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# Redox mechanism of *Trypanosoma cruzi* Resistance to Nitro Prodrugs Benznidazole and Nifurtimox

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**Abstract:** Chagas disease is an endemic infectious disease caused by parasite Trypanosoma cruzi (T. cruzi). Common symptoms include heart and gastrointestinal disorders. Conventional treatment consists in the administration of antiparasitics nitro prodrugs (e.g. benznidazole and nifurtimox), which are activated by the Nitroreductase (NTR) enzyme - Flavin Mononucleotide (FMN) complex in the parasite. To understand the parasite's mechanisms of resistance to the drugs, we studied a mutant enzyme (Pro46Leu) and used software UCSF Chimera to render a tridimensional image of it. Then, we computed its molecular electronic structure and the complex were optimized based on the data of global minimum geometry and energy using the Spartan 14' software for wave function, via semi-empirical method with the force field Austin Model 1 (AM1). The hybrid QM/MM structural relationships generated by the software allowed us to detect small changes to the system (distribution of charges, dipole interaction distances, potential energy surface, electrostatic potential map and shifting of angles in the wild-type and mutant enzymes). In addition, the integration between bioinformatics for the alignment and search of tertiary structures of a protein and quantum mechanics to analyse point changes of amino acids and protein folding are useful in explaining how the parasite develops a mechanism to resist the drugs and as a fast and accurate alternative to generate more effective antibiotics derivatives. Finally, using experimental analysis, we generated models to understand the mechanisms of adsorption of nitro prodrugs and the resistance of the parasite to these.

**Keywords:** Trypanosoma Cruzi, Benznidazole, Resistance, Quantum Mechanics, Molecular Mechanics

## 1. Introduction

American Trypanosomiasis (or Chagas disease) is a disease transmitted by parasite *Trypanosoma cruzi* [1-2]. This parasite is carried by an insect of the *Rhodnius* genus (Triatomine bug), which lives in the tropics and has spread the disease throughout South America [3-4]. Although prevalence is still high, treatment of Chagas disease has advanced considerably, and estimated cases dropped from 18 million in 1991 to 5.7 million in 2010 [5-7]; in Colombia, between 90000 and 900000 cases were reported by 2016 (WHO).

Antiprotozoal drugs (such as benznidazole and nifurtimox) are effective in the treatment of Chagas disease [8-10]. Benznidazole is considered the most effective due to its bioavailability, tolerability, and easy administration. This drug has several potential mechanisms of action, but like most nitroaromatic compounds its effectiveness depends on a previous activation process. This process is guided by free-radical reactions [1, 11], and redox reaction for the activation of nitro prodrugs in Chagas disease treatment mediated by the interaction between nitroreductase and Flavin mononucleotide (Figure 1). The latter produces the derivatization of nitro compounds via a flow of electrons. Then, after an anionic radical is obtained, radicals are reduced via an aerobic or anaerobic pathway. The conversion of the nitrous ion through the aerobic pathway takes advantage of the acidic conditions of the aqueous media that favor the reduction of the nitro functional group to an amine [12-14] (Equation 1A).

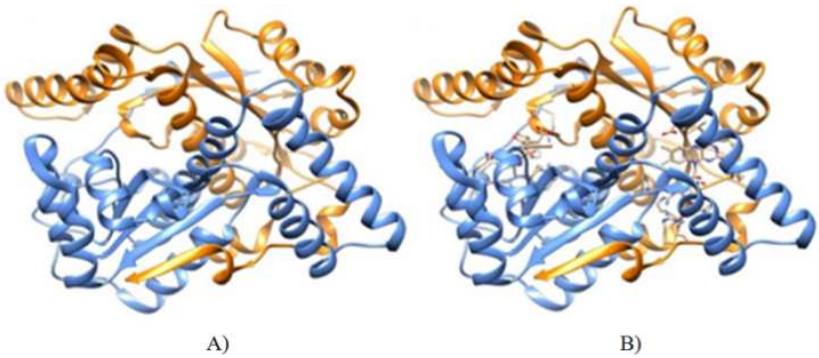
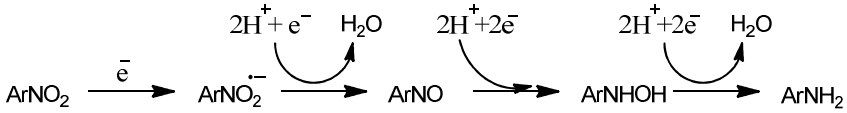



***Figure 1.*** *Three-dimensional representation of the NTR dimer in E. Coli (PDB ID 1DS7): A) NTR in ribbon contour; B) NTR with the FMN cofactor.*

In the aerobic pathway, the interaction between the radical of the nitro compound and the oxygen in the media produces superoxide reactive oxygen species, which can affect vital structures in the parasite [15] (Equation 1B).

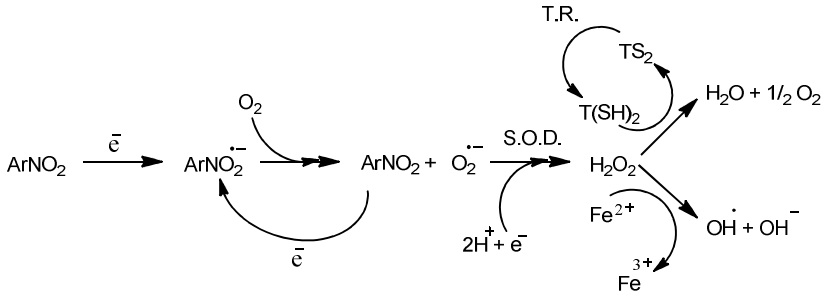
Equation 1. *Bioreductive pathway of nitro compounds, superoxide dismutase (SOD): A) Anaerobic pathway; B) Aerobic pathway* [15].

A)




(1)

B)




The compounds with a nitro functional group are reduced to an amino functional group and acquire high affinity toward nucleic acids in the parasite's structure, altering its metabolism [16-17] (Equation 2).

*Equation 2. Activation of nitro prodrugs by redox reaction.*

$$ArNO_2 \xrightarrow[\text{Coenzyme FMN}]{\text{Wildtype Ntr}} ArNH_2 \qquad (2)$$

When the disease becomes chronic, drugs lose their power, and the parasite can develop resistance. Resistance can either manifest as mutation on the orthosteric site, or as gene deletions that are translated into the nitroreductase enzyme [13, 18-21]. The concentration of this enzyme plays a key role in the parasite's metabolism: low concentrations contribute to a higher chance of developing resistance, while high concentrations make the parasite hypersensitive to the drug.

In this work, we carry out a computational approach (from a chemical perspective) to describe the mutation in enzyme NTR (Pro46Leu) that could affect its functionality and thus, favor the resistance of *T. cruzi* to nitro compounds [21-22].

## 2. Materials and Methods

Bioinformatic analysis

T. cruzi's NTR does not have a crystallized structure. For this reason, we built a hypothetical homology model using a predictor of I-TASSER structure and then refined it in ModRefiner software in PDB format [23-26] (Figure 2). We looked up the primary sequence in literature and then performed a multi alignment with software Jalview's ClustalX and T-Coffee tools [27-28]. Through this alignment, we located the mutation on the strain [19].

Visualization and alignment

The structures of the evaluated models were visualized in UCSF Chimera software v1.11 via ribbon visualization and density surface tools. The alignments were made in MatchMaker in a BLOSUM62 similarity matrix and we used a Needleman-Wunsch global alignment algorithm for the structures of wild type T. cruzi and E. coli [29].

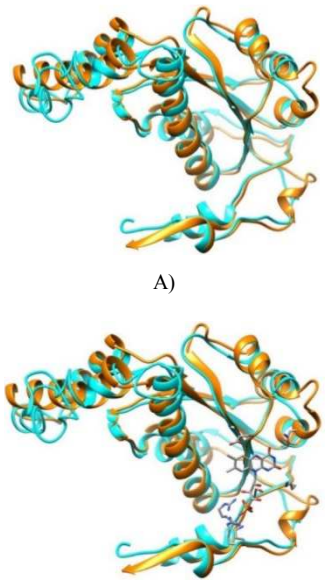
Calculations and parameters

Distance and angle measurements were obtained via a semi-empirical method using a hybrid Quantum

Mechanics/Molecular Mechanics (QM/MM) approach on the wild type protein and the mutant [30-32]. Then, these were visualized on Spartan 14 software tool. In addition, we identified the docking sites of FMN and NTR via computation of the surface potential aided by mesh visualization.

Analysis with the Quantum Mechanics/Molecular Mechanics hybrid

Energy and geometry on the structure of the catalytic site and in FMN were computed in Spartan 14 tool v1.2.0 (Acquired license) [33]. This tool allows the selection of a semi-empirical method of lower computational cost. This method requires shorter times of analysis and allows the integration of macromolecular systems such as enzymes and coenzymes. The bases used were Austin Model 1 (AM1) and Merck Molecular Force Field (MMFF), in aqueous phase. Given that studies on the use of these methods on biological systems have shown excellent results, we optimized the molecules by using parameters of minimum geometry and energy [31-32, 34].



A)

B)

***Figure 2.*** *E. coli's NTR enzyme alignment (Orange) and proposed model for T. cruzi's (Cyan) [23]: A) NTR alignment in ribbon contour; B) NTR alignment with the FMN cofactor.*

## 3. Results

Localization of the interaction sites in NTR's 3D structure

Type 1 NTR enzyme was chosen to study the resistance of T. cruzi to nitro prodrugs. We used a previously built model to generate a 3D structure of the enzyme [23]. Figure 3 shows the sites where T. cruzi's NTR interacts with FMN.

Structural analysis of Quantum Mechanics and Molecular Mechanics (QM/MM)

We expect T. cruzi's resistance to the antiprotozoal drugs to derive from the substitution of amino acid Leu46 for Pro46, which takes place in the amino acid chain between the beta and alpha structures on the catalytic site. The psi (ψ) (Trp47-Pro46), and phi (φ) (Pro46-Gln45) angles in the wild-type enzyme, which display values of + 44.21°, and - 88.60°, shift to values of + 59.44°, and - 89.43° when the enzyme variant is computed. In addition, enthalpy of formation changes from -2972.655kJ/mol (wild-type) to -2888.716kJ/mol (variant). Figure 4 shows the effect of the substitution on the distances and angles between amino acids, which results from the dihedral effect on the plane that is generated by the atoms adjacent to the modification. The modified bond lengths between amino acids Arg10, Ser12, Lys14, and the FMN cofactor are shown in Table 1.

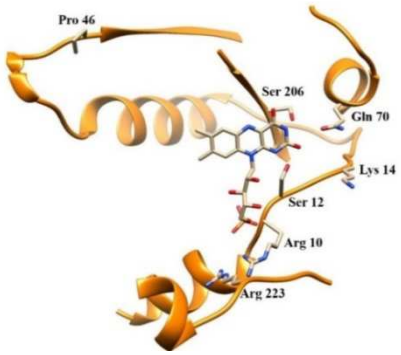
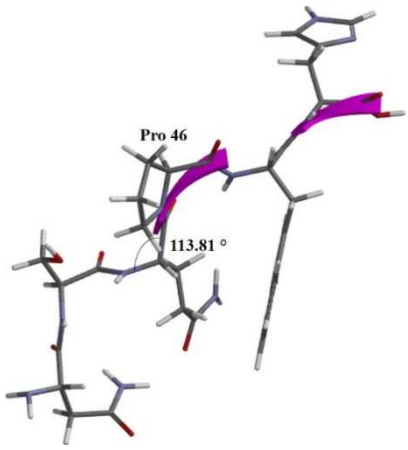
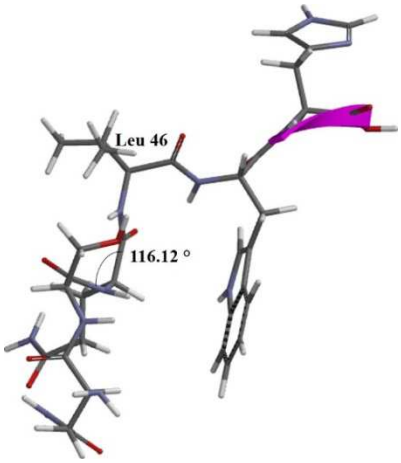



***Figure 3.*** *Representation of the sites where T cruzi's NTR interacts with FMN.*




A)




B)

***Figure 4.*** *Structural representation of the amino acid change in T. cruzi's NTR; A) Gln45-Pro46-Trp47 peptide; B) Gln45-Leu46-Trp47 peptide.*

***Table 1.*** *Bond lengths between the amino acids and FMN.*

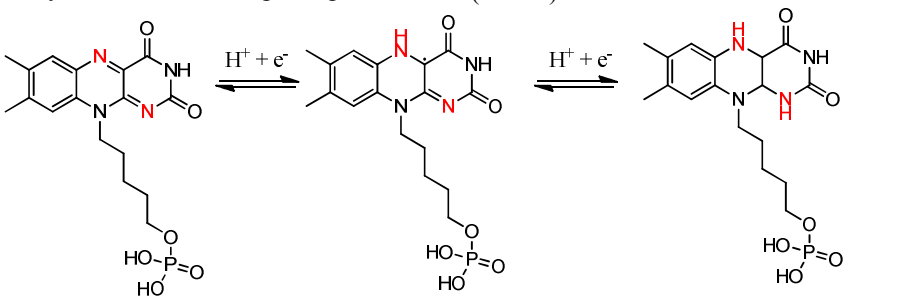
| T. cruzi | Wild type distances (Å) | Pro46Leu distances (Å) |
|---|---|---|
| Arg10 | 2.526 | 7.859 |
| | 2.100 | 6.979 |
| Ser12 | 2.325 | 8.801 |
| | 2.121 | 7.540 |
| Lys14 | 1.843 | 5.857 |
| Gln70 | 2.484 | 2.484 |
| | 2.457 | 2.457 |
| Ser206 | 2.144 | 2.071 |
| Arg223 | 1.847 | 1.847 |

## 4. Discussion

Mechanism of oxidation/reduction and drug activation

In T. cruzi, the enzyme-substrate complex promotes oxidation-reduction reactions [19-20], which are mediated by the electronic balance generated by the reduction of FMN. This coenzyme participates in pyrimidine biosynthesis but the parasite can dispense with its functionality and adopt a different mechanism for survival [35-38]. Equation 3 shows the structural representation of the FMN or riboflavin-5'-phosphate, which is derivative of riboflavin (vitamin B12) that acts as a coenzyme of diverse oxidoreductases. The reversible interconversion between the oxidized (FMN), semiquinone (FMNH•) and reduced semiquinone ($FMNH_2$) forms of the coenzyme occurs during the catalytic cycle. Equation 4 shows the representation of the redox reaction that activates the nitro prodrugs.

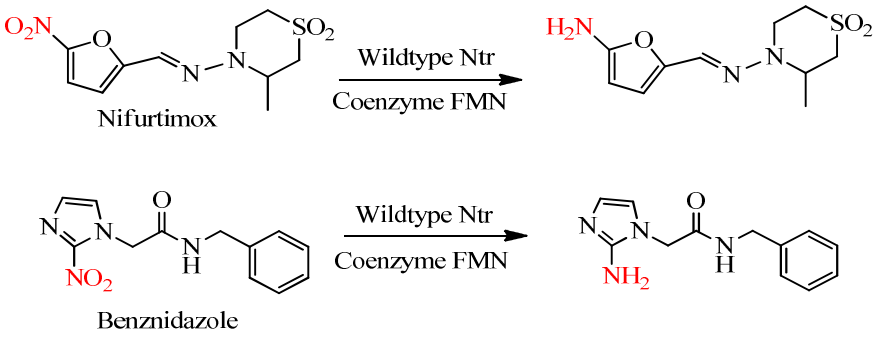
Equation 3. Representation of the interconversion reaction (Redox) of FMN




(3)

FMN (Flavin Mononucleotide) FMNH• (Semiquinone) $FMNH_2$(Hidroquinone)

Equation 4. Redox reaction for the activation of nitro prodrugs in Chagas disease treatment.



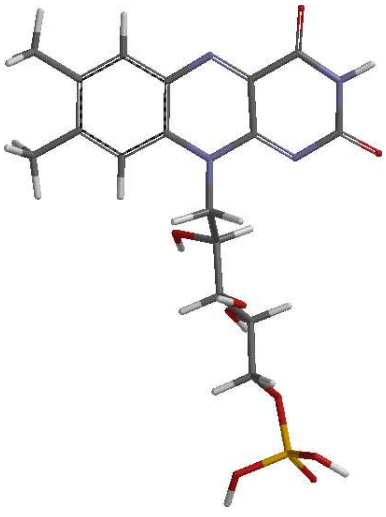

(4)

Energetic and geometric optimization of interaction NTR-FMN

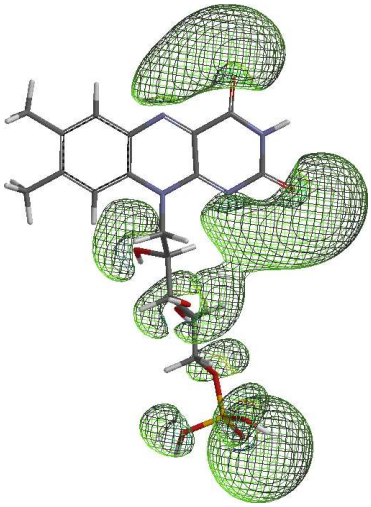
We used a hybrid Quantum Mechanics-Molecular Mechanics approach (QM-MM) to determine the interaction points and how these change after substitution of Leu46 for Pro46.

By considering the electrostatic potentials, we highlighted the areas with a higher contribution of electronic charge (negative charge) (Figure 5). These indicate the regions where interaction and docking with other molecules take place. We computed these potentials for FMN docking to the orthosteric site in NTR.

FMN mainly interacts with seven amino acids, which we identified and labeled using Swiss PDB viewer software (Figure 6). In addition, we described the electrostatic potentials of these amino acids for NTR (Figure 6A) and the enzyme-coenzyme complex (Figure 6B).

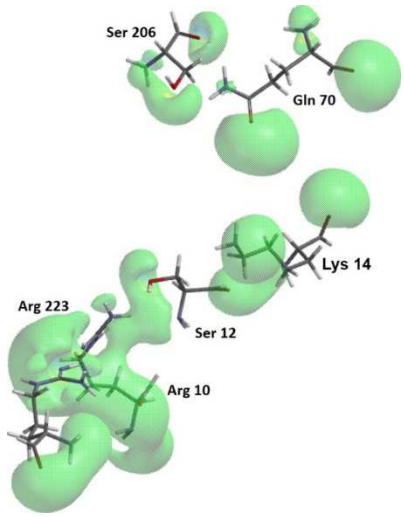


A)

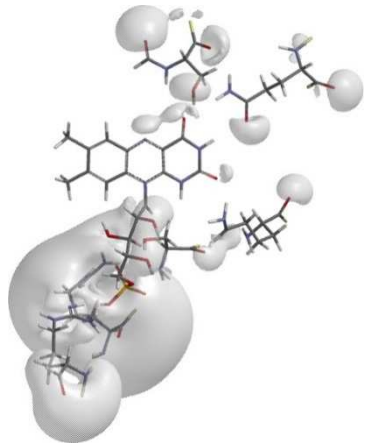


B)

***Figure 5.*** *Structural representation of FMN: A) Three-dimensional representation of FMN; B) Representation of the molecular electrostatic potentials (MEPs) (green region).*




A)



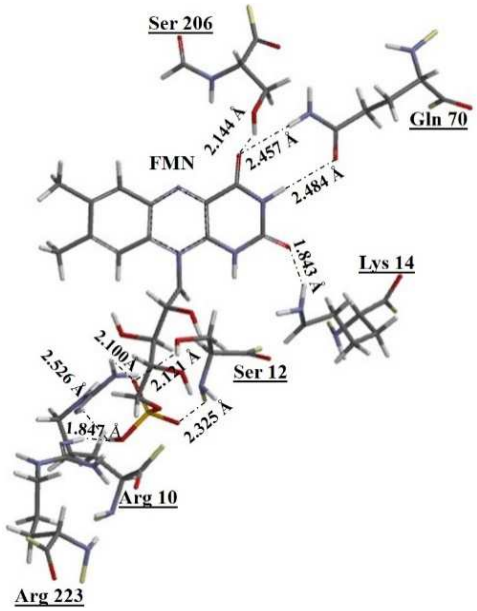
B)

***Figure 6.*** *Representation of the molecular electrostatic potentials (MEPs): A) amino acids on the catalytic site (green region); B) interaction of amino acids with FMN.*

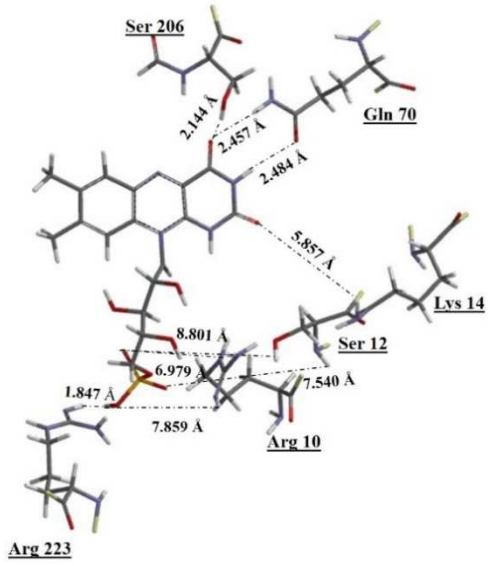
Quantitative analysis according to the semi-empirical method in the catalytic site.

We used the distance-measuring tool to compute the bond distances between FMN and NTR. With this tool, we determined that distances lower than 2.7 Å favor the interaction via hydrogen bonds. We used a semi-empirical method to consider the mutation and understand its effect. We found that the substitution of amino acid 46 on the catalytic site generates a spatial variation by modifying the reversible interconversion between the oxidized (FMN), semiquinone (FMNH•) and reduced forms (FMNH2) of the coenzyme.

Quantitative comparison in the effect between the anchoring of wildtype- and mutated-NTR with FMN coenzyme.




A)

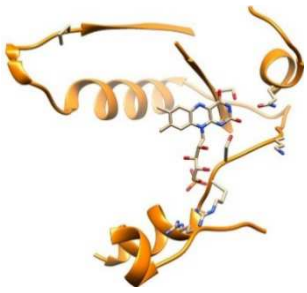



B)

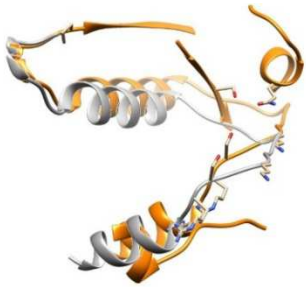
***Figure 7.*** *Structural representation of FMN interacting with the amino acids on the catalytic site of T. cruzi's NTR: A) Wild-type, B) Mutant.*

As shown in the generated model (Figure 4), a change of approximately 3° alters the interaction of the enzyme and the FMN cofactor. The quality of the interaction is affected, and the functionality of the complex is reduced. In addition, the change cripples the mechanism of the nitro prodrugs, which needs electrons to reduce its nitro chemical function and transform it into an amine. Figure 7 presents a comparison between the interaction of the enzyme and the coenzyme before (Figure 7A) and after Pro46Leu mutation (Figure 7B). Amino acids Arg10, Ser12 and Lys14 relocate and the bond distances between the enzyme and FMN increase as a result.

Finally, Figure 8 shows a ribbon model of wildtype NTR Figure 8A and its variant Pro46Leu. After mutation, FMN cannot dock to the enzyme and produce the electron flow needed to activate the antiparasitic prodrug Figure 8B. Shows the displacement of the orthosteric site in variant Pro46Leu Figure8C.



A)



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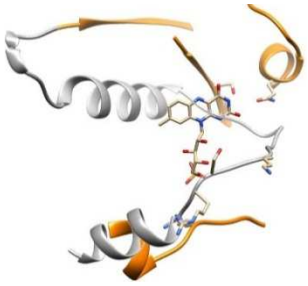

C)

***Figure 8.*** *Orthogonal view of T. cruzi's NTR enzyme: A) NTR and site of interaction with FMN; B) sequence of displaced amino acids due to amino acid substitution; C) alignment between wild-type NTR and the mutant.*

## 5. Conclusions

The computational hybrids that consider semi-empirical methodologies for polyatomic systems allow an insight into how nonsynonymous mutation in the translated enzyme produce changes in the docking of FMN, which alter the redox mechanism by which antiparasitic nitro prodrugs are activated. The analysis of the mutation Pro46Leu performed with quantum mechanics, presented conformational differences regarding changes on angle and distances between the amino acids that anchored to the active site of the FMN coenzyme and NTR enzyme. In addition, the integration between bioinformatics for the alignment and search of tertiary structures of a protein and quantum mechanics to analyze point changes of amino acids considering electronic densities, conformational changes in dihedral angles, distances, and protein foldings are useful in explaining how the parasite develops a mechanism to resist the drugs and as a fast and accurate alternative to generate more effective antibiotics derivatives.

## Equations

Software: Equation editor ChemBiodraw Ultra 12.0.

## Abbreviations

AM1: Austin Model 1; bzn: Benznidazole; CD: Chagas disease; FMN: Flavin mononucleotide; MM: Molecular mechanics; $MMFF_{aq}$ Merck Molecular Force Field in aqueous phase; nfx: Nifurtimox; NTR: Nitroreductase; PDB: Protein data bank; QM: Quantum mechanics.

## Conflicts of Interest

The authors declare no conflict of interest.

## Acknowledgements

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