Acta Tropica 172 (2017) 113–121

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Curcumin alters the cytoskeleton and microtubule organization on trophozoites of *Giardia lamblia*

Filiberto Gutiérrez-Gutiérrez[a], Lissethe Palomo-Ligas[a], José Manuel Hernández-Hernández[b], Armando Pérez-Rangel[b], Rodrigo Aguayo-Ortiz[c], Alicia Hernández-Campos[c], Rafael Castillo[c], Sirenia González-Pozos[d], Rafael Cortés-Zárate[a], Mario Alberto Ramírez-Herrera[a], María Luisa Mendoza-Magaña[a], Araceli Castillo-Romero[a,*]

[a] Departamento de Fisiología, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Guadalajara, Jalisco, Mexico
[b] Departamento de Biología Celular, Centro De Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México City, Mexico
[c] Facultad de Química, Departamento de Farmacia, Universidad Nacional Autónoma de México, México City, Mexico
[d] Unidad de microscopia electrónica, Centro De Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México City, Mexico

ARTICLE INFO

Keywords:
Parasite
Giardia lamblia
Cytoskeleton
Microtubules
Curcumin
Tubulin

ABSTRACT

Giardia lamblia is a worldwide protozoan responsible for a significant number of intestinal infections. There are several drugs for the treatment of giardiasis, but they often cause side effects. Curcumin, a component of turmeric, has antigiardial activity; however, the molecular target and mechanism of antiproliferative activity are not clear. The effects of curcumin on cellular microtubules have been widely investigated. Since tubulin is the most abundant protein in the cytoskeleton of *Giardia*, to elucidate whether curcumin has activity against the microtubules of this parasite, we treated trophozoites with curcumin and the cells were analyzed by scanning electron microscopy and confocal microscopy. Curcumin inhibited *Giardia* proliferation and adhesion in a time-concentration-dependent mode. The higher inhibitory concentrations of curcumin (3 and 15 μM) disrupted the cytoskeletal structures of trophozoites; the damage was evident on the ventral disk, flagella and in the caudal region, also the membrane was affected. The immunofluorescence images showed altered distribution of tubulin staining on ventral disk and flagella. Additionally, we found that curcumin caused a clear reduction of tubulin expression. By docking analysis and molecular dynamics we showed that curcumin has a high probability to bind at the interface of the tubulin dimer close to the vinblastine binding site. All the data presented indicate that curcumin may inhibit *Giardia* proliferation by perturbing microtubules.

1. Introduction

Giardiasis is one of the most common parasitic infections worldwide and is highly prevalent in developing countries (Koehler et al., 2013; Almirall et al., 2013). This parasitosis is characterized by diarrhea, cramps, headache, dehydration, malabsorption syndrome, vomiting and weight loss (Koehler et al., 2013; Almirall et al., 2013; Espelage et al., 2010). There are many drugs for the treatment of this parasitosis exhibiting variable efficacies and adverse effects (Lalle, 2010; Gardner and Hill, 2001; Granados et al., 2012). In the search for new antigiardial therapies, several natural substances have been tested (Freitas et al., 2006; Harris et al., 2000; Machado et al., 2010; Perez-Arriaga et al., 2006; Rufino-Gonzalez et al., 2012). Turmeric has been used for centuries for the treatment of different pathologies (Aggarwal et al., 2007). Its medicinal properties have been attributed mainly to the curcuminoids present in turmeric (Araujo and Leon, 2001). Curcumin, one of the principal bioactive components of rhizomes, possesses a wide range of pharmacological properties including anti-oxidant and anti-inflammatory activities (Aggarwal et al., 2007; Araujo and Leon, 2001; Jang et al., 2007; Cheng et al., 2001; Padilla et al., 2013). Several studies have reported the antiparasitic effect of curcumin against *Leishmania* spp., *Cryptosporidium parvum, Plasmodium falciparum, Giardia lamblia* and others. The effects were observed in growth, viability and cell differentiation (Perez-Arriaga et al., 2006; Saleheen et al., 2002; Chan et al., 2005; Chakrabarti et al., 2013; Cui and Miao, 2007; Magalhães et al., 2009; Shahiduzzaman et al., 2009). Besides, in animal models infected with *Giardia lamblia*, curcumin caused a considerable reduction in the number of trophozoites in intestinal sections and less cysts in feces (Said et al., 2012). However, the curcumiń s mechanism of action is still unknown.

* Corresponding author.
E-mail address: araceli.castillo@cucs.udg.mx (A. Castillo-Romero).

http://dx.doi.org/10.1016/j.actatropica.2017.04.027
Received 26 August 2016; Received in revised form 6 April 2017; Accepted 17 April 2017
Available online 30 April 2017

Curcumin has multiple therapeutic effects due to its ability to affect many intracellular targets. In a previous study Gupta et al. (2006) demonstrated that colchicine partly inhibits the binding of curcumin to tubulin; however, competition studies of curcumin with colchicine for tubulin binding indicated that curcumin inhibits microtubule polymerization by a different mechanism. The partial inhibition could be due to a conformational change in tubulin that reduces curcumin binding. Furthermore, the authors showed that vinblastine had no effect on the curcumin-tubulin interactions. Additionally to this study, Chakraborti et al. (2011) experimentally determined, by fluorescence resonance energy transfer (FRET), that the curcumin binding site is about 32 Å away from the colchicine-binding site, close to the vinblastine binding site. This study suggests that curcumin binding site is located at the interface, between the α- and β- tubulins of two α/β dimers, close to the vinblastine binding site. Based on these findings, we decided to investigate the mechanism of the curcumin effect on *Giardia* trophozoites, because in *Giardia*, tubulin is one of the major proteins of its cytoskeleton (Crossley and Holberton, 1983a, 1983b; Holberton and Ward, 1981; Jimenez-Cardoso et al., 1997). The microtubular cytoskeleton is comprised by median body, ventral disk, funis and flagella; essential for motility, attachment, intracellular transport, cell division and encystation/excystation (Dawson, 2010). Previous reports have shown that curcumin inhibits the growth of *Giardia lamblia* and causes morphological alterations (Perez-Arriaga et al., 2006). In the present study, we examined the cytotoxic effect of curcumin on *Giardia lamblia* trophozoites, analyzing the rearrangement of microtubular structures and tubulin expression. Concurrently, we evaluated changes in cell morphology by scanning electron microscopy. In addition, molecular docking and molecular dynamics simulation studies were accomplished to identify the binding site of curcumin on tubulin from *Giardia*.

2. Materials and methods

2.1. Culture of Giardia lamblia

Trophozoites of *Giardia lamblia* (WB clone C6) were grown axenically at 37 °C in borosilicate culture tubes containing Diamond's TYI-S-33 medium, pH 7.1 (Keister, 1983). Cultures were maintained by subculturing the cells twice a week.

2.2. Curcumin susceptibility assay

In order to evaluate the effect of curcumin on *Giardia lamblia* growth, an inoculum of 50,000 parasites/mL was grown in the presence of 0.3, 3 or 15 μM of curcumin (Sigma-Aldrich, C1386) in TYI-S-33 medium at 37 °C for 12, 24, 48 and 72 h. The diluent of curcumin, 0.5% DMSO (Sigma-Aldrich), and 0.5 μM of albendazole were used as a negative and positive controls. The cells were harvested by cooling the culture tubes and the cells were counted using a Neubauer chamber. All experiments were performed by triplicate. Data were analyzed by ANOVA (GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA) and P values of ≤ 0.05 were considered significantly different to the untreated cells.

2.3. Effect of curcumin on attachment

To evaluate the curcumin effect on adherence, 50,000 parasites/mL were grown at concentrations and time described above. After incubation, medium containing non-adherent cells was removed and kept on ice; tubes were filled with cold phosphate buffered saline (PBS) and placed on ice bath for 30 min to dislodge the adherent cells. The numbers of adherent and non-adherent trophozoites were determined by counting in a Neubauer chamber. The results were expressed as percentage of adhered trophozoites in relation to the total number of cells. Experiments were performed by triplicate, the variance was determined using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA.

2.4. Effect of curcumin on morphology and microtubule cytoskeleton

2.4.1. Scanning electron microscopy (SEM)

To analyze the morphology of trophozoites after curcumin, DMSO or albendazole treatment, parasites were washed with PBS, fixed for 1 h with 2.5% glutaraldehyde in PBS and adhered to poly-L-lysine-coated cover slips. The fixed cells were washed three times with PBS and post-fixed for 1 h in 1% OsO4 in dH_2O. Next, cells were washed with PBS, dehydrated in alcohol, subjected to critical-point drying with CO_2, coated with gold and analyzed by SEM (JOEL-JSM6510LV).

2.4.2. Immunofluorescence

To determine the possible effect on microtubules, parasites exposed to curcumin or DMSO were added to coverslips and allowed to adhere for 15 min at 37 °C. Adherent cells were fixed for 10 min in cold methanol (−20 °C) (J.T Baker), dried, permeabilized in 0.5% Triton X-100 for 30 min, and blocked for 1 h in 1% bovine serum albumin (BSA). The coverslips with fixed parasites were incubated with 1/200 mouse anti-α-tubulin (Invitrogen) for 1 h, washed with PBS, incubated with 1/200 anti-mouse IgG DyLight 594 conjugate (Thermo Scientific) for 1 h, and washed by five times with PBS. The preparations were mounted with the reagent Prolong Gold with DAPI (Invitrogen), and the fluorescence was analyzed by confocal microscopy (Leica TCS SP8). Images were processed using Leica Lite software.

2.4.3. Western blotting

For Western Blot assays, total protein extracts of parasites treated with curcumin or DMSO were obtained by sonication for 30 s (3 cycles) (Ultrasonic processor, Sonics and Material INC). Cell debris were removed by centrifugation (10,000 × g for 10 min). The protein concentration was determined by the Bradford method (Biorad). Soluble proteins (10 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and transferred to PVDF membranes (Towbin, 1979). After the transfer of the proteins, membranes were blocked for 1 h in PBS supplemented with 5% low fat milk and 0.05% Tween-20 (PBST), then incubated for 1 h with 1/200 mouse anti-α-tubulin (Life Technologies). Membranes were washed twice with PBST and incubated for 1 h with 1/5000 anti-mouse IgG-HRP (horseradish peroxidase; Thermo Scientific). Finally, membranes were thoroughly washed with PBST and the signal was detected by chemiluminescence (ECL Western Immobilon, Millipore). Taglin (1/500) was used as protein loading control (Ward et al., 1987). Semi-quantitative evaluation was performed by densitometry using ImageJ Software (Image Processing and Analysis in Java, U.S. NIH) (Rasband, 1999).

3. Identification of the predicted curcumin-binding site in *Giardia lamblia*

3.1. Homology modeling

Since at present time the crystallographic structure of *G. lamblia* tubulin is not available, the three-dimensional structures of α and β-tubulin were constructed by the homology modeling principle. In this study, we carried out the homology modeling process with SWISS-MODEL server (Biasini et al., 2014), using the amino acid sequences of *G. lamblia* α-tubulin (GenBank accession no. ESU39295.1) (Adam et al., 2013) and β-tubulin (GenBank accession no. EDO79714.1) (Kirk-Mason et al., 1988) and employing the *Bos taurus* tubulin (PDB: 1SA0) (Ravelli et al., 2004) crystallographic structure as template. Both monomer models were evaluated by the QMEANscore6 (Benkert et al., 2009) to obtain an estimation of the global and local quality of the model; whereas the quality of the protein geometry was assessed using PROCHECK (Laskowski et al., 1993). The tetrameric assembly of the