Research Article

Study of Ethinyl Estradiol Activity Against Promastigotes, Axenic and Macrophage-Dwelling Amastigotes of *Leishmania infantum* by Using Atomic Force Microscopy and Methyl Thiazolyl Tetrazolium Methods

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Abstract

Background: In the era of drug resistant organisms, there exists a huge need to overcome this issue. Drug repositioning is the action of repurposing current drugs against alternative targets.

Objectives: Herein, we assessed the antileishmanial activity of ethinyl estradiol and tolmetin on the promastigotes and amastigotes of *Leishmania infantum*, using methyl thiazolyl tetrazolium (MTT) and atomic force microscopy (AFM) methods.

Methods: Following previous in silico evaluation of 3358 FDA-approved compounds, ethinyl estradiol and tolmetin drugs were selected. Promastigote assay was done in RPMI 1640 culture in 96-well plates (10^5/100 μL). Also, axenic amastigotes cultured in acidic RPMI 1640 were seeded in 96-well plates (5×10^4 parasites). Raw 264.7 macrophages were seeded at a density of 3×10^6 cells/well. Then, the macrophages were infected with metacyclic promastigotes of *L. infantum*. Also, drug solvent and amphotericin B were considered as control wells. Drug challenge was performed in triplicate with different concentrations of ethinyl estradiol (250, 125, 62.5, 31.25, 15.62 and 7.81 μg/mL) and tolmetin (200, 100, 50, 25, 12.5 and 6.25 μg/mL) and cell viability was assessed by MTT assay. AFM imaging was done to observe morphological changes.

Results: The main finding of our investigation was the substantial effect of ethinyl estradiol on promastigotes, and amastigotes of *L. infantum* during 24, 48 and 72 h, according to MTT results and cellular morphology observations. Thus, the IC_{50} of this drug on promastigote, axenic amastigotes and macrophage-dwelling amastigotes during 72 h was 23, 45 and 32 μg/mL, respectively. However, tolmetin was not effective against *L. infantum* parasites. Cellular alteration, was observed by AFM technique.

Conclusions: This study showed that ethinyl estradiol had good anti-leishmanial activity and it is recommended to test this drug under *in vivo* condition. With the aid of drug repurposing we could substitute the old drugs with novel compounds for a specific disease.

Keywords: Drug Repositioning, Microscopy, Atomic Force, Ethinyl Estradiol, Tolmetin, *Leishmania infantum*

1. Background

Leishmaniasis is wide-spectrum disease, which is caused by the intracellular protozoan parasites of genus *Leishmania* (1, 2). It causes various clinical syndromes including cutaneous, mucocutaneous involvement as well as the serious visceral leishmaniasis (VL) (3, 4). *Leishmania infantum* and *L. donovani* in the Old World and *L. chagasi* in the New World are the causative organisms for Kala-Azar (5). It is approximated that an annual number of 500,000 cases are affected by VL globally (6). Humans, particularly children and immunosuppressed patients in Iran are mostly parasitized by *L. infantum* (7). Visceral leishmania-sis has been endemic in Iran and the most reported cases are from Ardabil, Fars and Bushehr provinces (4, 6, 8). Visceral leishmaniasis may manifest with various clinical features (4, 9). Accordingly, it is a requisite to base novel treatment options for this clinically important parasitic infection.

Several risk factors such as travel to endemic areas, decreased immunity, absence of an efficient vaccine enhance the occurrence of leishmaniasis (10). The primary medication for leishmaniasis mostly relies on Glucantime and Pentostam (first-line therapies) as well as Pentamidine and amphotericin B (second-line therapies). However, the long-term treatment course of this infection has invoked many

concerns regarding uncommon expensive drugs, development of side effects and emergence of drug resistant parasites ([11]). With respect to this, the discovery of innovative drugs and/or utilization of old drugs with novel formulations against parasites are key steps towards better future medications. During the last decades, web-based software has comprehensively aided us to assess molecule-receptor interactions and discovery of new treatment options ([12]). More recently, the antileishmanial experiments have utilized the drugs specific for other diseases such as anticancer and anti-inflammatory compounds ([13], [14]). For example, doxorubicin is an antineoplastic agent which has shown to have potent effects on *L. donovani* ([15]). Moreover, considerable inhibition of *L. infantum* proliferation in murine model was incurred following acetyl salicylic acid (Aspirin) treatment ([16]).

In this case atomic force microscopy (AFM) is a high-resolution imaging to detect subtle textural alterations on cell surface. Because of its versatility, AFM has become a tool in order to investigate the intrinsic mechanical properties of cells ([17], [18]). By, AFM we can examine whole and subcellular compartments and as well as carry out top-down mechanical characterization ([17], [19]). Besides, the AFM enables us to provide images from mechanical responses of single cell to drugs or other compounds ([17], [20]). We used AFM to investigate the Leishamnicidal properties of ethinyl estradiol and tolmetin on promastigotes and axenic amastigotes of *L. infantum*. Previously, we contrived an in silico investigation of 3358 compounds and deduced that ethinyl estradiol as a contraceptive with anticancer activity and tolmetin as a nonsteroidal anti-inflammatory drug (NSAID) are the appropriate drugs for antileishmanial assay. Accordingly, we designed an experiment to assess the antileishmanial activity of ethinyl estradiol and tolmetin on the promastigotes, axenic amastigotes and macrophage-dwelling amastigotes of *L. infantum* using (MTT) and AFM microscopy methods.

2. Objectives

In this study, we investigate the effects of ethinyl estradiol and tolmetin on promastigotes and amastigotes of *L. infantum* with MTT assay and morphological changes induced by these two drugs by AFM microscopy.

3. Methods

3.1. Experimental Drugs

Regarding our previous in silico investigation, two drugs, comprising tolmetin as NSAID and ethinyl estradiol as a contraceptive having antitumor function were se-

lected. These drugs were obtained from Faculty of Pharmacy, Ahvaz Jundishapur University of Medical Sciences.

3.2. Parasite Cultivation

A standard strain of *L. infantum* parasites (MCAN/IR/96/LONDON49) provided from Pasteur Institue of Iran. In brief, RPMI 1640 medium enriched by 10% heat deactivated fetal bovine serum (FBS) (Gibco, USA) 2 mM L-glutamine, 100 U/mL and 100 mg/mL streptomycin was used to culture 5×10^5 promastigotes per ml in a $24 \pm 1°C$ incubator (Shimaz, Iran). All chemicals were obtained from a Sigma (Sigma, Chemical Co., St. Louis, MO, USA). Subculture procedure was also done each 96 h at 2×10^7 cell densities/mL (10).

3.3. Promastigote Assay

The 96-well culture plates were seeded by a density of $10^5/100 \mu L$ *L. infantum* promastigotes. In addition, two control wells, one containing drug solvent and the other encompassing 40 μg/mL amphotericin B were considered. Subsequently, a 100 μL volume of various concentrations of tolmetin (200, 100, 50, 25, 12.5 and 6.25 μg/mL) and ethinyl estradiol (250, 125, 62.5, 31.25, 15.62 and 7.81 μg/mL) were added to respective wells, the examination was done in triplicates in $24 \pm 1°C$ incubation condition. Parasite count was performed following 24 h, 48 h and 72 h time periods using a Neubauer chamber slide. Next, 20 μL of prepared and filtered MTT solution was added to each well and incubation for 3 h at $24 \pm 1°C$ was carried out. After centrifugation (Sigma, Germany) the supernatant was discarded and 100 μL of dimethyl sulfoxide (DMSO) appended to all wells. Following shaking, the rate of absorbance was read at 570 nm using an ELISA reader device (Elx800, Bio Tek, USA). The percentage of viable promastigotes was calculated by the following equation:

Viable promastigotes (%) = $(A_T - A_B) / (A_C - A_B) \times 100$

A_C, A_T and A_B are the mean absorbance of untreated, treated and blank wells. Finally, the linear regression analysis was used to calculate $IC_{50(10)}$.

3.4. Amastigote Assay

In order to obtain axenic amastigotes, *L. infantum* promastigotes were cultured in RPMI 1640 medium with reduced pH (4.5 - 5.5), supplemented with heat deactivated fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin, under 5% CO_2 aeration and $37°C$ incubation (Memmert, Germany). A number of 5×10^4 amastigotes were placed in 96-well culture plate. Control wells were also considered as previously. One hundred μl of different concentrations of ethinyl estradiol and tolmetin were incorporated into respective wells. Then, triplicate plates

were incubated for 24, 48, and 72 hours at 37℃. Then, the MTT assay was performed as previously described to determine the effect of ethinyl estradiol on amastigotes of *L. infantum*. Finally, viable amastigote count was calculated using the aforementioned formula (21).

3.5. Macrophage-Dwelling Amastigotes Assay

The macrophage-dwelling amastigotes model was used to evaluate the leishmanicidal activity of ethinyl estradiol, Accordingly, RAW 264.7 macrophages were seeded at a density of 3×10^6 cells/well into a 24-well culture plate, and allowed to adhere following incubation at 37℃ and 5% CO_2. After 24 h incubation, the cells were washed to remove the unattached cells. Then, the macrophages were infected with metacyclic promastigotes of *L. infantum* at 10:1 (promastigote: macrophages) ratio. Further incubation was performed for 24 h at 37℃ and 5% CO_2, and then the infected cells were washed to remove un-phagocytosed promastigotes. Afterwards, infected macrophages were treated with different concentrations of ethinyl estradiol for a period of 72 h. Then, the MTT assay was performed as previously described to determine the effect of ethinyl estradiol on macrophage-dwelling amastigotes of *L. infantum* (21).

3.6. Atomic Force Microscopy

Seventy-two hours following ethinyl estradiol challenge and incubation, cultured promastigotes and axenic amastigotes were centrifuged, then the precipitated, washed, fixed and dried. Ultimately, AFM imaging of the treated and prepared promastigotes and amastigotes was performed by an atomic force microscope (AFM Workshop, Signal Hill, USA) (17).

3.7. Statistical Analysis

Results were analyzed finally using one-way analysis of variance (ANOVA) test and provided as the mean value of triplicate experiments ± standard deviation (SD) by linear regression. Data were analyzed using SPSS© ver.22 software.

4. Results

In this study the cytotoxic activity of different concentrations of tolmetin and ethinyl estradiol on cultured promastigotes and axenic amastigotes of *L. infantum* were evaluated for 24, 48 and 72 h in triplicate. The results of MTT test and morphology observations during 24, 48 and 72 h didn't demonstrate a significant impact of tolmetin on *L. infantum*. However, ethinyl estradiol, showed statistically significant activity on both promastigotes and amastigotes during 72 h. Consequently, IC_{50} of this drug on promastigotes and axenic amastigotes during 72 h was 23 and 45 μg/mL, respectively (Figure 1). Moreover, AFM imaging demonstrated considerable impacts of ethinyl estradiol on morphology of *L. infantum* promastigotes and amastigotes. However, considering the result, it is obvious that some changes occurred morphologically (Figures 2 and 3).

The incubation of *L. infantum* promastigotes with ethinyl estradiol inhibited the parasite growth efficiently (Figure 1). The IC_{50} concentration of ethinyl estradiol and amphotericin B as control on macrophage-dwelling amastigotes was 30 μg/mL and 32 μg/mL, respectively. Our results showed that treatment of amastigotes with the different concentrations of ethinyl estradiol lead to a significant decrease (P < 0.01) of intracellular amastigotes compared with control. We assume that besides the direct actions of ethinyl estradiol, it may also act indirectly through the activation of macrophages. As illustrated in Figures 2 and 3 we investigated the effects of ethinyl estradiol on promastigotes and axenic amastigotes of *L. infantum* by atomic force microscopy. The analyses showed that treatment of *L. infantum* promastigotes and amastigotes with subjected drugs cause ultrastructural changes. These results show that the morphology of parasite was changed and the cell shape was altered.

5. Discussion

The inconvenience, toxicity and resistance issue are main complications associated with the drug therapy for the leishmaniasis (22). The VL-specific therapy mostly relies on Glucantime and Pentostam (3, 23). Toxic medications used against VL, implicating higher risk of death following treatment (23). On the other hand, leishmaniasis has co-evolved with first-line drugs beyond 70 years of administration and, to some extent, have developed novel metabolic pathways to bypass drug actions (24). Therefore, bioinformatics, can be used to revisit different drug compounds to be administered against VL (25). Khademvatan et al. showed, in silico and *in vitro* study could lead to find potential drugs against leishmaniasis (10).

In present study, the MTT result confirmed the antileishmanial activity of ethinyl estradiol against *L. infantum*. This compound is able to inhibit intracellular amastigotes growth in 50% at 30 μg/mL concentration of ethinyl estradiol similar to 32 μg/mL concentration of amphotericin B as a control. Our results show that these compounds exert similar antileishmanial effects on promastigotes as well as amastigotes. Similar to previous studies (26, 27). Our results confirmed that the ethinyl estradiol has
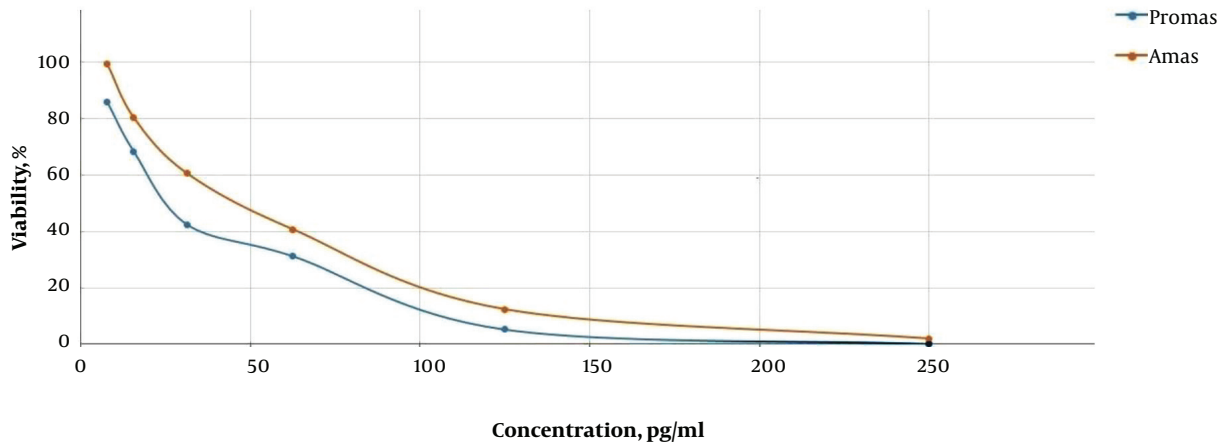
Figure 1. MTT assay to determine IC$_{50}$ value of the ethinyl estradiol on promastigote and axenic amastigote of *Leishmania infantum* after 72 hours of incubation
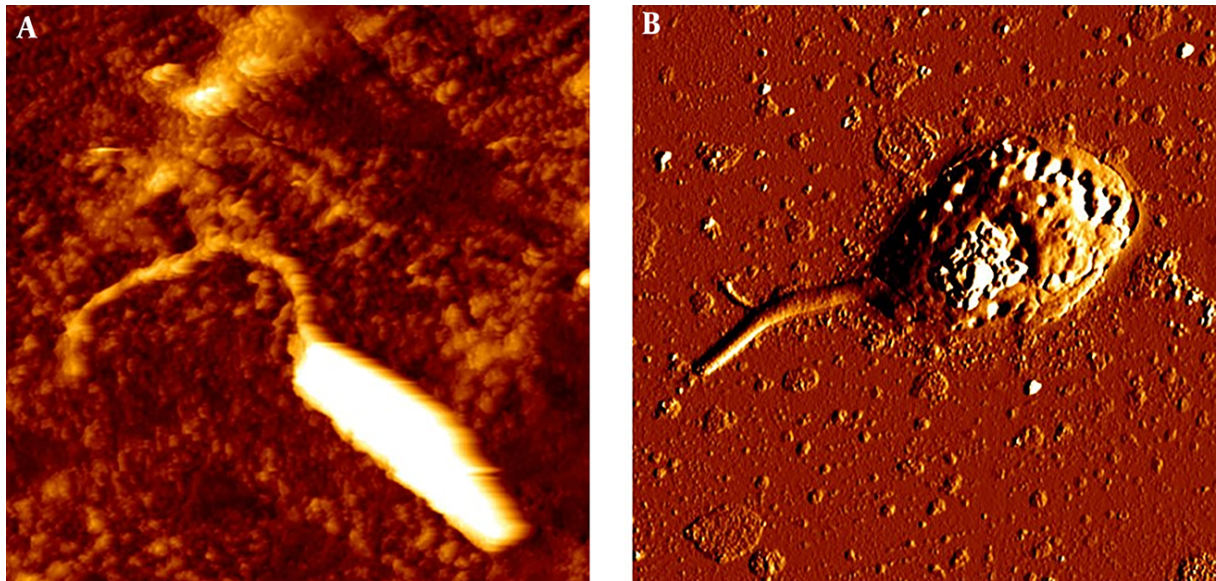

Figure 2. Comparison of untreated (A) and (B) treated *L. infantum* promastigotes with ethinyl estradiol

cytotoxicity which leads to parasite destruction. Two substantial concepts support drug repositioning strategies: addressing new uses of a compound having a known target or seeking novel utilization of a drug acting via an unconventional target (28). Based on our results, ethinyl estradiol possessed dose-dependent antileishmanial effects, in contrast to tolmetin. Also, parasite count demonstrated remarkable variation between sample and control groups. On the basis of measured optical density and IC$_{50}$ following 72 h, the 23, 45 and 30 μg/mL concentration of ethinyl estradiol was calculated to possess inhibitory effect on pro-

mastigotes, axenic amastigotes and intracellular parts of *L. infantum*, respectively.

Previously, it has been shown tamoxifen as anticancer drug has antileishmanial activity (29). Miguel et al. assessed the antileishmanial activity of an anti-estrogen drug, tamoxifen, suggesting that it can kill *L. infantum* promastigotes and macrophage-dwelling amastigotes (30). The cancer chemotherapy drug, cisplatin was shown to possess antileishmanial traits on *L. infantum* (31). The efficacy of some anticancer protein kinase inhibitors was evaluated by Sanderson and colleagues, showing sarafenib, la-
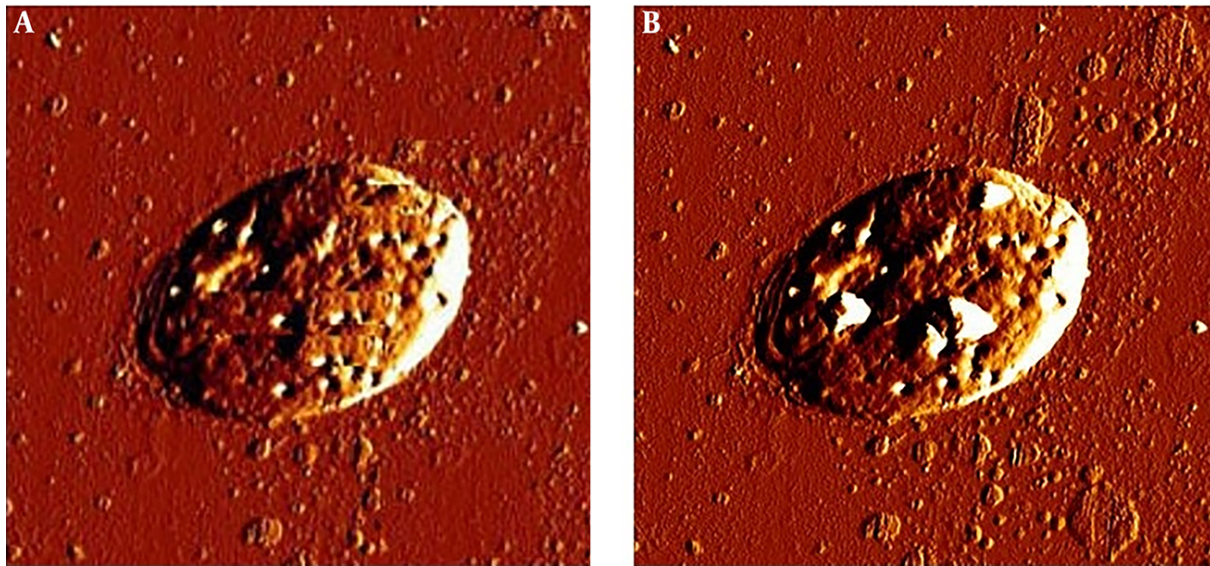
Figure 3. Comparison of *L. infantum* amastigotes without exposure (A) and (B) exposure to ethinyl estradiol

patinib and sunitinib as bioactive compounds against *L. donovani* amastigotes in murine macrophages (32). Raloxifene, an estrogen receptor modulator, was assessed on *L. donovani* promastigotes and *L. infantum* promastigotes and amastigotes (33). Cyclosporine as an immunosuppressant agent was also tested against *L. donovani* (34). Moreover, in this study we find that ethinyl estradiol as an anticancer drug can kill *L. infantum* with the maximum effect on promastigotes. The result of atomic force microscopy showed cell alterations in promastigotes and axenic amastigots of *L. infantum* treated with ethinyl estradiol. In the other study with AFM showed, drugs produce severe disturbances on membrane, leading to the death of parasites (35).

Findings from other antileishmanial agents on *Leishmania* sps are consistent with our AFM imaging results, suggesting cell length reduction (35-37). Herein, we selected two drugs from, 3358 FDA-approved compounds with software-based analyses, i.e. ethinyl estradiol and tolmetin for assay against *L. infantum* promastigotes and amastigotes. Our results asserted that only ethinyl estradiol was efficacious against the parasite with IC_{50} scores of 23 and 45, 32 μg/mL on promastigotes, axenic and macrophage-dwelling amastigotes, respectively, after 72 h incubation. Convincing evidence from our study demonstrate that drug repositioning could be a promising, affordable alternative for better treatment of leishmaniasis. Similar to our study Khademvatan et al. showed virtual screening and drug repositioning is good strategies for the discovery of new drug against leishmaniasis (38).

In contrast to our study, Valpato et al. has used conventional electron microscopy to illustrate the effect of T6 compound on *L. infantum*. So, their results were not illustrative of the exact leishamnicidal activity of the subjected compound (39). In another study was conducted by Rottini et al. they were used (-)α-bisabolol as antileishmanial agent (26), however they did not provide atomic force microscopy images to clarify the morphological changes. Therefore, we strictly suggest the use of AFM, which can provide information about the how the subjected drugs can affect parasite cells. Similar to our results Valpato et al. and Rottini et al. (26, 39) have showed the leishmanicidal activity of subjected compounds, while compared to their results, our findings suggest that ethinyl estradiol can be used as a selective antileishmanial agent.

5.1. Conclusions

In this study we showed that ehinyl estradiol had good antileishmanial activity and it is recommended to be tested under *in vivo* condition. Furthermore, we strictly suggest the use of AFM which can provide information on how drugs can affect parasite cells.

Acknowledgments

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