

Article

Structural Relationship of Flavin Mononucleotide (FMN) and Type I Nitroreductase (NTR) of *Trypanosoma cruzi* in Resistance Testing with Benznidazole and Nifurtimox

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Abstract: The Chagas disease (CD) is an endemic infectious disease in a large part of Latin America. This disease is characterized by the fact of being caused by the parasite *Trypanosoma cruzi*. Some symptoms of this disease are cardiopathies and gastrointestinal problems. Therefore, the commonly used treatment consists of nitro drugs such as benznidazole and nifurtimox, which target the nitroreductase enzyme that docks to the coenzyme flavin mononucleotide (FMN) and an oxidation-reduction reaction is generated. From this reaction, the nitro chemical function of benznidazole and nifurtimox is reduced to amine. This amine in turn interacts with the parasite's DNA. In vitro tests experimentally help in the study of the parasite resistance to the drug benznidazole. This fact could explain how nonsynonymous mutations in the nitroreductase (NTR) enzyme do not allow the anchorage of the FMN coenzyme and as a consequence the parasite susceptibility to benznidazole decreases as the drug cannot be reduced. The structural relations from the hybrid Quantum Mechanics-Molecular Mechanics (QM/MM) allow to detect the small changes generated in the system, considering a charge distribution associated with the structure, angle changes, interaction distances, potential surface calculations between the wildtype enzyme and the mutation found.

Keywords: *Trypanosoma cruzi*; benznidazole; resistance; quantum mechanics; molecular mechanics

1. Introduction

The American Trypanosomiasis is the name given to the CD which is transmitted by the parasite *Trypanosoma cruzi* [1], [2]. Its vector is the triatomine bug from the *Rhodnius* family. This bug is commonly found in a tropical climate and has spread this disease throughout the South American countries [3], [4]; there is a high proportion of reported cases for infected patients in Latin America but the treatment of these cases has considerably advanced and there is an increasing in the prevalence cases of the disease with an estimation between 18 millions in 1991 and 5.7 millions in 2010 [5]–[7]. Besides, there is a calculation between 90,000 and 900,000 reported cases in Colombia by the year 2016 according to the World Health Organization (WHO). This is the reason why the attention to this disease may be defined as a matter of priority [6]–[8].

The most commonly used antiprotozoal drugs to combat this disease are benznidazole (bnz) and nifurtimox (nfx) [8]–[10]. Likewise, bnz is considered as the best drug for the treatment because of its bioavailability, tolerability, easiness in the dosage administration and efficacy. These nitroaromatic compounds have various mechanisms of action but most of these compounds depend on a previous activation, which is described from free-radical reactions [1], [11]. These reactions are mediated by the docking of nitroreductase (NTR) enzyme-FMN coenzyme (**Figure 1**). This enzyme produce a derivatization of the nitro compounds from a flow of electrons. After the anionic radical is obtained for the nitro compounds, a reduction of these anionic radicals is possible through an aerobic or anaerobic pathway. The conversión of nitrous ion through an aerobic pathway taking advantage of aqueous and acidic conditions of the environment that favor the reduction of the nitro functional group to the amine function [12]–[14] (**Equation 1A**).

A free radical reaction in non-polar mechanism is produced for the aerobic pathway between the radical of the nitro compound and the oxygen of the environment. This reaction makes reactive oxygen species be produced like superoxides, which can modify vital structures inside the parasite [15] (**Equation 1B**).

The compounds with nitro functional group experience a reduction and as an amino functional group have a high affinity with component structures of nucleic acids of the parasite by altering its metabolism [16], [17] (**Equation 2**).

These drugs lose their efficacy when the stages of the CD are chronic and that is when the resistance occurs. This resistance manifests as mutations in the orthosteric site or gen deletions that translate into the NTR enzyme [13], [18]–[21]. The concentration of this enzyme in the metabolism of the parasite has a fundamental role since low concentrations of this enzyme result in a higher probability of resistance to nitro drugs as nfx and bnz. On the contrary, this enzyme makes the parasite be hypersensitive to these two drugs when its concentration increases.

This work is aimed at carrying out a computational approach from a chemical perspective for the mutation in the NTR enzyme of *T.cruzi* since the NTR enzyme variant (Pro46Leu) could affect the functionality of this enzyme and favor the resistance of the parasite to nitro compounds affecting the activation of the drug [21], [22].

2. Materials and Methods

2.1 Bioinformatic analysis

The NTR does not have a crystallized structure. For this reason, the hypothetical model constructed by homology and threading with the predictor of I-TASSER structure and refined with the ModRefiner software in PDB format will be used[23]–[26] (**Figure 2**). From information of the primary sequence obtained from literature, a multi alignment with the software Jalview is made with the ClustalX and T-Coffee tool [27], [28]. Through this alignment, the mutation studied in the strains evaluated is located [19].

2.2 Visualization and alignment

The structures of the evaluated models were visualized and understood by illustrating their characteristic spatial distribution from the UCSF Chimera Version 1.11 software since this software provides an illustrative idea about the obtained models with ribbon visualization tools and density surfaces. The alignments with three-dimensional structures can also be made with this software. These alignments can be made through the comparison of the MatchMaker with a BLOSUM62 matrix and a Needleman-Wunsch global alignment algorithm for the wildtype structures of *T.cruzi* and *E.coli* [29].

2.3 Calculations and parameters

Calculations are made through semi-empirical method with the hybrid Quantum Mechanics/Molecular Mechanics (QM/MM) for the wild protein and the mutation Pro46Leu [30]–[32]. The distance and angle measurements were made with a Spartan 14 software tool for the previous protein and its mutation. Besides, a potential surface is calculated with a mesh visualization in order to identify the anchorage sites of the FMN coenzyme and NTR enzyme.

2.4 Analysis with the hybrid Quantum Mechanics-Molecular Mechanics

The calculations of energetic optimization of equilibrium geometry of the catalytic site and FMN structures were carried out by using the Spartan 14 version 1.2.0 (Purchased license) [33]. This tool allowed to select the semi-empirical method of low computational cost. This method requires shorter analysis times and allows the work with macromolecular systems like enzymes and coenzymes. The bases used were Austin Model 1 (AM1) and Merck Molecular Force Field (MMFF) in aqueous phase. Given the fact that the studies about the use of these methods for biological systems with excellent results have been presented, the molecules were optimized by using parameters of minimum geometry and energy with 12000 optimal cycles of these methods [31], [34], [32].

3. Results

3.1. Alignment of primary sequences and characterization of NTR with clustalX and T-coffee 2.0

Experimentally, the primary sequences of the constituent amino acids of the NTR enzymes for the strains of resistant and susceptible parasites were amplified and sequenced [19], [18]. This is the reason why a structural alignment is made from bioinformatics, taking into account the sequences characterized by the Jalview software and its tool T-Coffee and ClustalX. Thus, obtaining a high degree of similarity for the sequences of the parasites. These results are shown in the following (Figure3).

The NTR mutation of *T. cruzi* was found in the fragment that is located inside the FMN receptor for the amino acids that are in the position Ala 41 to Val 50, as can be seen in (Table 1).

3.2. Localization of interaction site in tridimensional structure of NTR

Once the function of the flavins and their derivatives as cofactors in the redox reactions have been understood, the NTR enzyme of *T. cruzi* has been selected as a system for the study of the resistance in the CD. However, this NTR enzyme does not have crystallized structure but a theoretical model by homology and threading is used from fold recognition to generate a structure proposed in the literature [23]. The interaction site of the NTR enzyme of *T. cruzi* with FMN, as presented for *E. coli* NTR (Figure 4A), can be found among the following amino acids: Arg10, Ser12, Lys14, Asn71, Glu165, Lys205 and Arg207. Concerning *T. cruzi* NTR, the interaction can be found among the amino acids: Arg10, Ser12, Lys14, Gln70, Ser206, Arg223 (Figure 4B). The docking of the FMN with the previous amino acids will be studied further.

3.3 Structural analysis of Quantum Mechanics and Molecular Mechanics (QM-MM)

The *T. cruzi* resistance to the antiparasitic nitro drugs may be attributed to the change from the amino acid Pro46 to Leu46. Such a change can be found in the amino acid sequence of the chain between the beta and alpha structures of the amino acids that make part of the catalytic site. The structural change make the psi (ψ) and phi (φ) angles: between Trp47-Pro46 and Pro46-Gln45 be 44.21° and -88.60° respectively. When the variant in the NTR enzyme from Pro46 to Leu46 is obtained, the psi (ψ) and phi (φ) angles have values of 59.44° and -89.43° respectively. The (Figure 5) shows the effect of amino acid change in distances and angles between amino acids because of the dihedral effect of the plane

which is generated in the atoms adjacent to the modification. This structure represents the effect in the angle change from the amino acid Pro46 to Leu46 in the interaction of FMN and the catalytic site of the NTR enzyme of *T. cruzi*. Here, a modification of the bond lengths between the amino acids Arg10, Ser12 and Lys14 was carried out as shown in **Table 2**. This table also shows the values of the distances between the amino acids of the catalytic site and the cofactor before and after the structural variation. Besides, the heat formation indicates that the variant Pro46Leu destabilizes the system.

4. Discussion

4.1 Mechanism of oxidation/reduction reaction and the activation of antiparasitic pro-drugs

The FMN is considered the coenzyme of the NTR enzyme of the parasite *T. cruzi*. The function of enzyme-substrate complex lies in being the promoter of some oxidation-reduction reactions inside the parasite whose metabolic functions are determined by the flavins as progressive mediators of the protein reductions in approximately 1% of the processes [19], [20]. As for the parasite, some examples of metabolic functions mediated with these reactions are given by the oxidation of dihydroorotate to orotate. This oxidation is docked to the reduction of fumarate to succinate. Both oxidation and reduction reactions were mediated by the electronic balance generated by the reduction of the FMN cofactor which participates in pyrimidine biosynthesis. This biosynthesis is a pathway of the parasite for the obtention of nucleic acids of pyrimidine base and is in relation to the growth kinetics in the infected cells. Likewise, the parasite dispensed with the functionality of this enzyme and adopted other mechanism that allowed it to survive at the moment of generating resistance since it could colonize the petri dish again in vitro experimental analysis with antiparasitic nitro drugs [35]–[38]. The **Equation 3** shows the structural representation of the FMN or riboflavin-5'-phosphate. This is a derivative of riboflavin (vitamin B12) that acts as a coenzyme of diverse oxidoreductases. The reversible interconversion among the oxidized form (FMN), semiquinone (FMNH•) and reduced semiquinone (FMNH₂) of the coenzyme occurs during the catalytic cycle. In the **Equation 4** shows the representation of the redox reaction of the nitro-drugs that generate effects in the parasite *T. cruzi*.

4.2 Energetic and geometric optimization of interaction NTR-FMN

The study of this interaction between the coenzyme and wildtype NTR structure is key in the recognition of the interaction points that favor the cofactor anchorage and the subsequent reduction for the viability of the redox reactions in the system. In addition, the mutation found by amplifying and sequencing the NTR enzyme was located in the amino acid Pro46Leu. Therefore, it is subsequently intended to make an energetic and geometric optimization of the structural changes at the moment of generating the mutation that can be measured with a hybrid Quantum Mechanics-Molecular Mechanics (QM-MM). The electrostatic potentials are a way of considering the charge distribution in a molecule (**Figure 6**). This fact allows to identify the areas with higher contribution of electronic charge (negative charge) than others. This is particularly important in the SAR analysis since it indicates the interaction and docking region with other molecules. Therefore, the respective calculation is carried out by only taking into account the FMN as coenzyme and anchorage in the orthosteric site of the NTR.

The FMN mainly interacts with seven amino acids that were identified and labeled with the Swiss PDB viewer software (**Figure 7**). Moreover, the electrostatic potentials of these amino acids are described (**Figure 7A**). These electrostatic potentials are of a great interest to the approach of the interaction during catalytic cycle between the FMN and the NTR (**Figure 7B**).

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

When the bond distances between the FMN coenzyme and the NTR enzyme are quantified, the distance measuring tool of the software is used. Through this tool, values within the interaction range

of the hydrogen bond are obtained; this range shows that distances lower than 2.7 Å favor the plausible interaction. The mutation was evaluated through a calculation by semi-empirical method which was used to carry out the respective structural mutation to better understand its effect. In addition, the modification of the amino acid 46 in the catalytic site that can be found in the amino acids Arg10, Ser12 and Lys14 generates a spatial variation by modifying the reversible interconversion between the oxidised form (FMN), semiquinone (FMNH•) and the reduced form (FMNH₂) of the coenzyme.

4.4 Quantitative comparison in the effect between the anchored of wildtype and mutation NTR enzyme with FMN coenzyme.

As can be seen in the generated model (**Figure 5**), a change of approximately 3° significantly alters the interaction with the FMN cofactor. For that reason, the anchorage of this cofactor would not be so efficient and the redox functionality of the enzyme-coenzyme system is directly affected. This fact does not favor the reduction mechanism of the nitro drug bzn or nfx that needs the electrons generated by that docking so that its nitro chemical function can be reduced and transformed into amine, which potential affinity with the nucleic acids of the parasite. The (**Figure 8**) presents the comparison between the wildtype interaction with the coenzyme (**Figure 8A**) and the effect of the variation Pro46Leu (**Figure 8B**). The displacement of the amino acids Arg10, Ser12 and Lys14 can be seen and thus, the bond distances between the enzyme and the FMN increase.

From another structural perspective (**Figure 9**), the ribbon visualization for the wildtype NTR enzyme is presented (**Figure 9A**) and the variant Pro46Leu, by which the effect of the angle change related to the side chain of the amino acid in the position 46 can be denoted. The manifestation of this angle change is shown by a lack of docking of the FMN coenzyme and it doesn't produce the electron flow to activate the antiparasitic prodrug (**Figure 9B**). The (**Figure 9C**) shows the displacement of the amino acids of the catalytic site when an alignment of the wildtype enzyme and the variant Pro46Leu is made.

4.5. Figures, Tables and Schemes

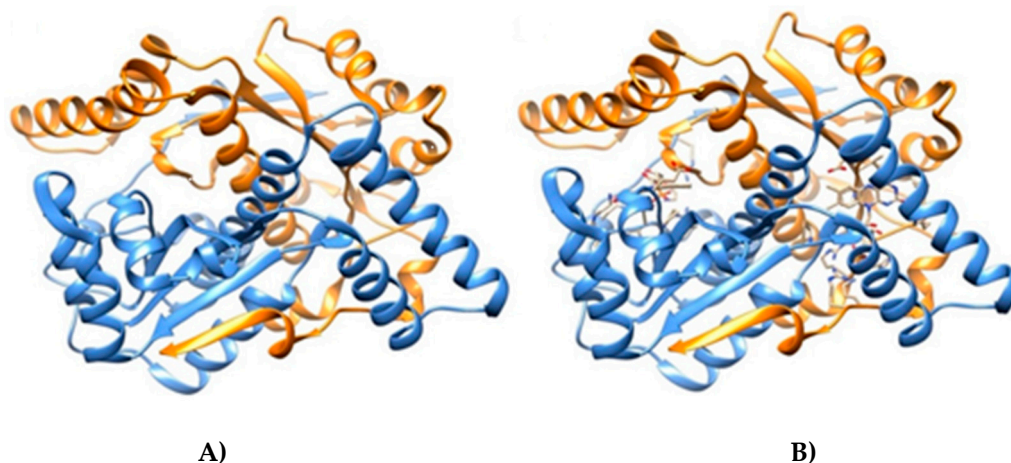


Figure 1. Three-dimensional representation of the NTR dimer of E. coli (PDB ID 1DS7): A) NTR in ribbon contour; B) NTR with the FMN cofactor.

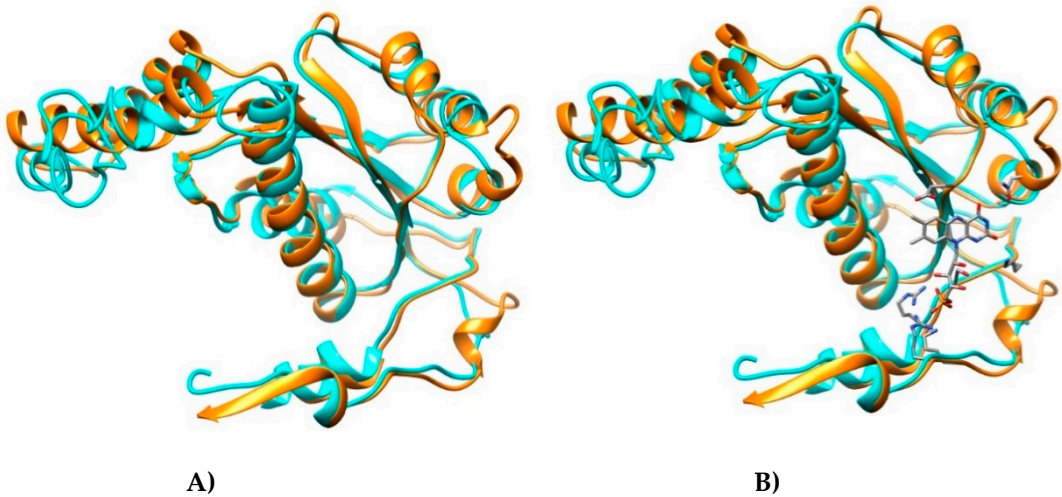


Figure 2. *E.coli* NTR alignment (Orange) and proposed model for *T.cruzi* NTR (Cyan) [23]: A) NTR alignment in ribbon contour; B) NTR alignment with the FMN cofactor.

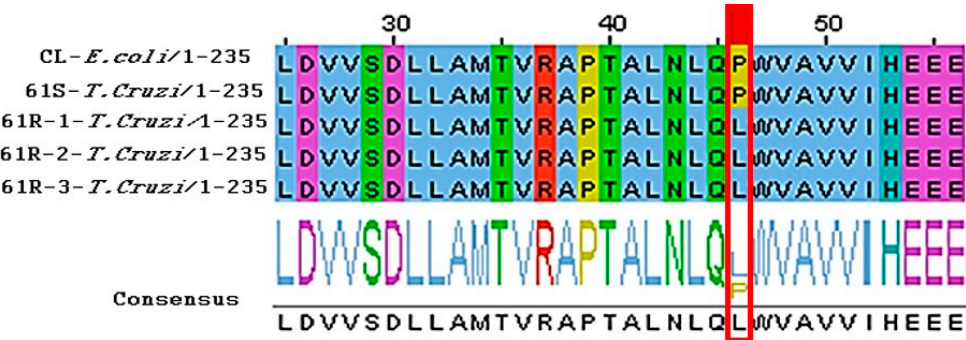


Figure 3. Primary sequence NTR alignment of the *T. cruzi* strains: wildtype NTR of the parasite that is sensible and resistant to benznidazole. The underlying mutation (Pro46Leu) in the resistant NTR enzyme can be seen in this image [19].

Table 1. Primary sequence of the FMN receptor: there is a mutation that causes resistance to bzn and nfx in the NTR enzyme in the position 46.

Organism	Sequence of amino acids
	46
Trypanosoma cruzi	41-ALNLQProWVAV-50
	Leu

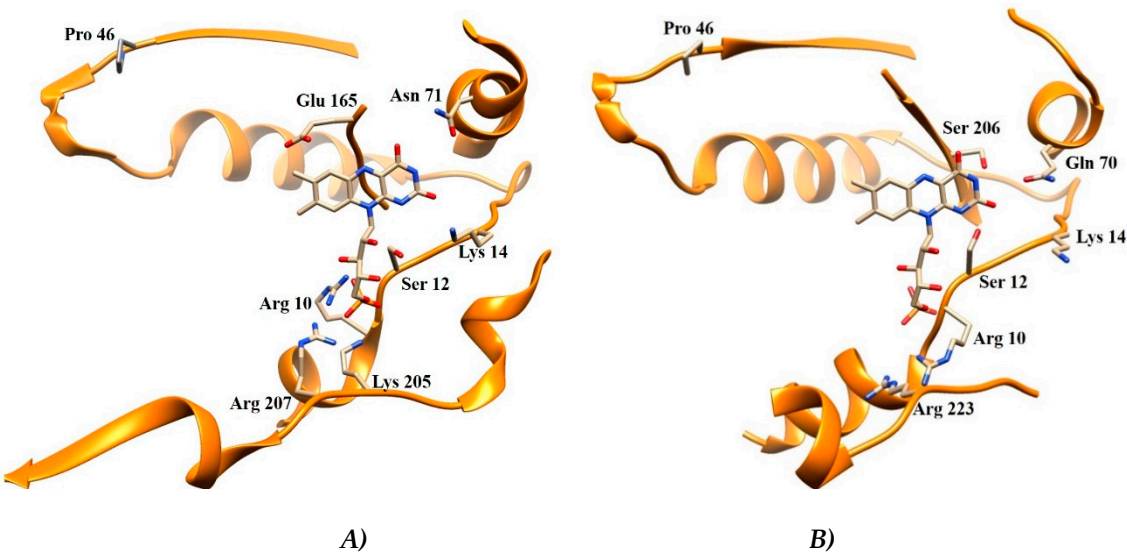


Figure 4. Representation of the interaction site with the FMN cofactor: **A)** Type I NTR enzyme of *E.coli*; **B)** Type I NTR enzyme of *T.cruzi*.

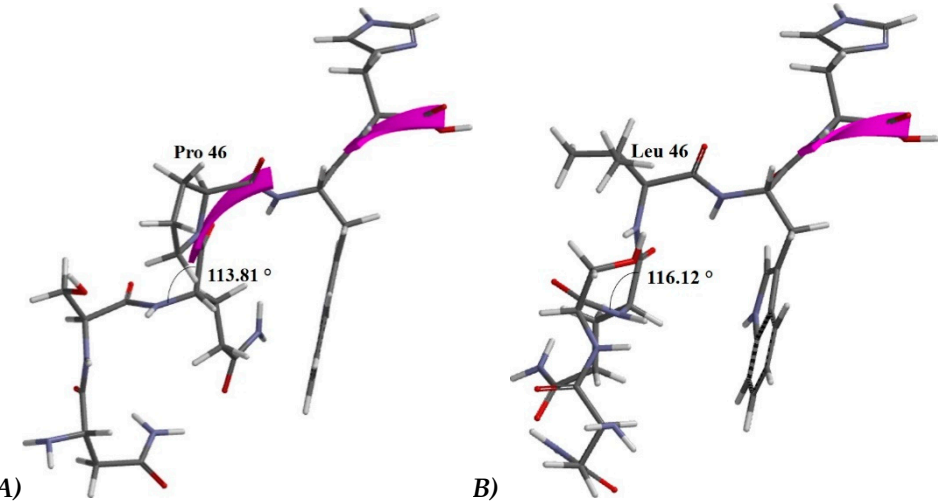


Figure 5. Structural representation of the amino acid change in the NTR enzyme of *T.cruzi*; **A)** peptide Gln45-Pro46-Trp47; **B)** peptide Gln45-Leu46-Trp47.

Table 2. Bond length between the amino acids of the catalytic site and the FMN.

<i>T. Cruzi</i>	Wildtype Distances (Å)	Pro46Leu Distances (Å)
<i>Arg10</i>	2.526	7.859
	2.100	6.979
<i>Ser12</i>	2.325	8.801
	2.121	7.540

<i>Lys14</i>	1.843	5.857
<i>Gln70</i>	2.484	2.484
	2.457	2.457
<i>Ser206</i>	2.144	2.071
<i>Arg223</i>	1.847	1.847
<i>Energy</i>	-2972.655kJ/mol	-2888.716kJ/mol

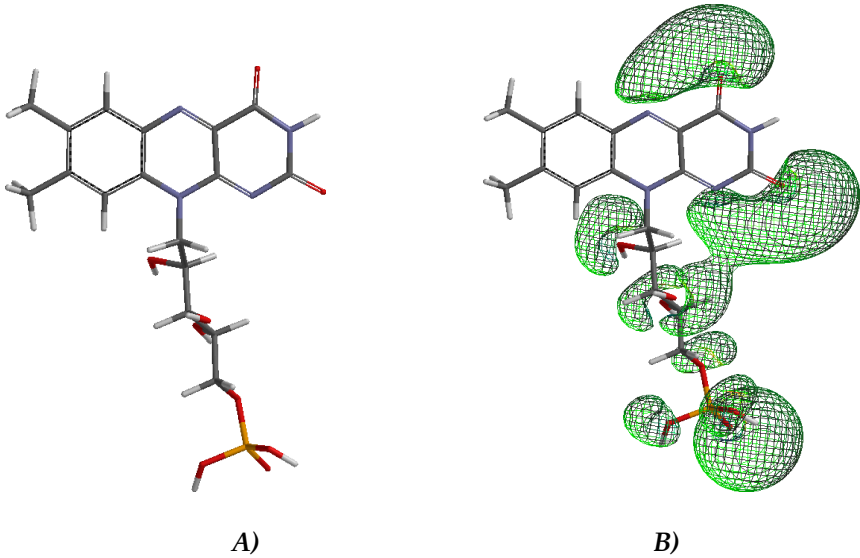


Figure 6. Structural representation of the FMN molecule: A) Three-dimensional representation of FMN; B) Representation of the molecular electrostatic potentials (MEPs) (green region).

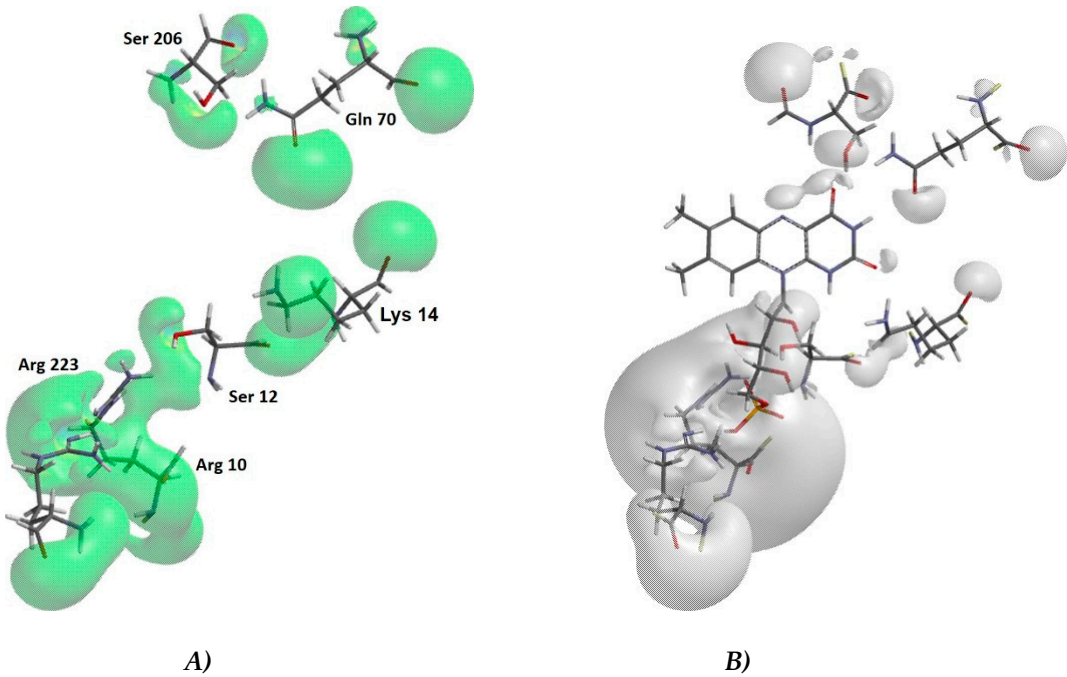


Figure 7. Representation of the molecular electrostatic potentials (MEPs): **A)** amino acids of the catalytic site (green region); **B)** interaction of amino acids with FMN.

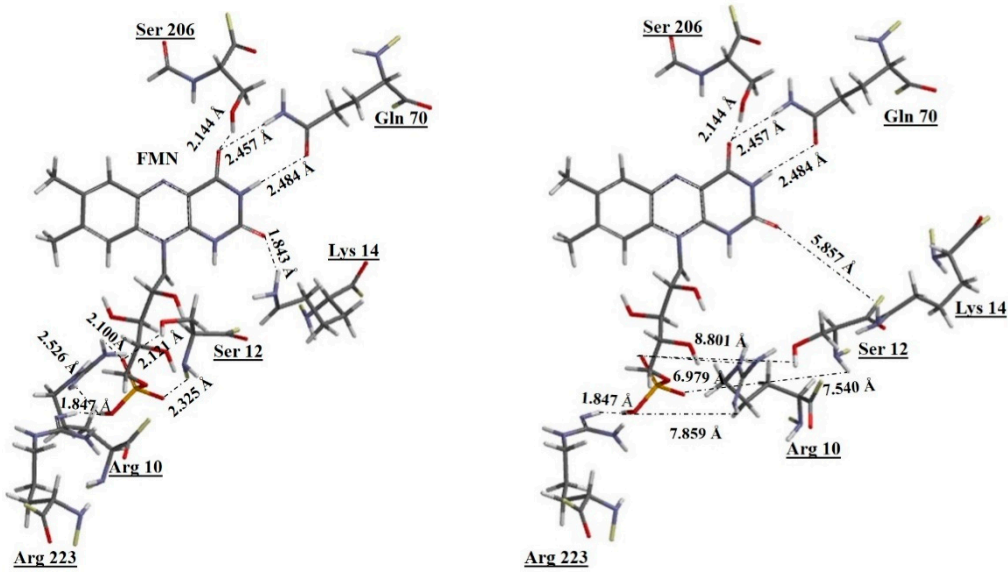


Figure 8. Structural representation of the flavin mononucleotide molecule interacting with the amino acids of the catalytic site of the NTR enzyme of *T.cruzi*: **A)** Without modification of Pro46, **B)** With modification of Pro46Leu

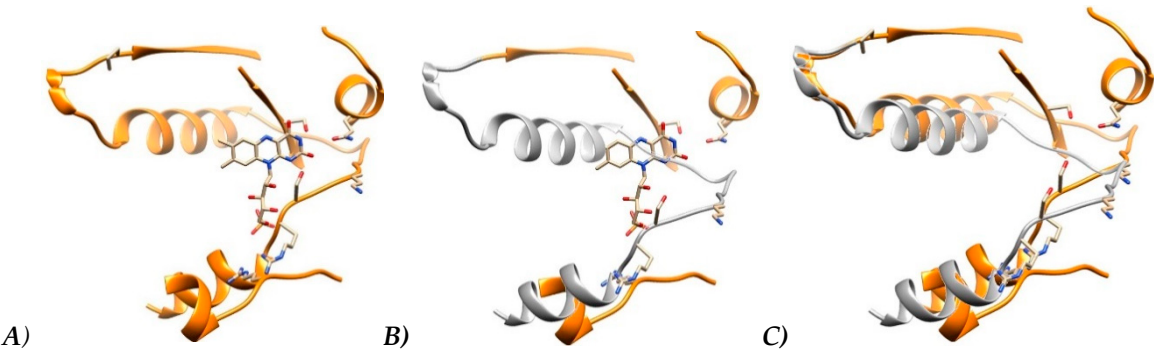
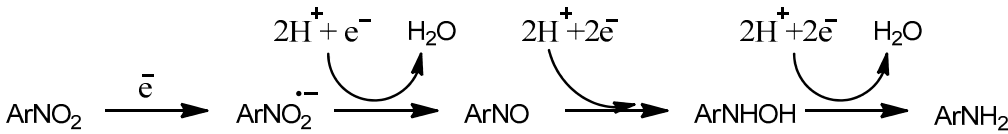


Figure 9. Orthogonal view of the NTR enzyme of *T.cruzi*: **A)** NTR enzyme of *T.cruzi* and the FMN interaction site; **B)** sequence of displaced amino acids due to the amino acid change; **C)** alignment between the wildtype NTR and the mutation.

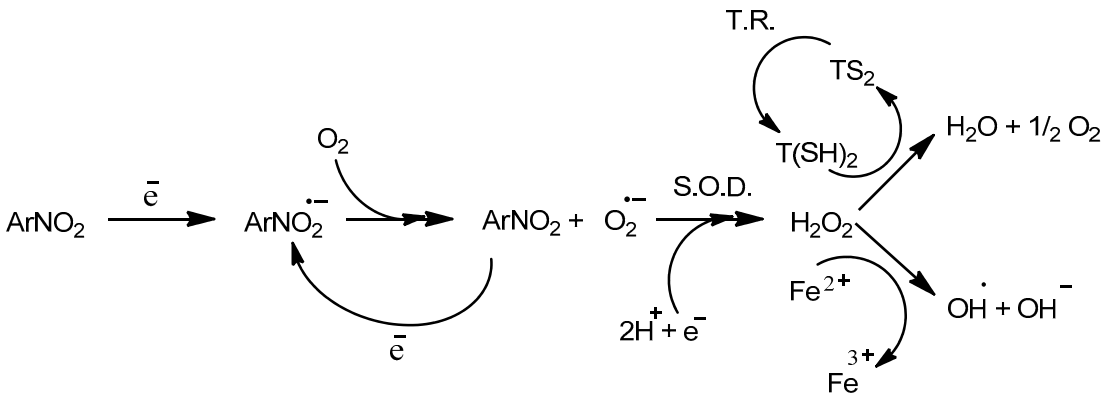
3.4. Software: Equation editor ChemBiodraw Ultra 12.0

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

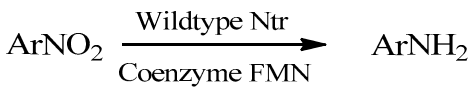
A) Aerobic pathway



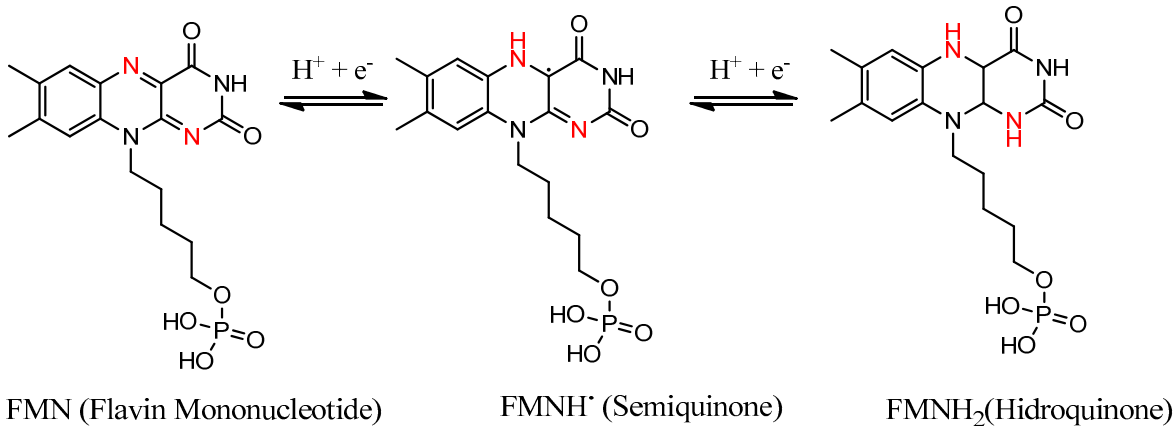
245 **B) Anaerobic pathway**



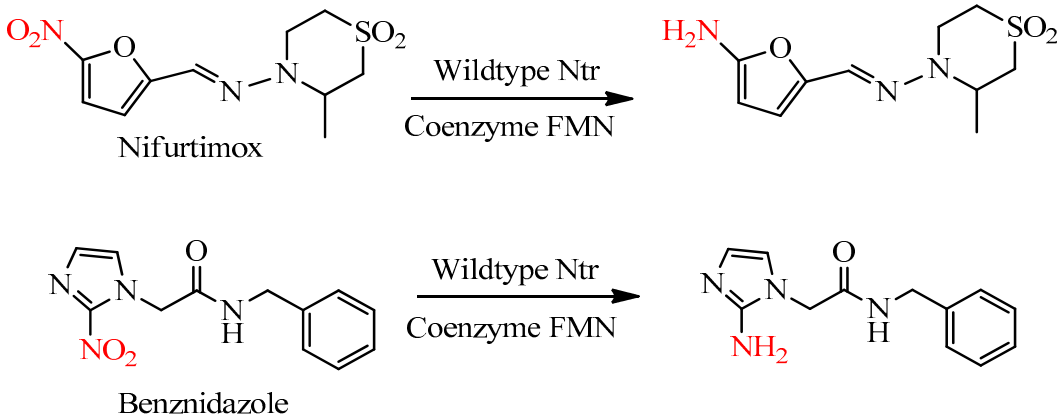
247 **Equation 2.** Activation of nitro drugs from a redox reaction.



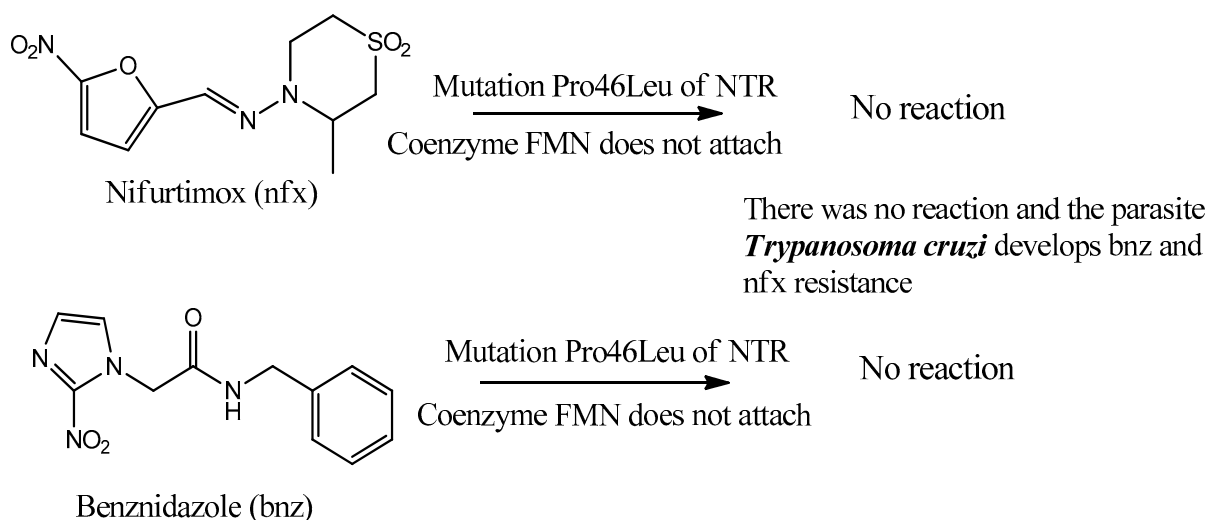
250 **Equation 3.** Representation of interconversion reaction (Redox) of the flavin mononucleotide molecule



253 **Equation 4.** Redox reaction of nitro drugs that generate effects in the parasite *T. cruzi* (Activation)



255 **Equation 5.** Redox reaction of resistance of the *T. cruzi* to antiparasitic nitro drugs (No activation)



256

257 5. Conclusions

258 The computational methods applied to the study and understanding of macromolecular biological
 259 systems have raised potential importance in the study of diseases since these methods allow to
 260 understand and explain biological processes at a molecular level from a functional-structural
 261 approach. The CD caused by the parasite *T. cruzi* and the antiprotozoal treatments such as
 262 benznidazole or nifurtimox have therapeutical ranges that work very well in early phases of the
 263 disease but their efficacy decreases. Therefore, it is very likely that survival mechanisms of the
 264 parasite can be activated so that it strives to survive in the adverse conditions. For that reason,
 265 understanding the role of the NTR enzyme and the FMN coenzyme (in charge of the redox reaction
 266 of the nitro drug bzn and nfx when there is an adverse environment for the parasite) and the
 267 mutations related to the survival in an environment of selective chemical stress gain real
 268 importance and are crucial in the study of the resistance and pharmacology in the treatments of
 269 infectious tropical diseases (**Equation 5**).

270 The computational hybrids that take into account semiempirical methodologies for polyatomic
 271 systems are an approximate approach that allowed to understand how a nonsynonymous mutation
 272 in the translated NTR enzyme can cause effects in the anchorage of a coenzyme such as the FMN
 273 and alter the redox mechanism of the activation of the antiparasitic nitro pro-drugs in the treatment
 274 of the Chagas disease. Therefore, the changes of dihedral angles, the changes of distances and the
 275 alterations in the protein foldings are arguments used to explain the experimentally obtained
 276 resistance from the molecular conceptualization.

277 **Author Contributions:** Araque and Soto-Ospina elucidated the tridimensional structures and
 278 designed the experiments with geometrical and energetical optimization; Araque and Soto-Ospina
 279 performed the experiments; Araque and Soto-Ospina wrote and edited the paper.

280

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283 **Conflicts of Interest:** The authors declare no conflict of interest

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