ORIGINAL ARTICLE

Drug-protein *in vitro* and *in silico* interaction analysis with hemoglobin as a study model

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ABSTRACT

Study model: It is an in vitro experimental study with a computational approach. **Objective**: Analyze the presence of interaction between hydrophobic drugs bezafibrate and hydrochlorothiazide and hemoglobin to predict bioavailability changes as well as in the protein function. **Metodology:** The in vitro tests to evaluate the interaction between the bovine hemoglobin and bezafibrate and hydrochlorothiazide were perfomed by spectrophotometry; bioinformatic tools made interaction analysis and extrapolation for human hemoglobin. **Results:** The in vitro tests showed a decrease in the absorbance (k) at 405 nm equal to 8.75 x 10-4 min-1 for bezafibrate and 6.25 x 10-4 min-1 for hydrochlorothiazide. The decrease suggests an interaction between the drugs and hemoglobin, for bezafibrate this interaction seems to be stronger than hydrochlorothiazide. The in silico analysis showed that the drugs bind to the protein portion of the hemoglobin. The binding affinity constant obtained by molecular docking from bezafibrate and bovine hemoglobin (-8.3 Kcal/mol) sustain the experimental value of k and the greater number of interactions observed in relation to hydrochlorothiazide (-6.6 kcal/mol). The same pattern was observed for interaction of bezafibrate (-7.6 kcal/mol) and hydrochlorothiazide (-6.7 kcal/mol) with human hemoglobin. **Conclusion:** The spectrophotometry and bioinformatic methods suggested the possibility of hemoglobin interaction with hydrophobic drugs such as bezafibrate and hydrochlorothiazide; this interaction could affect the normal function of hemoglobin and change the pharmacodynamics and pharmacokinetics of drugs impairing their therapeutic efficiency.

Keywords: Molecular Docking Simulation; Hemoglobin; Computer Simulation

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INTRODUCTION

Hemoproteins, such as hemoglobin, have been used as a model to study drug-protein interactions for understanding the dynamics of the drug in the body. Although hemoglobins are present in erythrocytes, many therapeutic drugs can cross membrane barriers and reach them[1].

It is known that the interaction between drugs and proteins is responsible for the desired action of the drug but also for its side effects. In general, these interactions can influence the distribution of the drug in the body causing its inactivation, not allowing a free drug therapeutic concentration to activate a receptor, or even delay its excretion[2]. In addition, drug-protein binding can lead to changes in both secondary and tertiary protein stilettos[3,4].

The association of drugs with blood components is a reversible process that usually involves albumin and α1 acid glycoprotein. However, more complex components such as lipoproteins, erythrocytes or platelets may be involved[5,6].

Proteins such as albumin and hemoglobin have been studied due to interactions with drugs used in clinical practice; it is important to be aware of how these interactions can affect the primary function of these proteins and how biodistribution and drug effects on target tissues are affected[3].

Hemoglobin is an important hemeprotein associated with oxygen transport, being the main protein of vertebrate erythrocytes. Each milliliter of blood has approximately five billion erythrocytes and each erythrocyte has approximately 280 million hemoglobin molecules[7]. Taking this protein as a study model, it should be taken into account that drugs can bind to their different subunits, in their globin and/or heme group, as occurs with some antimalarials[8].

Different disorders in hemoglobin or more specifically in the heme group may alter the functioning of hemoglobin and other proteins that have the heme group as a prosthetic group, such as protein complexes of cytochrome P450. This can lead to specific pathologies by altering the binding and metabolization of drugs and drugs, thus affecting the desired effects on the body.

Thus, it is clear the need to determine the existence of interactions between the different drugs of clinical use with proteins of the organism and analyze the nature of these interactions. Because it is a plasma protein, it is also extensively used for protein-binding interaction research in toxicity and safety studies[9,10]. This study suggests the use of hemoglobin as a model protein for the study of drug-protein interaction, specifically for predominantly hydrophobic drugs, such as bezafibrate and hydrochlorothiazide widely used in therapy.

The analysis of an interaction between molecules today has been greatly facilitated by computational methods, such as molecular docking that is useful and reliable for predicting possible interactions and affinity of ligands with macromolecules. *In silico* methods are gaining prominence since the experimental determination of complex three-dimensional structures is quite complex and costly[11].

MATERIAL AND METHODS

In vitro *drug-protein interaction analysis*

In this work, the holo form of purified bovine hemoglobin (Sigma®) was used as a model protein. For drug interaction experiments, hemoglobin was solubilized in PBS (Phosphate buffer saline) pH 7.4 at the time of use at a concentration of 6.7 mM. The drugs for therapeutic use in humans used as a model were bezafibrate (anti-lipemic) and hydrochlorothiazide (antihypertensive), both of a primarily hydrophobic chemical nature. The drugs were isolated from the commercial drug obtained in a pharmacy.

Both drugs, presented as a tablet, were macerated and solubilized in PBS with the aid of a magnetic stirrer. Then the mixture was centrifuged at 5000 rpm for separation and disposal of excipients. The presence of bezafibrate and hydrochlorothiazide was detected by absorption at 230nm and 273nm, respectively. For the interaction analyses, bovine hemoglobin was incubated with bezafibrate 0.01 g/mL and with hydrochlorothiazide 0.01 g/mL separately.

The drug-protein interaction *in vitro* was evaluated by analyzing of reaction kinetics based on absorbance variation at 405 nm (Soret Band) by spectrophotometry at room temperature (25ºC). Protein and drug concentrations were adjusted to simulate physiological and therapeutic proportions, respectively. As a negative control of the reaction, bovine hemoglobin in PBS was incubated under the same experimental conditions, however in the absence of drugs.

The variation in absorbance is a decrease (k), which was calculated using the equation k = ln[(hemoglobin)t/(hemoglobin)0]. Data were collected every 2 min for 40 min. Kinetic reactions were performed in triplicate and submitted to statistical analysis using Rstudio® software. When a reduction in light absorption was observed during the enzymatic kinetics reaction, the Wilcoxon-Mann-Whitney test was applied, comparing the results obtained in the first 10 min with the data collected between minutes 30 and 40, adjusted for control variation.

In silico *analysis of drug-protein interaction*

The primary sequences of both bovine and human hemoglobins were aligned using the ClustalX software[12]. The EMBL-EBI online global alignment tool was also used to assess the similarity of the sequences studied[13].

Both bovine and human hemoglobins have a three-dimensional structure solved by crystallography and X-ray diffraction deposited in the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do). For the *in silico* analyses, the 2QSS (Bovine Hb) and 2H35 (Human Hb) structures were used. The three-dimensional chemical structures of bezafibrate and hydrochlorothiazide used for molecular docking were obtained in ZINC12, a virtual database of chemical structures (http://zinc.docking.org).

Molecular docking was performed using AutoDock Tools (http://autodock.scripps.edu/) coupled to Vina software (http://vina.scripps.edu/). The analysis of amino acid residues involved in drug-protein interactions and those involved in the positioning of the heme group after docking were made using PyMol software.

RESULTS AND DISCUSSION

In vitro *analyses*

UV-Vis absorption spectroscopy allows exploring structural changes in a protein and investigating the formation of protein-ligand complexes. Thus, spectroscopy is widely used because it is a simple and useful technique for measuring structural changes in proteins[14].

In the case of hemoglobin the absorption spectra of the heme group are very sensitive, which makes the technique suitable for investigating drug-protein interaction. Hemoglobin in ferrous state is characterized by the Soret band at 405-407 nm[15]. *In vitro* tests showed a decrease in light absorption at 405 nm for the two drugs analyzed ($p=0.045$ for bezafibrate and $p=0.022$ for hydrochlorothiazide), as shown in Figures 1 and 2.

The absorbance variation calculation resulted in $k=8.75 \times 10^{-4}$ min^{-1} for bezafibrate and $k=6.25 \times 10^{-4}$ min^{-1} for hydrochlorothiazide. According to these values, bezafibrate seems to interact with a slightly higher affinity with bovine hemoglobin than hydrochlorothiazide. According to Sugihara (16), the presence of bezafibrate reduces the oxygen affinity of erythrocytes, as it binds to different binding sites of 2,3-bifosfoglicerate, an allosteric regulator of hemoglobin affinity. According to these results, we can infer that there was an interaction between the drugs tested and hemoglobin, modifying the structure of bovine hemoglobin[17].

The decrease in absorbance at 405nm indicates a change in the positioning of the heme group, possibly due to the interaction of hemoglobin with the drug. Only with spectrophotometric analyses it is impossible to determine whether the drug connected directly to the heme group or to some nearby amino acid residue, and thus the change in its positioning was transferred to the prosthetic group. Therefore, molecular imaging techniques were used to evaluate the interaction of the ligand with the target protein.

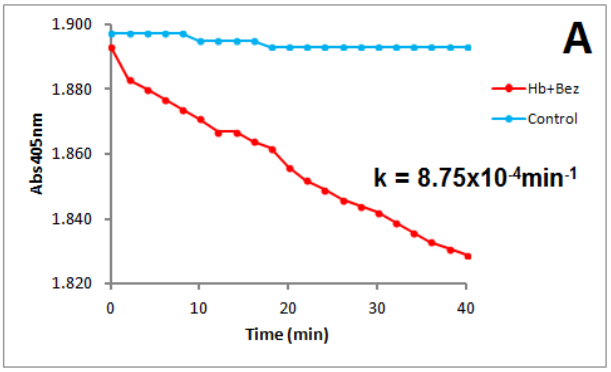

Figure 1: Kinetics of reaction of the interaction between bovine hemoglobin and bezafibrate.

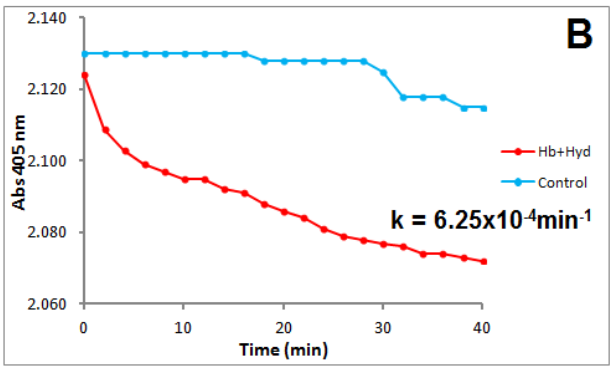

Figure 2: Kinetics of reaction of the interaction between bovine hemoglobin and hydrochlorothiazide.

In silico *analysis*

Sequence and alignment analysis

Bovine hemoglobin shows small structural and functional differences in comparison to human hemoglobin[18]. Thus, bovine and human hemoglobin were analyzed by bioinformatics tools to obtain similarity data between them.

Amino acid composition analysis showed that the alpha and beta chains of the two hemoglobins have a similar content. Alpha chains have the same number of residues (141) and in the beta chain of human hemoglobin there is an even more residue (146) than bovine hemoglobin (145).

The alignment of the primary sequences of hemoglobins showed that the position of amino acids is conserved (Figures 3 and 4). The alpha chains showed 92.2% sequence similarity and the beta chains showed 90.4%.

There are 17 different residues in the alpha chains. Among these 17 residues, 12 were replaced by other residues with a similar side chain (in load or size). The remaining five have completely different side chains in size or load. In position eight of the primary sequence of alpha chains a Gly/Thr substitution (2QSS/2H35) can be observed; a Ala/Lys replacement (2QSS/2H35) at position 60, Ala/Asp (2QSS/2H35) at position 64, Glu/Ala (2QSS/2H35) at positions 71 and 82. In summary, there was a substitution of not charged residues by charged ones or hydrophobic residues by hydrophilic ones.

In beta chains there are 24 different residues, of which 18 are similar substitutions (in load and size). There is no alignment in the first amino acid because there is a gap in bovine hemoglobin when pairing with the Val1 of human hemoglobin, then the first amino acid of bovine hemoglobin aligns with the second of human hemoglobin and so on. The other replacements are Met1/His2 (2QSS/2H35), Lys75/Ala76 (2QSS/2H35), Val111/Cys112 (2QSS/2H35), Val124/Pro125 (2QSS/2H35) and Asp128/Ala129 (2QSS/2H35).

These changes are significant and can confer different isoelectric points for hemoglobins and cause changes in a three-dimensional structure. However, the analysis of the positioning of the altered residues in the three-dimensional structure showed that they are not close to the heme group, so such residues do not significantly interfere with the position of this group (Figure 5 and 6).

```
                    10        20        30        40        50        60        70        80
                    .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
2QSS                VLSAADKGNVKAAWGKVGGHAAEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAALTKAVEHLDDLPGAL
2H35                VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGKKVADALTNAVAHVDDMPNAL
Clustal Consensus   ***.*** **********.**.**************************************** *** ***:** *:**:*.**

                    90        100       110       120       130       140
                    .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.
2QSS                SELSDLHAHKLRVDPVNFKLLSHSLLVTLASHLPSDFTPAVHASLDKFLANVSTVLTSKYR
2H35                SALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLTSKYR
Clustal Consensus   * ********************.******:***::**************.**********
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Figure 3. Alignment of alpha chains. 2QSS (Bovine Hb), 2H35 (Human Hb). One and two points show similar substitutions; Nonsimilar overrides are not marked. * = No substitutions.

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                    10        20        30        40        50        60        70        80
                    .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
2QSS                -MLTAEEKAAVTAFWGKVKVDEVGGEALGRLLVVYPWTQRFFESFGDLSTADAVMNNPKVKAHGKKVLDSFSNGMKHLDD
2H35                VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDN
Clustal Consensus     **.***:****:****:**********************************.****.************.:**:*: ***:

                    90        100       110       120       130       140
                    .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.
2QSS                LKGTFAALSELHCDKLHVDPENFKLLGNVLVVVLARNFGKEFTPVLQADFQKVVAGVANALAHRYH
2H35                LKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYH
Clustal Consensus   ******:****************:******** ***::******* :** :**************:**
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Figure 4. Alignment of beta chains. 2QSS (Bovine Hb), 2H35 (Human Hb). One and two points show similar substitutions; Nonsimilar overrides are not marked. * = No substitutions.

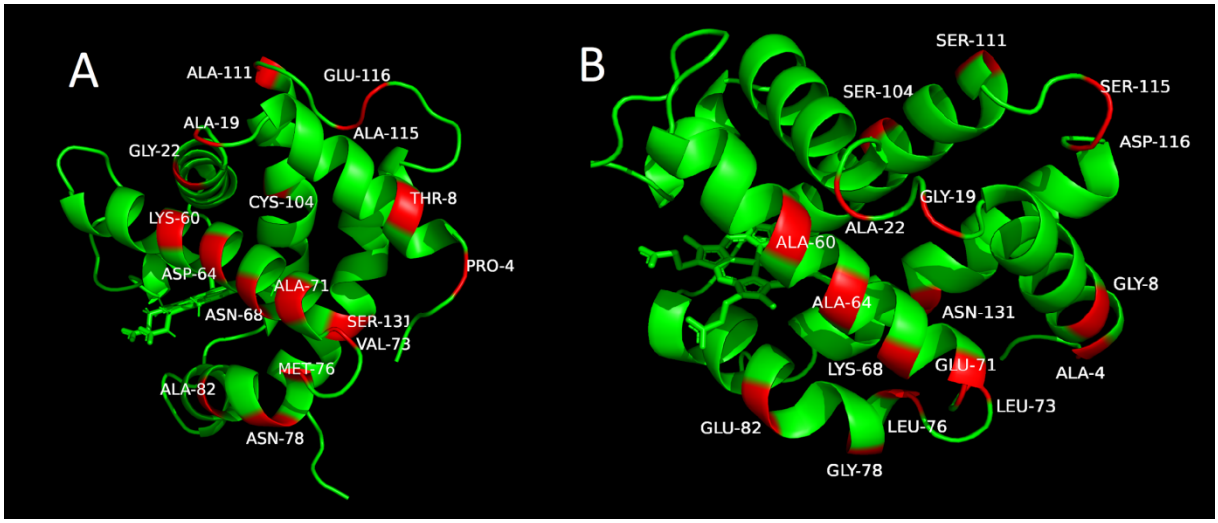

Figure 5. Position of the most significant residue substitutions in the alpha chain of human (A) and bovine hemoglobin (B). Different residues are in evidence; the name and position in the primary sequence are shown.

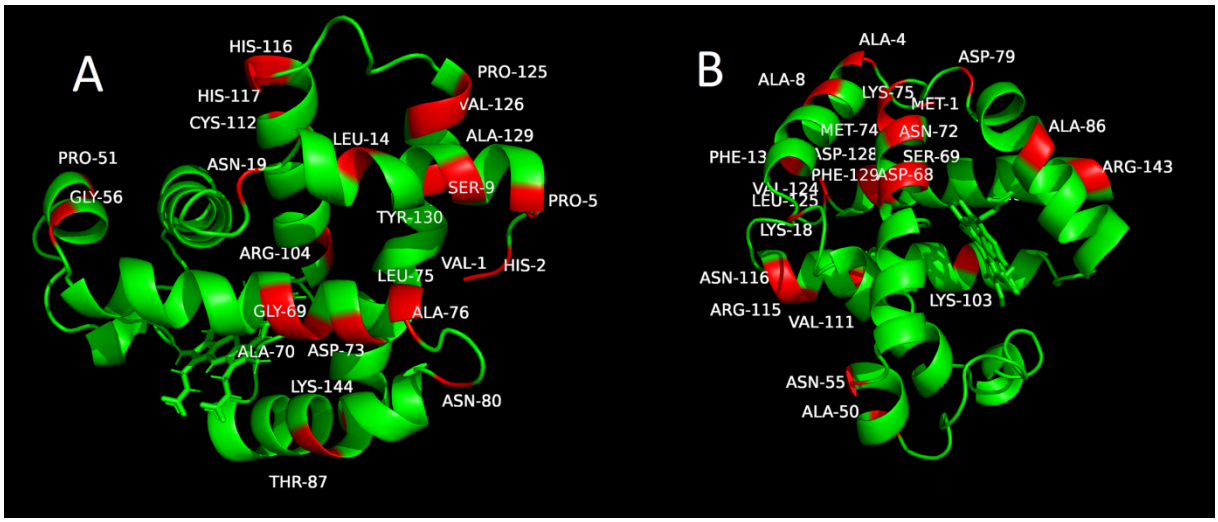

Figure 6. Position of the most significant residue substitutions in the beta chain of human (A) and bovine hemoglobin (B). Different residues are in evidence; the name and position in the primary sequence are shown.

Molecular docking

Molecular docking was used to analyze, at the molecular level, the place of interaction of hydrochlorothiazide and bezafibrate with human (2H35) and bovine (2QSS) hemoglobins. Simulation is necessary to better understand the dynamics of the interaction between protein-ligand[19].

Molecular docking of human hemoglobin demonstrated that bezafibrate and hydrochlorothiazide do not interact directly with the heme group, but with the protein part. The bezafibrate showed polar interactions with the length of 2. Å with the residue Thr38 of chain α1 (chain A), 2.4Å with the residue Lys132 of chain β1 (chain B) and 2.1Å with the His97 of the β2 chain (chain D) (Figure 7). Hydrochlorothiazide made interactions of 2.1 Å with thr38 residue of chain α2 (chain C) and 2.3Å with Arg104 of chain β2 (chain D) (Figure 8).

The interaction affinity, calculated in kcal/mol by AutoDock Vina, was -7.6 kcal/mol for the complex formed by human hemoglobin with bezafibrate and -6.7 kcal/mol for human hemoglobin with hydrochlorothiazide.

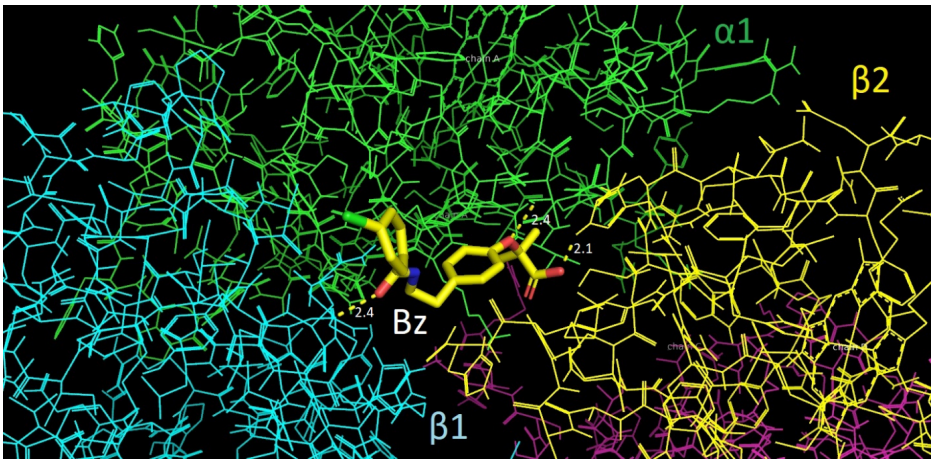

Figure 7: Interactions between bezafibrate (Bz) and human hemoglobin. Polar interactions with residues Thr38 (α1), Lys132 (β1) and His97 (β2) of human hemoglobin are presented by yellow dashed lines.

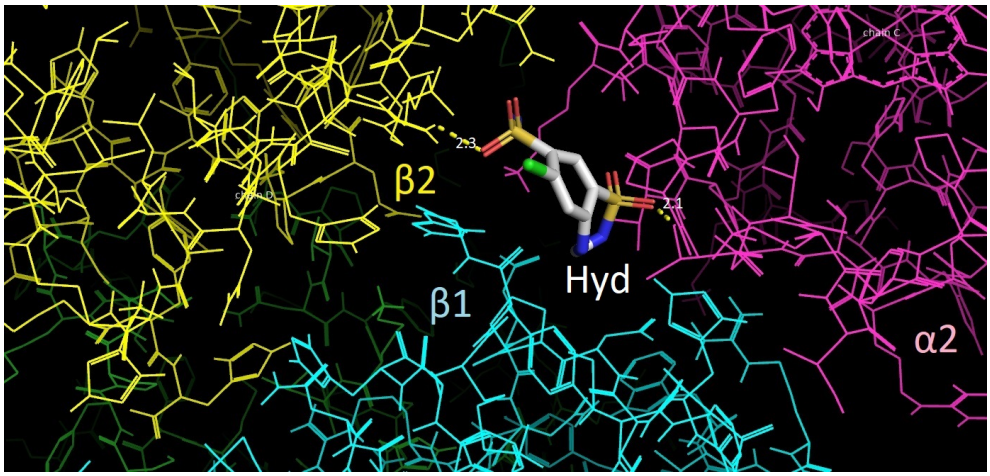

Figure 8: Interactions between hydrochlorothiazide (Hyd) and human hemoglobin. Polar interactions with the amino acids Thr38 (α2) and Arg104 (β2) of human hemoglobin are represented by yellow dashed lines.

Bezafibrate performed two polar interactions of 2.1Å and 2.5Å with arg39 residue of chain β1 (chain B), a 3.5Å connection with Tyr42 and another interaction of 2.2Å with the Arg92 residue of chain α2 (chain C) (Figure 9), while hydrochlorothiazide performed a polar interaction of 3.4 Å with the residue Ser138 in chain α1 (chain A), an interaction of 3.4Å with Asp126 and another 3.5Å interaction with Asn131, both from chain α2 (chain C).

The binding affinity was -8.3 kcal/mol for the complex formed between bovine hemoglobin and bezafibrate, and -6.6 kcal/mol for bovine hemoglobin and hydrochlorothiazide. The fact that bezafibrate performs four polar bonds with bovine hemoglobin (Figure 9) indicates the reason for the higher interaction affinity (shown by k value and affinity in kcal/mol) when compared to hydrochlorothiazide. The same is observed for complexes formed with bezafibrate and hydrochlorothiazide linked to human hemoglobin, since they present three and two polar interactions, respectively.

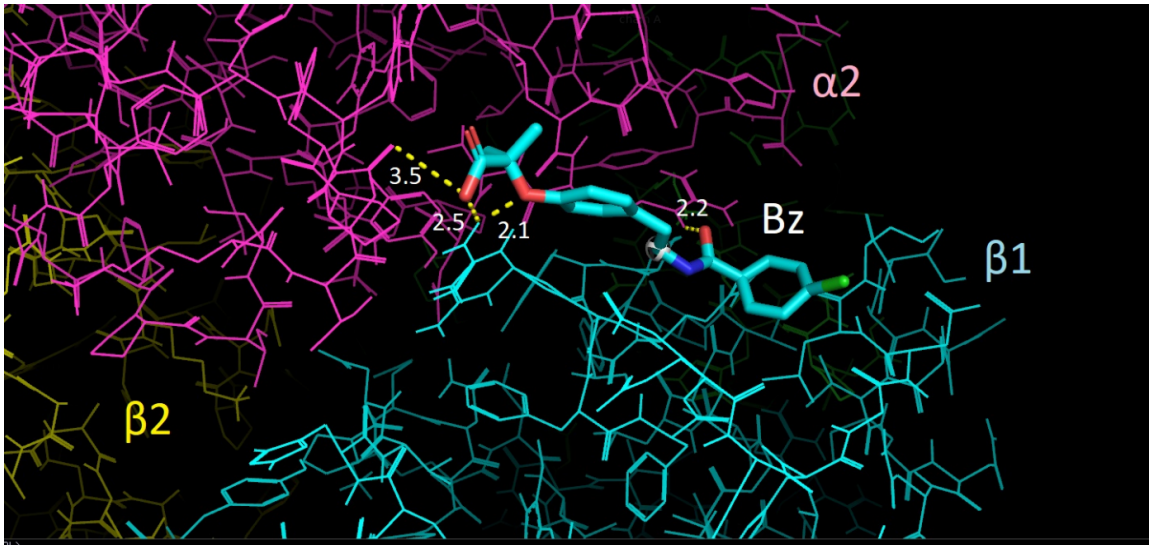

Figure 9. Interactions between bezafibrate (Bz) and bovine hemoglobin. Polar interactions with the amino acids Arg39(β1), Tyr42 (α2) and Arg92 (α2) of bovine hemoglobin with the residues are represented by yellow dashed lines.

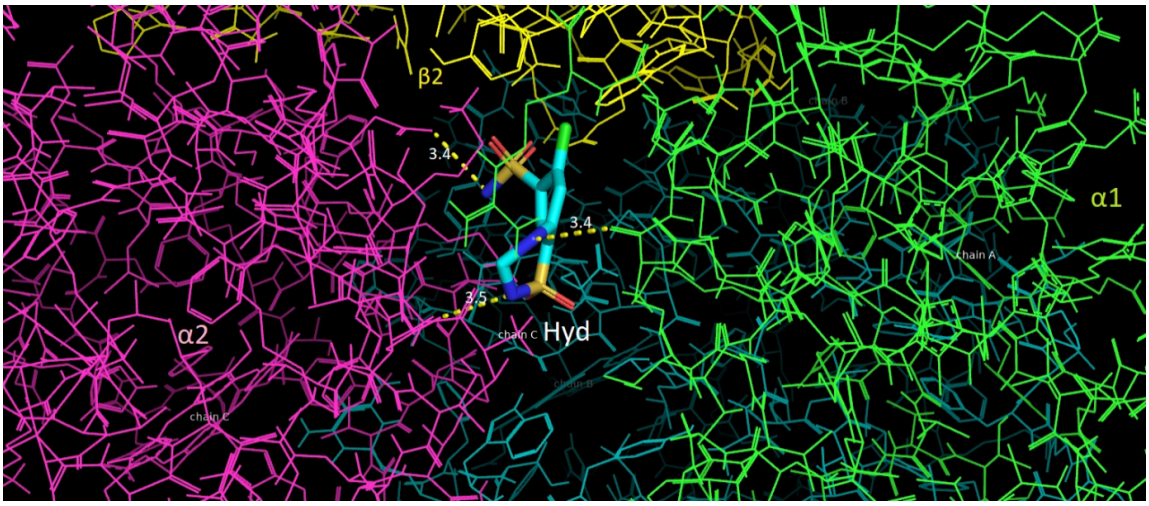

Figure 10: Interactions between hydrochlorothiazide (Hyd) and bovine hemoglobin. Polar interactions with the residues Ser138 (α1), Asp126 (α2) and Asn131 (α2) of bovine hemoglobin are represented by yellow dashed lines.

RMSD

In bioinformatics, the root mean square deviation (RMSD) from atomic positions is useful in the validation of molecular input and represents the measurement of the mean distance between the atoms of overlapping proteins; RMSD is calculated in relation to the best conformum obtained in the dosage and use only mobile heavy atoms. As a result of the docking, we obtained two metric variants of RMSD: rmsd/lb (lower limit of RMSD) and rmsd/ub (upper limit RMSD), which differ by how atoms are matched in the distance calculation. While in rmsd/ub each atom is combined in a conformation with itself in the other conformation, ignoring any symmetry, in rmsd/lb each atom is combined into a conformation with the nearest atom of the same element type in the other conformation[20]. In this work, the molecular docking performed presented zero value for both metrics.

CONCLUSION

In the present study, spectroscopy and molecular anchorage techniques were used to elucidate the nature of the interactions between the drugs bezafibrate and hydrochlorothiazide with bovine and human hemoglobin. It was verified by decreasing absorbance in the Soret band that these drugs interact with bovine hemoglobin *in vitro*, and due to the sequence similarity between both proteins, possibly this interaction should occur with human hemoglobin. Molecular anchorage analyses showed that hemoglobin can to bind to these therapeutic drugs in the living organism, through its protein part. The results suggest that bezafibrate binds more strongly to human and bovine hemoglobin when compared to hydrochlorothiazide. The *in silico* results corroborate the *in vitro* results, and the inference of greater bezafibrate affinity can be explained by the greater number of chemical interactions observed in relation to hydrochlorothiazide. It is suggested, therefore, that the normal function of hemoglobin can be modified since the interaction with the drugs can change its conformation; this can alter the microenvironment of the heme pocket, thus affecting the oxidation state of the central iron atom, interfering in the interaction with molecular oxygen. In addition, when connecting to hemoglobin the free fraction of the drug is altered, and consequently its dosage and therapeutic efficiency.

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Conflict of interest Statement

There is no conflict of interest between the authors of this study.

Authors' Contributions

Bianca Luiza Melo de Assis - Responsible for the analysis and choice of bioinformatics models, in vitro spectrophotometric analyses and manuscript writing - 25%; Anderson Dillmann Groto - Responsible for molecular docking analysis, statistical analysis and manuscript writing - 22%; Vítor Hugo Mota - Responsible for molecular docking analysis and manuscript writing - 20%; Gabriele Caroline Peiter - Responsible for the critical analysis of the manuscript, writing and correction of the manuscript - 20%; Kádima Nayara Teixeira - Author of the idea of the research. Supervision of all stages of the study. Translation of the article into English - 13%

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