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Chemoprevention of Leishmaniasis: *In-vitro* antiparasitic activity of dibenzalacetone, a synthetic curcumin analog leads to apoptotic cell death in *Leishmania donovani*☆

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ABSTRACT

Curcumin is the major phenolic compound found in turmeric, a dry powder of rhizomes and roots of the plant, *Curcuma longa* L., which is widely used as spice and food colorant around the world, and in herbal medicinal practice in Asian countries. The present study reports the leishmanicidal activity of trans-dibenzalacetone (DBA), a synthetic monoketone analog of curcumin, against Leishmania donovani parasites. We for the first time report the antiproliferative effect of a curcumin analog (DBA) on the intracellular amastigotes of *L. donovani*, the clinically more relevant stage of the parasite than its promastigotes stage. The leishmanicidal effect of DBA was further confirmed by scanning and transmission electron microscopies. Cell growth was arrested in G0/G1 phase with increased concentration of cytosolic calcium and dissipation of mitochondrial membrane potential. Further, the unique trypanothione/trypanothione reductase (TR) system of Leishmania cells was significantly inhibited by DBA. This economically synthesizable simple monoketone analog of curcumin has the potential for field use against visceral leishmaniasis which is currently widespread in tropical and subtropical developing countries of the world. In conclusion, we have identified an analog of curcumin for potential applications against leishmaniasis, based on its strong antiparasitic activity and low toxicity. This curcumin analog compares favorably, at least in vitro, with the existing medication miltefosine.

1. Introduction

Trypanosomatids are a family of kinetoplastid protozoa comprised of flagellated protists characterized by the presence of a single flagellum [1]. Leishmaniasis is one of the three major human parasitic diseases caused by trypanosomatids, the other two being sleeping sickness caused by Trypanosoma brucei and Chagas disease caused by *T. cruzi*. *Leishmania donovani* is transmitted to humans by sandflies and responsible for visceral leishmaniasis (VL), the fatal form if untreated, in 88 tropical and subtropical countries in every continent except Australia and Antarctica [2, 3]. There is evidence for its prevalence among early Egyptians dating back to 3500 BC by recent detection of *L. donovani* DNA in the bone tissue samples from ancient mummies of the era [4]. In 1903, Leishman and Donovan were the first to report that leishmaniasis is caused by a protozoan parasite which they named *Leishmania donovani* [5]. Being an opportunistic infection with up to 70% of adult leishmaniasis related to HIV infection, it is the second-largest parasitic disease in the world (after malaria) with 500,000 new cases of VL reported annually [6]. VL has been classified by WHO as one of the most neglected tropical diseases which impacts mostly the poorest populations and for which no adequate therapy currently exists [7]. The orally effective drugs, such as miltefosine, a phosphocholine derivative and sitamaquine, an aminoquinoline have found limited success in India, Brazil and Kenya, all exhibit serious toxic side-effects and develop resistance [8–11]. Recent trend has been to employ combination therapy, such as antimonials or miltefosine with antileismanial antibiotics to overcome drug resistance [12]. However, most of these therapies are not practical for field use in treating VL in economically-

☆ This article is dedicated to Prof. Govind J. Kapadia

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challenged developing countries. Thus, there is urgent need for new orally effective, affordable drugs without toxicity and drug resistance in managing VL infection and improving quality of life of large populations afflicted with this debilitating parasitic disease in both developing and developed countries of the world.

Curcumin [(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the principal phenolic compound responsible for the yellow color of turmeric, a dry powder of rhizomes and roots of the perennial plant, *Curcuma longa* L. belonging to the ginger family, Zingiberaceae [13]. Over the centuries, turmeric has been used extensively as a spice; food colorant and fabric dye, and in herbal medical practice in India, China and other Asian countries [14]. The European Union and the US Food and Drug Administration consider curcumin, designated as yellow food color E100, safe for human consumption (http://www.feingold.org/Research/PDFstudies/colors.pdf). It is a strong antioxidant exhibiting remarkable pharmacological properties, such as anti-inflammatory, antimicrobial and cancer chemoprevention through a variety of mechanisms involving multiple targets [15–17]. In clinical trials, curcumin exhibited no adverse effects in humans at high doses (8-12 g/day) [16, 18]. However, instability, poor cellular uptake and bioavailability of curcumin and its naturally occurring analogs, curcumin II (demethoxycurcumin) and curcumin III (bisdemethoxycurcumin) have hindered their development into clinically useful drugs to treat systemic leishmaniasis [19]. Since antioxidant capacity of curcumin and its analogs with β-diketone [20–23] or monoketone [24, 25] moiety in their structure is considered essential for their biological activities, numerous curcumin analogs with improved stability, antioxidant capacity, cell penetration and bioavailability profile have been synthesized for structure-antioxidant/biological activity studies [19, 25–27]. Previous investigations have demonstrated antiparasitic activities of curcumin [21, 28–30] and related synthetic curcuminoids [31, 32].

The average daily intake of turmeric in India is estimated to be 38–190 mg (equivalent to 0.38–9.5 mg/day of curcumin) where it is widely used in popular curry dishes for its unique flavor and signature bright yellow color [33, 34]. In some rural areas of this country, its consumption is as high as 3.8 g/day. Effect of curcumin on systemic leishmaniasis appears to be dose dependent: in high doses it has antiparasitic effect and in low doses it increases parasite load in critical organs and exacerbates VL [34, 35]. Formation of reactive oxygen species (ROS) and imbalance of calcium homeostasis account for high doses of curcumin inducing cell death of *L. donovani* [21]. In contrast, curcumin in low doses suppresses interferon γ and nitric oxide production by activation of the anti-inflammatory protein called PPARγ which results in suppression of the body's initial acute inflammatory immune response against invading parasite and helps them evade the host immune attack [35]. Thus, currently there is an inconclusive research on the effect of dietary intake of curcumin in systemically Leishmania infected population in India, especially those in rural areas. However, topical ointments and skin injections of turmeric and curcumin have proven to be helpful in the treatment and prevention of cutaneous leishmaniasis [36].

The present study describes the leishmanicidal activity of (1E, 4E)-1,5-diphenyl-1,4-pentadien-3-one (trans-dibenzalacetone, DBA), a simple, synthetic monoketone analog of curcumin, against promastigotes and intracellular amastigotes of *L. donovani*. The potential mechanism of leishmanicidal activity of DBA was studied by cell cycle progression and ultrastructure of treated parasites. These leishmanicidal results are of particular interest since DBA is a simple monoketone curcumin analog easily synthesized in high yield (> 85%) from inexpensive ingredients by a one-step process at room temperature and has potential for economically treating VL currently wide-spread among poor populations of the world.

2. Materials and methods

2.1. Synthesis of trans-Dibenzalacetone (DBA)

DBA was synthesized as described earlier [37, 38] by the one-step Aldol condensation reaction of benzaldehyde in ethanol with acetone under basic conditions utilizing aqueous sodium hydroxide solution at room temperature with a yield of > 85%. Recrystallization from aqueous alcohol twice yielded DBA as bright yellow crystals, melting point 103–104 °C and its purity established by thin-layer chromatography, as described earlier [39]. The chemical structure of DBA was confirmed by infrared (strong signal for the presence of carbonyl group at 1649 $cm^{-1}$with none attributable to hydroxyl group), ultraviolet (λ_{max} 326 nm, log ε_{max}4.53 for conjugated carbonyl compound), ^{1}H-nuclear magnetic resonance (vinyl protons doublet of doublets, 16 Hz at 7.74 and 7.09 ppm, each for 2H for the benzylic protons and for the protons α to the carbonyl carbon, respectively) and mass spectra (molecular ion, M^{+} at *m/z* 234, M^{+}–H ion at m/z 233 and ion at m/z 131 from cleavage at carbonyl carbon leading to ion at m/z 103 from loss of CO) [39, 40].

2.2. Parasite culture and measurement of cell viability

Promastigotes and intracellular amastigotes of *L. donovani* strain (MHOM/IN/80/DD8) used in this study were routinely cultured as described previously [41]. Stock solution of DBA (1 mg/ml) was made in 0.1% (*v*/v) of DMSO and stored in aliquots at 4 °C. The drug was diluted in culture media immediately prior to each experiment to provide series of drug concentrations (0, 10, 20, 40, 80 and 160 μg/ml), as needed.

Resazurindye/AlamarBlue® (7-hydroxy-3H-phenoxazin-3-one-10-oxide) (Invitrogen, Cat. No. DAL1025, Carisbad, CA) was employed for measuring parasite (promastigotes) viability. Logarithmic phase promastigotes of *L. donovani* (50,000 cells/200 μl/well) were seeded in 96-well microtiter plates (Greiner, Bio-one, Germany) in the presence of increasing concentrations (0–40 μg/ml) of DBA, incubated at 25 °C for 24 h. Miltefosine was used as the standard drug. AlamarBlue® (20 μl) was added to each well and the plate was further incubated at 25 °C for 4 h. Absorbance was measured in a microplatereader (Biotek, Epoch) using λ = 570 nm as test wavelength (resorufin) and λ = 600 nm as reference wavelength (resazurin) serving as blank (normalized to the 600 nm value). The percent of cell-viability of DBA treated parasites was analyzed by following equation: [untreated control λ (570–600 nm) – treated set λ (570–600 nm)]/untreated control λ (570–600 nm) × 100.

2.3. In vitro evaluation of antileishmanial activity of DBA in intracellular amastigotes

The BALB/c mouse macrophage cell line J774A.1 was infected with stationary phase promastigotes of *L. donovani* as previously described [41]. Infected macrophages were treated with increasing concentrations (0–20 μg/ml) of DBA, incubated at 37 °C in 5% CO_2 for 24 h. After indicated incubation time, infected macrophages treated with or without DBA were fixed with ice-cold methanol on slides which were then submerged in 10% (*v*/v) Giemsa staining solution (Thomas Baker) for 45 min, followed by water rinse and let dry. The slides were viewed on an inverted bright field microscope (IX73 Inverted Microscope, Olympus). At least 100 macrophages per well were counted from duplicate cultures and the percentage of infected macrophages were calculated using the formula [42]: % reduction = number of amastigotes per 100 macrophages (treated samples)/number of amastigotes per 100 macrophages (infected control) × 100.

In vitro antileishmanial activity was expressed as the concentration inhibiting parasite growth by 50% (IC_{50}) and was analyzed by plotting percentage of cell viability versus log growth concentration of DBA.