-administration of picroliv (10 mg/kg, 12 days, po) has further enhanced antileishmanial efficacy from 72% to 82%. Significant generation of ROS, RNS and H_2O_2 and increased phagocytosis was observed in animals treated with ketoconazole + miltefosine; however, addition of picroliv to this combination did not alter the level of metabolites and phagocytosis due to its antioxidative and nonleishmanicidal characteristics, respectively. Significant rise in cell mediated immunity witnessed in this group reveals the role played by the immunomodulator, picroliv and justifies the significance of enhanced cell mediated immunity in the therapy. These findings suggest a new strategy for leishmanial chemotherapy at reduced cost and toxicity.

1. Introduction

Visceral leishmaniasis (VL) also known as kala-azar is the most serious form of leishmaniasis, a disease caused by a sand fly borne protozoan parasite. VL causes bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia and is fatal, if not treated. Although the disease is endemic in more than 60 countries, around 59,000 deaths, with 200 million people at risk, 90% of the 500,000 cases every year happen in five countries: India, Bangladesh, Nepal, Sudan, and Brazil (Pandey et al., 2009; Griensven et al., 2010).

The spreading resistance of the parasite towards the standby antimonial drugs, the severe toxicity of most drugs in use, including orally effective drug miltefosine and the emergence of Leishmania/HIV co-infection as a new disease entity has triggered a continuous search for alternative therapies (Seifert and Croft, 2006). One possible strategy that has been successfully used for malaria and tuberculosis is 'combination therapies'. It is also a cost-effective alternative to current monotherapy for VL. For example according to the Meheus et al. (2010), co-administration of miltefosine with paromomycin exhibited a cost of $72.9 per patient treated whereas drug cost of individual treatment with miltefosine and paromomycin is $130.2 and $96.6 per patient treated, respectively. Another approach is 'therapeutic switching' that has been applied for sleeping sickness and chagas disease (Croft, 2008). It refers to "alternative drug use" discoveries which differ from the original intent of the drug. Amphotericin B, paromomycin and miltefosine are very successful examples of "new drugs from old" in VL (Croft and Sutherland, 2002).

Azole antifungal agents have been used as antileishmanial agents since 1980s as they can specifically inhibit ergosterol synthesis by blocking 14α-demethylase. N-substituted azoles (ketoconazole, miconazole, econazole, fluconazole, and itraconazole) are well-tolerated drugs that are potentially active against

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Leishmania, but their use in the treatment of cutaneous leishmaniasis (CL) and VL has produced conflicting results (Srinivas et al., 2009). Both ketoconazole and fluconazole have undergone clinical evaluation against VL in India (Sundar and Chatterjee, 2006). However, combination of ketoconazole and allopurinol was used clinically by Hueso et al. (1999) and Llorente et al. (2000) in the patients of VL treated with glucantime who developed abdominal pain, nausea, vomiting and acute pancreatitis respectively. Halim et al. (1993) also reported that a case of a renal transplant recipient who developed pancreatitis during stibogluconate treatment for VL was also successfully treated with this combination.

Cure of leishmaniasis, even during chemotherapy, appears to be dependent upon the development of an effective immune response that activates macrophages. Combination therapy employing immunomodulators with antileishmanials can boost immune system to fight against this devious pathogen that evades the immune response by attenuating pro-inflammatory signalling pathway. Most frequently used immunomodulators were BCG (Bacille Calmette–Guèrin), MDP (muramyl dipeptide), trehalose mycolate, glucan, tuftsin, and protein-A which have a direct effect on the macrophages (Croft and Yardley, 2002). Previous reports showed that biological immunomodulators such as interferon (IFN)-γ (Murray et al., 1988) and imiquimod (Buates and Matlashewski, 1999; Arevalo et al., 2001) have enhanced the activity of antimonials in the treatment of VL. Antileishmanial efficacy of miltefosine was also found to be enhanced when given in combination with a potent immunomodulator, picroliv (Gupta et al., 2005). Based on these findings, we have explored the effect of picroliv on a combination of ketoconazole and miltefosine. Picroliv, standardized fraction from the alcoholic extract of root and rhizome of *Picrorhiza kurroa*, is a mixture of iridoid glycosides containing 55–60% of picroside-I and kutkoside in a ratio of 1:1.5. Constituents of picrorhiza with hepatoprotective, immunomodulatory and related activities have been described in detail recently by Verma et al. (2009). The immunostimulant activity and hepatoprotective potential of picroliv was demonstrated in mice by Puri et al. (1992) and Rajeshkumar and Kuttan (2000) respectively. It is under Phase III trial and is expected to be in market very soon for human use. The objective of the present study was to assess the antileishmanial effects of miltefosine, ketoconazole and picroliv individually and in different combinations in *in vitro* and *in vivo* conditions. Study also includes monitoring of alterations in biochemical parameters like production of toxic oxygen metabolites, lymphocyte transformation test and phagocytosis.

2. Materials and methods

2.1. Parasite

The WHO reference strain of *Leishmania donovani* (MHOM/IN/80/Dd8), obtained from Imperial College, London (UK) was maintained as promastigotes *in vitro* and as amastigotes in golden hamsters (*Mesocricetus auratus*) (Gupta et al., 2005). Luciferase transfected *L. donovani* promastigotes are also being maintained in this laboratory (Div. of Parasitology, CDRI, Lucknow) since 2005 as detailed by Sunduru et al. (2009).

2.2. Animals

Inbred hamsters weighing 40–45 g of both sexes were used for the study. All experiments were conducted with the Institutional Animal Ethics Committee guidelines for the use of handling animals. Throughout the study animals were housed in climate ($23 \pm 2\,^{\circ}C$; relative humidity 60%) and photoperiod controlled (12 h light–dark cycles) animal quarters. They were fed standard rodent pellet supplemented with grain and had free access to drinking water.

2.3. Compounds

Ketoconazole was received from Sigma–Aldrich, India, whereas miltefosine was purchased from Synpha*B*ase AG, Switzerland. Picroliv was obtained from ethanolic extract of *P. kurroa* (Dwivedi et al., 1992). It contains several compounds out of which picroside-I and kutkoside are two major bioactive constituents (together not less than 55% of total in the dried substance). It is a light yellowish-brown amorphous powder with characteristic odour, bitter in taste. It is insoluble in hexane, sparingly soluble in benzene, chloroform and ethyl acetate but soluble in acetone, ethanol, methanol and water.

For *in vitro* evaluation, ketoconazole stock was prepared in dimethyl sulfoxide (DMSO) and miltefosine stock was prepared in deionized water and test concentrations were diluted from stocks in appropriate medium immediately before the assays. For the *in vivo* part of the study, picroliv and miltefosine was dissolved in deionized water and aqueous solution of ketoconazole was prepared by suspending the accurately weighed drug in a standard suspension vehicle of 0.5% carboxymethyl cellulose, 0.60% benzyl alcohol, 0.48% Tween-80 in 0.9% sodium chloride.

2.4. Cell line

Mouse macrophage cell line (J774.A-1) was maintained in RPMI medium (Sigma), supplemented with 10% foetal calf serum and 4 μg/ml gentamycin.

2.5. In vitro screening

2.5.1. Anti amastigote activity

For assessing the activity of compounds against the amastigote stage of the parasite, mouse macrophage cell line (J774.A-1) infected with promastigotes (expressing luciferase firefly reporter gene) was used. The test material in appropriate concentrations (400–1.56 μM) in complete medium was added after replacing the previous medium and the plates were incubated at 37 °C in a CO_2 incubator for 72 h. Inhibition of the parasite growth was determined by comparison of luciferase activity of drug treated with that of untreated controls as described by Porwal et al. (2009).

2.5.2. Evaluation of individual drug responses in vitro

In order to assess IC_{50} (50% inhibition of parasite), all the drugs were tested against intracellular amastigotes in various concentrations. Miltefosine and picroliv were tested at concentrations ranging from 1.56 μM to 12.5 μM and ketoconazole from 50 to 400 μM. Two replicates of each experiment were carried out. Picroliv did not show any antileishmanial efficacy against amastigotes and therefore it was not included in *in vitro* combination trial. Sub curative doses of miltefosine and ketoconazole (3.12 μM and 200 μM) were used for further combination study.

2.6. In vivo screening

The evaluation in hamsters was carried out in accordance with the method described by Bhatnagar et al. (1989). Five to six animals were used for each agent and the same numbers were kept as untreated controls. The drug treatment was given by oral route. To assess the effect of drugs, spleen biopsy was performed on each animal on 7th day post treatment and amastigote counts were assessed by Giemsa staining (Gupta et al., 2005). The percent inhibition