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Original Article

Evaluation of antileishmanial activity of valproic acid against *Leishmania donovani*: An integrated *in silico* and *in vitro* study

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ABSTRACT

The antileishmanial activity of valproic acid was evaluated in an *in vitro* culture of *Leishmania donovani* promastigotes using amphotericin B as standard. Both valproic acid and amphotericin B showed antileishmanial activity at IC_{50}s of 0.183 and 0.138 µg/ml, respectively. A homology model of *Leishmania donovani* histone deacetylase I (*Ld*HDACI) was built and validated using *in silico* tools. Docking valproic acid into the active site of this Zn–dependent *Ld*HDACI added a bidentate coordination to the tricoordinated metal. The resultant pentacordinated Zn^{+2} is no longer available for the natural substrate. Thus, the antileishmanial mechanism of valporic acid is thought to be via competitive inhibition of *Ld*HDACI.

Key Words: *Ld*HDAC1, leishmania, valproic acid, amphotericin B, histone deacetylase inhibitor, homology model, drug-repurposing.

INTRODUCTION

Leishmaniases are group of clinically diverse vector-borne diseases, caused by a protozoa parasite of the genus Leishmania. There are three main forms of leishmaniases, visceral, cutaneous and mucocutaneous [1]. Visceral leishmaniasis (VL) is the most life threatening form which if untreated is fatal. The disease is caused by *Leishmania donovani* complex in East Africa and Indian subcontinent and by *Leishmania infantum* in many parts of the world including Latin America and Mediterranean Basin [2]. VL affects 200 to 400 thousands people worldwide annually. Ninety percent of the VL occurs in Sudan, Ethiopia, Bangladesh, India, Nepal, and Brazil [3]. Currently, there is no available effective vaccine and on the other hand treatment of leishamaniasis is based on pentavalent antimonials drugs and amphotericin B which are toxic and prone to drug resistance [4]. Therefore, it becomes mandatory to develop new drugs that would possibly be a key for safe, effective and affordable antileishmanial treatment. Drug development is tortuous, risky and financially burdensome with many compounds failing for every one that succeeds and reaches the market [5]. The application of known drugs to new indication, so-called drug-repurposing [6], has a significant advantage over traditional drug development. Having passed a significant number of toxicity studies and other tests, the repurposed drugs can bypass most of the early cost and time needed to bring a drug to a market. The identification of novel targets that interact with marketed drugs is the first step in assessing the repurposing potential [7].

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Cell proliferation and differentiation in protozoan parasites, like other eukaryotes, crucially depends on acetylation and deacetylation of histone and nonhistone proteins [8]. These transformations are catalyzed by histone acetyl transferases (HATs) and histone deacetylases (HDACs), respectively [9,10]. The genome of Leishmania parasite contains multiple genes that encode different HDACs which have been shown to be crucial for the survival and proliferation of the Leishmania parasite [11]. It was revealed that histone deacetylase inhibitors, specifically suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), apicidin analogues and others have *in vitro* activity against *L. donovani* parasites [12,13]. This antiparasitic activity could be mediated by histone deacetylase inhibition [14]. The old anticonvulsant and mood stabilizer, valproic acid, has been found to inhibit both class I and II human HDACs, *Toxoplasma gondii* and *Schistosoma mansoni* HDACs [15–17]. We have recently reported the *in silico* activity of valproic acid against *Plasmodium falciparum* histone deacetylase 1 [18]. It was proposed that valproic acid would also have antileishmanial activity mediated through inhibition of *L. donovani* histone deacetylase I. The objective of this study was to investigate the in *vitro* activity of valproic acid against *Leishmania donovani* promastigotes. It was also hoped to propose a molecular mechanism for the expected antileishmanial activity of valproic acid using *in silico* tools.

MATERIALS AND METHODS

Drugs: Amphotericin B (Ambisome$^{(R)}$) was commercially purchased. Valproic acid (sodium salt, SUN Pharmaceutical Industries, India) was kindly provided by Azal Pharmaceutical Company, Sudan. The sodium valproate stock solution (20mg/ml; DMSO) was diluted two-fold with liver infusion treptose (LIT) complete media to obtain serial dilutions ranging from 200µg/ml to 0.049 µg/ml.

Parasite isolation and cultivation: A confirmed-positive VL patient was subjected to lymph node aspiration for parasite isolation. Biopsies were aseptically inoculated into vacutainers containing Novy-MacNeal-Nicolle (NNN) medium. Cultures were incubated at 25°C and parasite growth was monitored daily. Promastigotes were transferred into tissue culture flasks containing LIT media supplemented with 10% fetal calf serum (FCS), genatmycin and benzylpenicillin. The medium was changed every 3 days until promastigotes reached their stationary phase [19,20].

In vitro evaluation of antileishmanial activity: Promastigote density was adjusted to 2×10^6 parasites/ml using LIT complete media. A volume of 100 µl from parasite culture was transferred into 96-well flat bottom microtiter plate. Various concentrations of freshly prepared valproic acid solution were added (100µl) in triplicates. A negative control (DMSO without any drug), and positive control (amphotericin B) were treated similarly. The plates were incubated at 25°C for 72 hours. Parasites were counted by using hemocytometer [19,20].

Statistical analysis of data: Each experiment was performed in triplicates. Results were expressed as mean ± standard deviation of the mean (SD) and analyzed using SPSS 18. The IC_{50} values were calculated using GraphPad Prism 6.0 software.

Model building: The 430 amino acids sequence of *Ld*HDACI (GI:193527475) was obtained from the NCBI protein database and YASARA structure program was used to build *Ld*HDACI homology model [21]. The target sequence was PSI-BLASTed against UniRef90 and the resultant position-specific scoring matrix (PSSM) was used to search the PDB database for potential modeling templates. A prewritten macro was used to build different models based on the top five ranked templates. Finally, the best parts of the generated models were combined to obtain a hybrid model with better accuracy.

Model refinement: The hybrid model was further refined using YASARA short Molecular Dynamics (MD) simulation. The model refinement was conducted using YASARA2 force-field for 500 picoseconds at 298°K using the NVT canonical ensemble [22].

Model quality assessment: The MD simulated model's quality was assessed using online bioinformatics tools. Stereochemical accuracy and absolute quality were assessed using RAMPAGE and QMEAN online servers, respectively [23,24].

Molecular Docking: The 3D structure of valproic acid (CID: 3121) was obtained from the PubChem database. The ligand protonation state, energy minimization and molecular docking of valproic acid into *Ld*HDACI were performed using MOE [25]. The plausible active site was identified using Alpha Site Finder. Valproic acid was docked into the identified active site using the triangle matcher placement method. Thirty docking poses were retained and scored using London *dG* scoring function. The retained poses were further refined to 0.1 kcal mol^{-1} $Å^{-1}$ rms using CHARMM27 molecular mechanics force field [26] and rescored using the *dG* function.

RESULTS AND DISCUSSION

In vitro antileishmanial activity: Valproic acid, at dose range 200–0.05µg/ml, showed 90.42±1.44–32.5±3.31% promastigote inhibitory activity. On the other hand, the standard antileishmanial drug amphotericin B showed 95.42±0.72–48.75±10.68% inhibitory activity at the same dose range (Table 1). The IC_{50} values for valproic acid and amphotericin B were found to be 0.183 and 0.138 µg/ml, respectively (Figure 1). At 200 and 100µg/ml, the inhibitory activity produced by valproic acid was significantly greater than that produced by amphotericin B at doses ≤ 1.56 µg/ml (One way ANOVA, $P \leq 0.05$). Treatment of the *L.donovani* promastigotes with a mixture of the two drugs (1:1), at concentration ≥ 3.13µg/ml, resulted in complete parasite growth inhibition. Further investigations are needed to develop a combination therapy of amphotericin B and valproic acid to increase the efficacy and reduce the dose and, thus, toxicity of each drug.

In silico study

Refined hybrid model: The hybrid model was found to be the best in terms of z-score (−0.967) and it was, therefore, further refined using YASARA2. The MD generated model with the lowest potential energy (−49946.70 kcal mol−1) was employed for further work. This model adopted a canonical *α*/*β* HDAC fold similar to human and *Schistosoma mansoni* HDAC solved structures [27–29]. Similar to other HDACs, the model formed a single *α*/*β* domain composed of a central eight strands parallel *β*-sheet sandwiched by 16 *α*-helices (Figure 2). The model contained a divalent zinc ion at the bottom of the active site. The most important feature of the enzyme active site was the presence of HDACs canonical catalytic triad, where Zn^{2+} of the free enzyme formed coordinates with three amino acids, Asp190, His192 and Asp279 (Figure 2) [30].

Model quality assessment: RAMPAGE based-assessment of the refined model revealed that 97.2% of the amino acids were located in the favored region, 2.5% in allowed region, and the rest 0.3% residues were outliers (Figure 3). These findings greatly indicated that the stereochemical accuracy of the model is accepted in terms of dihedral angles (ϕ,ψ). On the other hand, the absolute quality of a model was evaluated by QMEAN server. Six descriptors of the model were combined and the resultant z-score was compared to scores of a non-redundant set of high resolution x-ray crystallography structures of similar size to the model's. The normalized QMEAN6 z-score for the model was within the standard deviation value typically obtained for crystallography structures confirming that the model absolute quality was in match with them (Figure 4). With this model in hand, it was now time to conduct molecular docking of valproic acid to support the proposed *Ld*HDACI inhibitory action.

Molecular docking: In Zn–dependent class I HDACs, the metal ion is coordinated at five points with a pentacoordinate geometry. It forms three coordinations with two Asp residues and one His in addition to two coordinations with a bidentate ligand [27–29]. In our ligand–free *Ld*HDACI model, the metal forms three coordinates with Asp279, His192 and Asp190 (Figure 2). Docking valproic acid into the active site of *Ld*HDACI expanded the coordination sphere to pentacoordinate by adding a bidentate coordination to Zn^{2+} via the carboxyl carbonyl oxygen and the hydroxyl oxygen of valproic acid (Figure 5). Thus, the pentacoordinated Zn^{2+} will not be available to interact with the natural substrate resulting in a competitive inhibition. Other interactions of valproic acid with the active site have also been identified. The amino acids Tyr318 and His154 formed hydrogen bonds with the carboxyl carbonyl oxygen and hydroxyl oxygen of valproic acid, respectively. Furthermore, the aliphatic parts of valproic acid showed hydrophobic interaction with Phe164. All these interactions are considered potential to increase the valproic acid affinity to *Ld*HDACI active site. It is worth noting that, the well-known HDAC inhibitor trichostatin A (TSA) was reported to have a potent activity against *Leishmania donovani* [12]. However, its exact mechanism of action is not known. To support our proposed antileishmanial mechanism of valproic acid, we also docked TSA into *Ld*HDACI active site. The same valproic acid's interactions were revealed by TSA, where it showed a bidenate interaction with Zn^{2+}, hydrogen bonds with Tyr318 and His154, and hydrophobic interaction with Phe164 (Figure 5). Thus, the antileshmanial activity of valproic acid and TSA is very probably mediated through *Ld*HDACI inhibition.

CONCLUSION

The well-known mood stabilizer and non-expensive valproic acid showed promising activity against *Leishmania donovani* promastigotes when compared to amphotericin B. *In silico* study revealed that this activity could be mediated through LdHDACI inhibition. Knowing that *Ld*HDACI is crucial for the parasite throughout its life cycle [13], valproic acid could possibly be active against *Leishmania donovani* amastigotes. Further parasitological and efficacy studies should be conducted to confirm the *in vivo* antileishmanial efficacy of valproic acid.

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Conflict of interest: The authors declare no conflict of interest.

Table 1: Antileishmanial activity of valproic acid against amphotericin B.

Concentration (µg/ml)	Mean %Inhibition ± SD	
	Valproic acid	Amphotericin B
200	90.42 ± 1.44 *↓	95.42 ± 0.72
100	85.83 ± 3.15 *↓	92.92 ± 0.72
50	83.33 ± 6.89	91.67 ± 4.39
25	75.42 ± 0.72 *↓	89.17 ± 0.72
12.5	60.00 ± 1.25 *↓	89.17 ± 3.15
6.25	61.67 ± 6.29 *↓	86.67 ± 5.64
3.13	64.17 ± 7.11 *↓	86.25 ± 2.17
1.56	57.50 ± 10.23 *↓	76.67 ± 4.02
0.78	62.08 ± 0.72 *↓	75.83 ± 4.02
0.39	51.25 ± 0.00 *↓	63.33 ± 4.39
0.20	36.67 ± 1.44	41.67 ± 5.05
0.10	39.58 ± 1.91	50.00 ± 7.50
0.05	32.50 ± 3.31	48.75 ± 10.68

↓* Significantly less potent compared to amphotericin B at the same dose (Student t-test, $P \leq 0.05$).

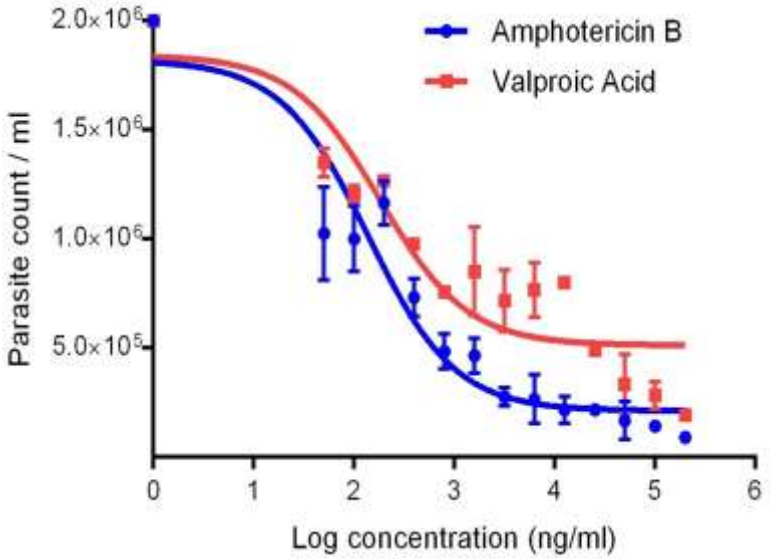

Figure 1. The viability of *L.donovani* promastigotes in the presence of different concentrations of valproic acid and amphotericin B.
The IC_{50} is the antilogarithm of the concentration at which the curve passes through the 50% parasite count.

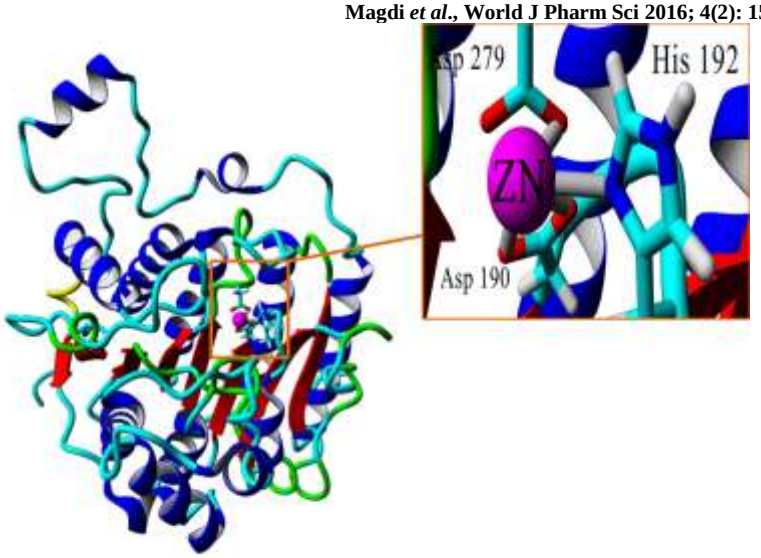

Figure 2. Ribbon representation of ligand-free *Ld*HDACI model.
The enlarged fragment shows zinc coordination state.

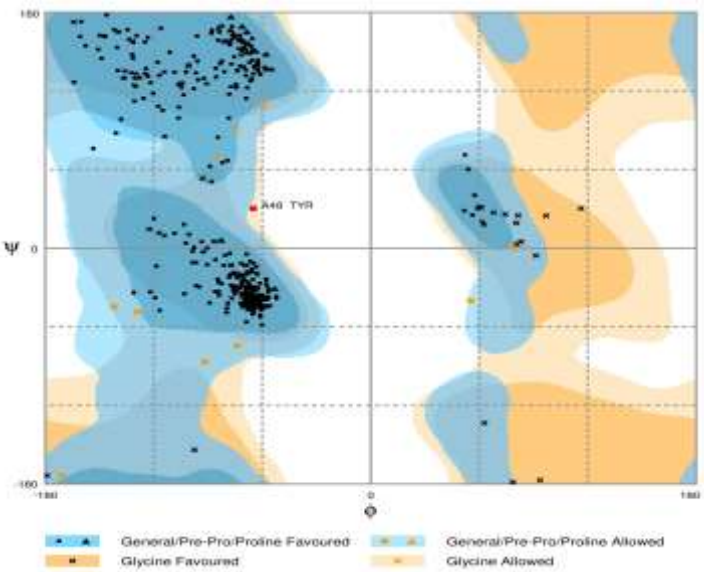

Figure 3. Ramachandran plot of dihedral angles (ϕ, ψ) of LdHDACI model.
Number of residues in favoured region (~98.0% expected) : 386 (97.2%)
Number of residues in allowed region (~2.0% expected) : 10 (2.5%)
Number of residues in outlier region : 1 (0.3%)

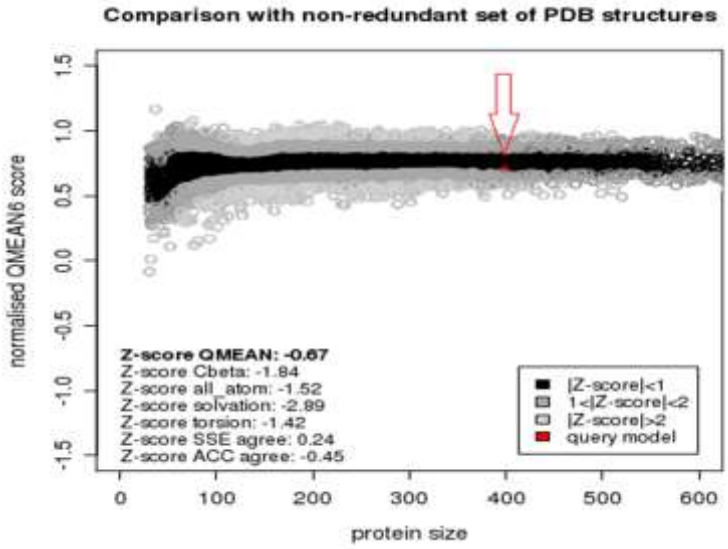

Figure 4. Normalized QMEAN6 z-score of *Ld*HDACI.
The red arrow highlights the position of the query model indicated by red 'X'.

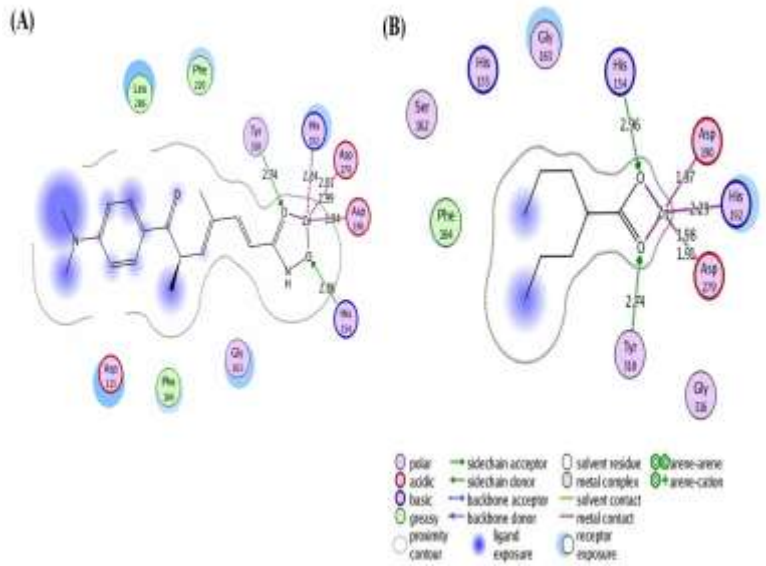

Figure 5. Ligand-model interactions.
A schematic diagram of *Ld*HDACI binding site showing the critical interactions undergone by (A) TSA and (B) valproic acid with zinc ion as well as the bonds' lengths.

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