Bioorganic Chemistry 109 (2021) 104709

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Antioxidant and anti-sickling activity of glucal-based triazoles compounds – An *in vitro* and *in silico* study

Rodinei Vieira Veloso [a], Anwar Shamim [b], Yann Lamarrey [c], Hélio A. Stefani [b,*], Juliana Mozer Sciani [a,*]

[a] Laboratório Multidisciplinar de Pesquisa, Universidade São Francisco, Bragança Paulista, SP, Brazil
[b] Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brazil
[c] Laboratório de Biologia Molecular, Hemocentro de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil

ARTICLE INFO

Keywords:
Sickle cell disease
Antioxidant
Glucal-based triazoles compounds

ABSTRACT

The sickle cell disease (SCD) has a genetic cause, characterized by a replacement of glutamic acid to valine in the β-chain of hemoglobin. The disease has no effective treatment so far, and patients suffer a range from acute to chronic complications that include chronic hemolytic anemia, vaso-occlusive ischemia, pain, acute thoracic syndrome, cerebrovascular accident, nephropathy, osteonecrosis and reduced lifetime. The oxidation in certain regions of the hemoglobin favors the reactive oxygen species (ROS) formation, which is the cause of many clinical manifestations. Antioxidants have been studied to reduce the hemoglobin ROS levels, and in this sense, we have searched for new antioxidants glucal-based triazoles compounds with anti-sickling activity. Thirty analogues were synthetized and tested in *in vitro* antioxidant assays. Two of them were selected based in their effects and concentration–response activity and conducted to *in cell* assays. Both molecules did not cause any hemolysis and could reduce the red blood cell damage caused by hydrogen peroxide, in a model of oxidative stress induction that mimics the SCD. Moreover, one molecule (termed **11m**), besides reducing the hemolysis, was able to prevent the cell damage caused by the hydrogen peroxide. Later on, by *in silico* pharmacokinetics analysis, we could see that **11m** has appropriated proprieties for druggability and the probable mechanism of action is the binding to Peroxiredoxin-5, an antioxidant enzyme that reduces the hydrogen peroxide levels, verified after molecular docking assays. Thus, starting from 30 glucal-based triazoles molecules in a structure–activity relationship, we could select one with antioxidant proprieties that could act on RBC to reduce the oxidative stress, being useful for the treatment of SCD.

1. Introduction

Free radicals or reactive oxygen species (ROS) are atoms with one or more unpaired electrons in their orbit, turning instable as highly reactive, by donating or receiving electrons [45].

Several diseases are related to the ROS formation, which can cause several cell damages, intra or extracellular protein alterations, and DNA modification. Thus, the harmful role of ROS has been associated to neurodegenerative and cardiovascular diseases, cancer, and sickle cell disease (SCD) [31].

The SCD has a genetic cause, characterized by the replacement of glutamic acid to a valine in the β-chain of hemoglobin, being this mutation homozygotic (HbSS) or heterozygotic (HbSC) [3].

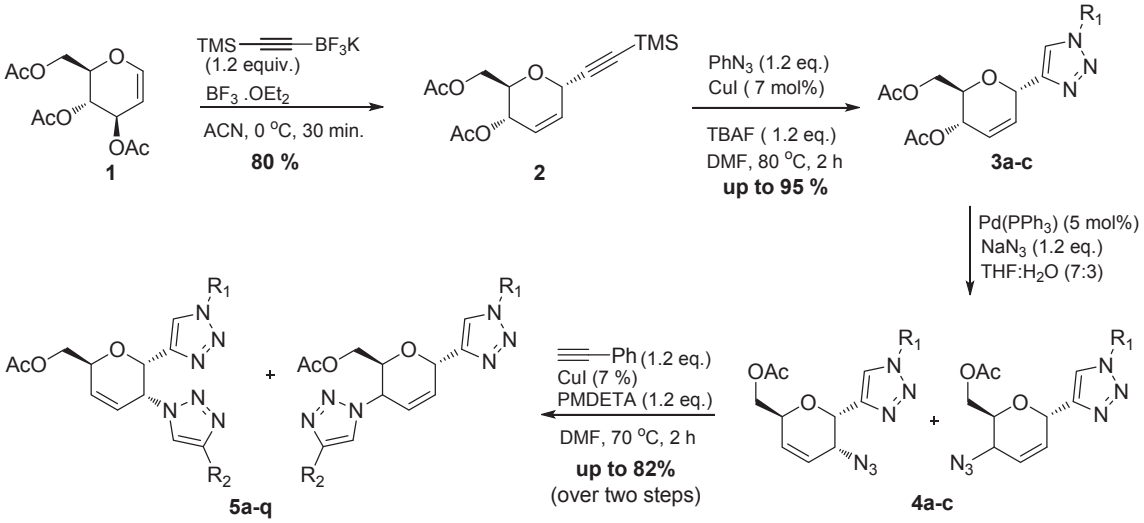
Hemoglobin is a 64.4 kDa vascular protein present in the red blood cells (RBC), constituted by a tetramer (2 subunits α e 2 subunits β), with function of oxygen transportation. Each subunit has a covalent-bound heme group, constituted by a protoporphyrin and one iron atom in its center. The oxygen binds irreversible to Fe^{2+} and produce Fe^{3+}. Nevertheless, the heme group protects the Fe^{2+} oxidation by make the bind reversible and restoring the protein oxygen binding capacity [59].

The hemoglobin mutation in the SCD causes protein polymerization of tetramers and consequently instability, due to the change of the protein electric charge. Consequently, the red blood cells (RBC) become deformed and rigid, in elongated and bending shape [41]. Moreover, the ability of RBC in transporting oxygen is reduced, especially in the microvasculature, which causes hemolysis, elimination of nitric oxide, and alteration in the RBC permeability [56,43,13].

The symptoms suffered by such patients range from acute to chronic

* Corresponding authorsat: Universidade São Francisco, Avenida São Francisco de Assis, 218, Bragança Paulista, São Paulo CEP: 12916-900. Brazil.
E-mail address: juliana.sciani@usf.edu.br (J. Mozer Sciani).

https://doi.org/10.1016/j.bioorg.2021.104709
Received 8 October 2020; Received in revised form 4 January 2021; Accepted 28 January 2021
Available online 9 February 2021



Scheme 1. Synthesis of bis-triazolyl glycosal derivatives from 3,4,6-tri-*O*-acetyl-D-glucal.

complications, and include chronic hemolytic anemia, vaso-occlusive ischemia, pain, acute thoracic syndrome, cerebrovascular accident, nephropathy, osteonecrosis and reduced lifetime [23,1].

Besides the alteration in RBC morphology, the HbS hemoglobin isoform results in the ROS production inside the RCB, in the prosthetic group heme containing iron atoms, a highly oxidative region in the hemoglobin. This region, called "oxidative hotspots", contains certain amino acids that favor the ROS formation, such as βCys93, able to be irreversibly oxidized, which contributes to the collapse, denaturation and degradation of the hemoglobin β subunit, causing the release of the heme group, essential for the oxygen binding. It is important to mention that this region is important for the transition of R (the relaxed state) to T (the tense state), representing a higher and lower affinity for oxygen, respectively [20].

Toxic radicals O_2 and H_2O_2 are frequently formed, which increases the antioxidant enzymes activity (catalase and dismutase superoxide) as a response for this oxidative stress. The increase of NADPH oxidase expression is another internal source for toxic ROS production, besides active mitochondria retention by mature mutated hemoglobin-RBCs [1].

The RBC membrane is a target for oxidative stress, as it is abundant in -SH groups, converted into disulfide binds (-R-SSG) from the oxidant agents, which causes proteins' denaturation and lipoperoxidation of lipidic components of the membrane, inducing cell hemolysis [9].

Due to the importance of oxidative stress in the SCD context, antioxidants have been considered for the treatment of such disease, which today is based on drugs to treat the symptoms (e.g. analgesics) and blood transfusion.

Hydroxyurea and L-glutamine are drugs approved by FDA for SCD, although the mechanism has not been completely elucidated yet [11]. It is known that the hydroxyurea inhibits the hemoglobin polymerization and protects the βCys93, while the L-glutamine increases the NAD synthesis, reducing the ROS production [39].

Classic antioxidants available, such as ascorbic acid, have inadequate physical–chemical properties, and consequently poor pharmacokinetics, impairing the molecule to reach the molecular target in a therapeutic concentration. Therefore, new druggable antioxidants need to be studied.

Triazoles are heterocyclic compounds with five-membered unsaturated ring containing 3 nitrogen atoms, with several pharmacological proprieties [21]. Antifungal activity is the most known, and the important functional groups for such structure are the 1,2,3-triazole and 1,2,4-triazole rings [29].

However, besides being antifungal, this scaffold has been used for the synthesis of molecules applied for antimicrobial, antiviral, antitumor and neuroregenerative activities [28]. Antioxidant activity has also been associated with this structure, with an unknown mechanism [2].

Its pharmacokinetics and pharmacodynamics properties are favorable, and generally binds easily with their therapeutic targets. For example, 1,4 disubstituted 1,2,3-triazol has a CH-bond as a hydrogen donor and N-3 pair as hydrogen acceptor [5].

Moreover, the presence of glucal in their structure tends to increase the molecule stability, increases the polarity, and makes them compatible with the biologic fluids due to the stereogenic center of carbohydrates [10].

Here we have evaluated the antioxidant activity of 30 glucal-based triazole compounds and studied their neutralization effect on the oxidative stress induced in RBC, simulating the SCD. Moreover, the pharmacokinetics and molecular target were studied, in order to check their druggability and the possible mechanism of action.

2. Material and methods

2.1. Synthesis

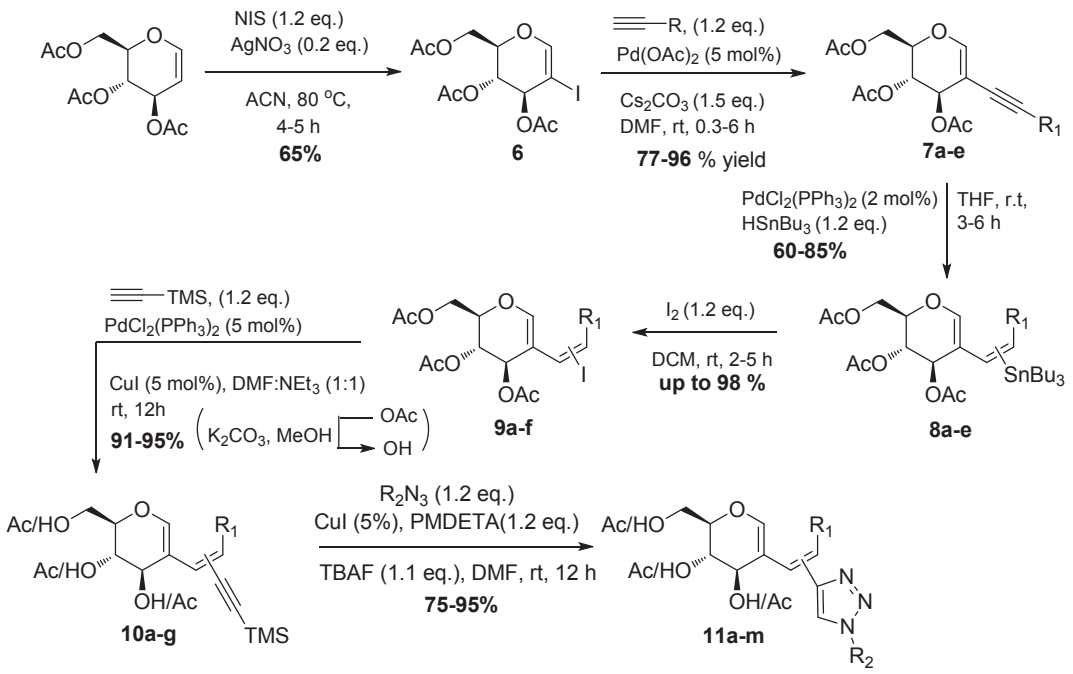
The synthesis of the libraries of compounds below are categorized based on the difference in synthetic procedures.

2.1.1. Synthesis of glycosal based bis-triazole derivatives

The synthesis of glycosal based bis-triazole derivatives started with 3,4,6-tri-*O*-acetyl-D-glucal **1**, using Ferrier rearrangement to obtain glycosal alkyne **2**, followed by coper catalyzed azide-alkyne cycloaddition (CuAAC) click reaction to obtain the glycosyl-triazoles **3a-c** [51,47]. These glycosyl triazoles **3a-c**, were then used in palladium-catalyzed Tsuji-Trost type allylic azidation to obtain regioisomeric glucal based allylic azides **4a-c** followed by a second CuAAC click reaction to obtain 17 bis-triazole compounds **5a-q** as shown in Scheme 1 [50].

2.1.2. Synthesis 2-alkenyl mono-triazole derivatives of D-glucal

The trimethylsilyl protected alkyne derivatives of glucal 10a-g were synthesized from 3,4,6-tri-*O*-acetyl-D-glucal based on the reported methods [49,46,58]. This includes the synthesis of 2-iodo-3,4,6-tri-*O*-acetyl-D-glucal **6** starting from the acetylated D-glucal, followed by the copper and ligand free Sonogashira coupling and regioselective palladium-catalyzed hydrostannation to obtain the corresponding alkynyl derivatives **7a-e** and stannyl derivatives **8a-e** of D-glucal, respectively. The stannyl derivatives were subjected to iodination to afford **9a-f** and Sonogashira coupling to obtain **10a-g** which were ultimately applied in the CuAAC click reaction to obtain the desired triazole



Scheme 2. Synthesis of 2-alkenyl mono-triazole derivative of D-glucal.

based glucal compounds **11a-m** as summarized in Scheme 2 [48].

2.2. Antioxidant activity

The antioxidant activity of molecules was determined by 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay and e ferric reducing antioxidant power (FRAP) assays.

The DPPH test was conducted in 96-well plate with 30 min incubation of 0.5, 1 or 2 mM of samples with 0.1 mM DPPH reagent, diluted in methanol, in the dark. After this period, the mixture had its absorbance read by a spectrophotometer at $\lambda = 515$ nm. The % of radical scavenging activity was calculated by: [(Ac - As)/ Ac)] × 100, where Ac = absorbance of the control and As = absorbance of the test sample.

For the PFRAP assay, phosphate-saline buffer (PBS 50 mM pH 7.3) was incubated with 1% potassium ferrocyanide and samples (0.5, 1 or 2 mM) for 20 min at 50 °C. After that, 10% trichloroacetic acid was added and the solution centrifuged at 3000 rpm for 10 min. An aliquot was added to distilled water and 0.1% ferric chloride, and the solution had its absorbance read by a spectrophotometer at $\lambda = 700$ nm. The relative % of reducing power was calculated as (A- Amin) / Amax - Amin) × 100, where A = absorbance of the sample, Amin = absorbance of control and Amax = Highest absorbance of standard.

For both assays, a control (methanol or buffer instead the sample) was added and ascorbic acid was used as reference standard. The samples were analyzed in triplicate and the results are shown as featuring potential antioxidant activity.

2.3. Hemolytic activity

The hemolysis was performed in human erythrocytes, obtained from a pool of 20 healthy volunteers (approved by the Ethics Committee from Universidade São Francisco, CAAE 25441719.0.0000.5514). The blood was collected in EDTA tubes and centrifuged at 1000*g* for 10 min at room temperature. Plasma was removed and red blood cells (RBC) were washed with phosphate-saline buffer (PBS, 50 mM, pH 7.3). One mL of RBC was diluted with 9 mL of PBS to give a 4% suspension. Samples (0.5 μM, diluted in PBS) were incubated to 20 μL of 4% RBC and 50 μL PBS for 60 min at 37 °C. The mixture was centrifuged at 4000 rpm for 5 min at room temperature and an aliquot of supernatant were transferred to 96-well plate for the hemoglobin measure by spectrophotometer at 414 nm. Data were compared with negative control (PBS buffer) and a positive control (Triton X-100 0.1%). Experiments were performed in triplicate and the percent hemolysis was calculated as follows: (%) = (Asample − Abuffer)/(Atriton − Abuffer) × 100.

2.4. Oxidative stress in RBC

The molecules were tested in RBC induced with oxidative stress in order to evaluate the prevention or treatment effect (impairment or removal of free radicals).

For prevention test, RBC (20 μL of 4% suspension) were incubated with 50 μL PBS and 1 mM molecules for 60 min at 37 °C. After that, 1% H_2O_2 was added for 10 min and then the mixture was centrifuged for 4000 rpm for 5 min at room temperature. An aliquot of supernatant was transferred to a 96-well plate and the absorbance was read at $\lambda = 414$ nm.

For treatment test, RBC (20 μL of 4% suspension) were incubated with 50 μL PBS and 1% H_2O_2 for 10 min. After that, 1 mM molecules were incubated for 60 min at 37 °C. The mixture was centrifuged and read at spectrophotometer at the same protocol described above.

Experiments were performed in triplicate and compared to a negative (PBS) and positive (H_2O_2) control.

2.5. In silico ADME parameters, toxicity, physicochemical descriptors and druglike nature of compounds

ADME parameters (gastrointestinal absorption, blood–brain barrier permeation and liver enzymes inhibition) of molecules were predicted by Swiss ADME. Moreover, the physicochemical properties and drug-likeness was predicted, following the Lipinski rules [30].

The toxicity was predicted by admetSAR 2.0 server [54]. Parameters evaluated were acute oral toxicity and carcinogenicity [57].

2.6. Molecular docking

The **11m** had its 2D structure drawn in OpenBabel Cheminformatics and saved into mol2 format after being converted into 3D with pH 7 and no changes in adding or deleting hydrogens.

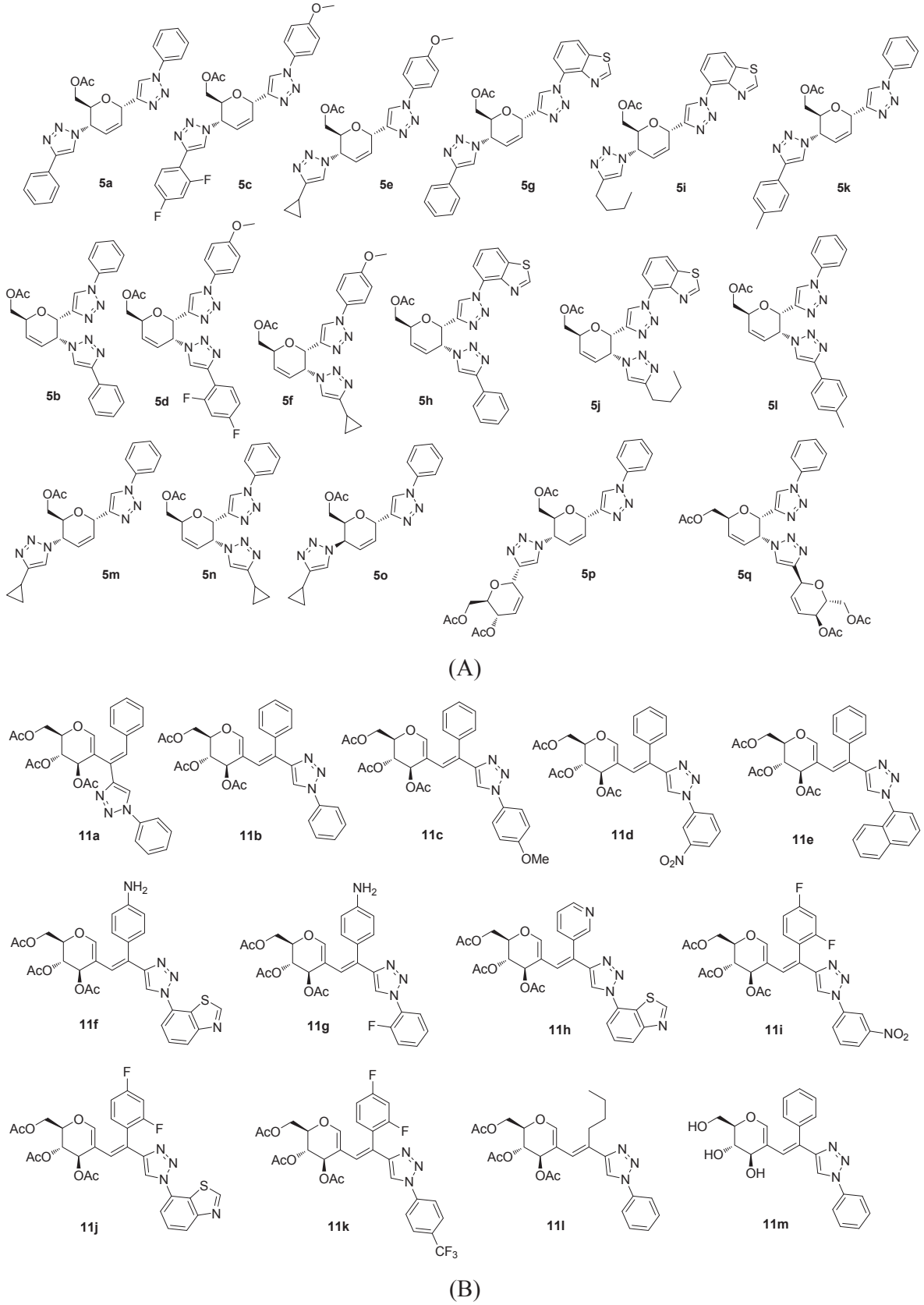


Fig. 1. Chemical structures of triazolyl glucal derivatives (A) bis-triazoles, (B) mono-triazoles.

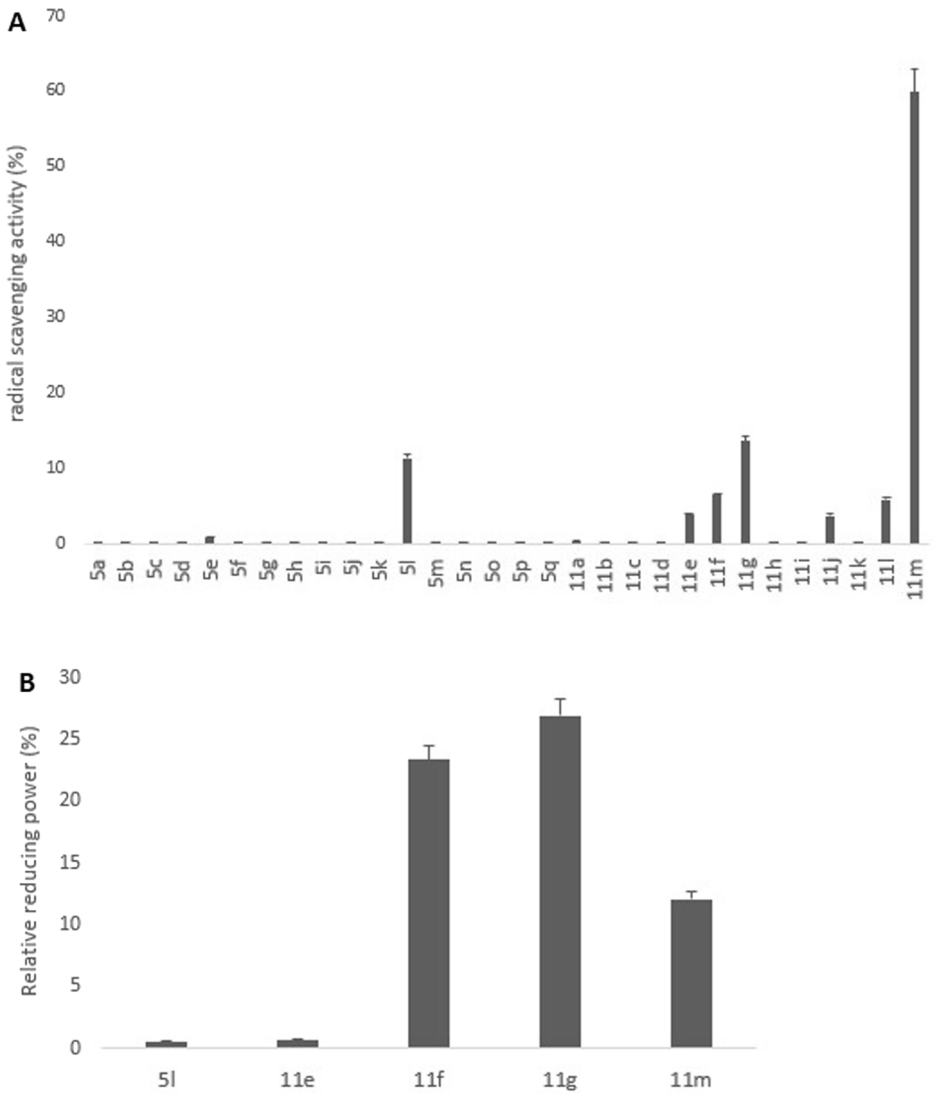


Fig. 2. Antioxidant tests for the molecule's evaluation. A - DPPH assay, shown as percentage of radical scavenging activity. B – PFRAP assay, shown as percentage of relative reducing power activity.

The molecular docking was performed in the Swiss Dock webserver (http://www.swissdock.ch/docking), with parameters set to automatic search, considering the CHARMM method. Receptors and ligands were protonated at pH 7.0 and proteins were chosen in the Protein Data Bank (PDB) as follows: Superoxide Dismutase (1CB4 -chain A + B), Catalase (1DGB - chain A), Glutathione peroxidase 3 (2R37 - chain A), NADPH oxidase (2CDU - chain B), Lipoxygenase (1N8Q - chain A) and Peroxiredoxin-5 (3MNG - chain A).

2.7. Statistical analysis

Cell experiments were submitted to statistical analysis for groups comparison. For that, ANOVA test was used, with Tukeýs post-test. Differences were considered statistically significant when $p < 0.05$, indicated by asterisks in the figures.

3. Results and discussion

3.1. Chemistry

The triazole's scaffold has been associated to the antioxidant activity due to functional groups that favors the radical scavenging, such as –OH, -SH, –COOH, -PO_3H_2, C═O, -NR_2, -S-, -O- [14].

In this sense, some triazole-derived molecules have been described: 4-amino-5-aryl-3H-1,2,4-triazole-3-tiones with methoxybenzyl and methoxyphenyl groups [16], 4,5-Dihydro-1H-1,2,4-triazole-5-one [15], 1,2,4-triazols [26,6] and 4-[3,4-di-(4-nitrobenzoxy)-benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-one [15].

TD3 (4,4′-di(1,2,3-triazolyl) disulfide) is under pre-clinical trials for its promising activity of saline-bind formations that is able to stabilize the R state of hemoglobin and destabilize the T state, which favors the oxygen bind to the protein [36].

TD1, a TD3 analogue(di(5-(2,3-di-hydro-1,4-benzodioxin-2-yl) −4H-1,2,4-triazol-3-yl) disulfide) was developed as an antioxidant focused on anemia, with mechanism of βCys93 protection [22].

Carbohydrates also have been used in several synthetic compounds, as it facilitates the cell recognition and can activate intracellular pathways for cell proliferation or apoptosis. Moreover, the addition of this group can add interesting proprieties, such as the increase of hydrophilicity, reduction of the toxicity and increase of the bioavailability [50].

Thirty compounds were synthesized starting from 3,4,6-tri-*O*-acetyl-D-glucal a commercially available reagent purchased from *Sigma-Aldrich*. The main reactions performed included Ferrier rearrangement, Stille reaction, copper(I) catalyzed azide alkyne cycloaddition (CuAAC), Tsuji-Trost azidation, Sonogashira coupling and various functional groups

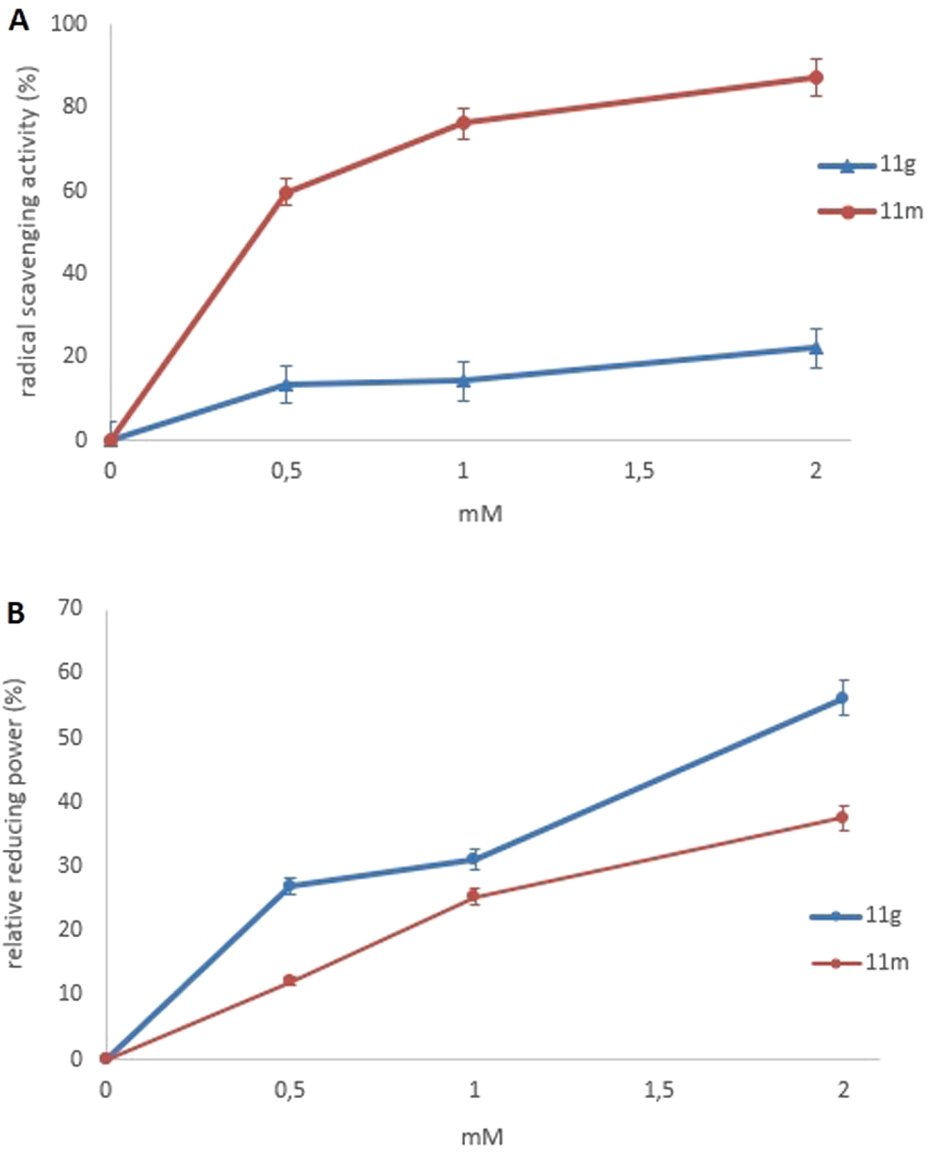


Fig. 3. Concentration-response effect of **11g** and **11m**: A – DPPH test and B – PFRAP test.

transformation to afford these multicyclic glucal based triazole compounds. All the obtained compounds were stable, easy to handle and were achieved with good to excellent yields. The structural characterization was performed using NMR, FTIR, and HRMS and the detailed experimental procedures and characterization is already reported in literature that is appropriately cited in the corresponding sections.

All the compounds are summarized in Fig. 1 and subdivided into glucal-derived bis-triazoles (Fig. 1A) and glucal based mono-triazoles (Fig. 1B). The analogues had variation in their chemical structures to increase stability and biological effect and were submitted to antioxidant activity in the first screening. This rationale in the medicinal chemistry was applied for the search of new molecules with better activity, selectivity, and low adverse effects, through the study of structure–activity relationship (SAR) [32].

3.2. Antioxidant activity

Antioxidants are being considered in the treatment of several diseases, including cancer, neurodegenerative and sickle cell diseases [1].

Due to their success in the clinics, new antioxidants have been searched for a better activity and appropriated pharmacokinetics features. Ascorbic acid is the most known but is highly hydrophilic and have physico-chemical proprieties that impairs their cell membrane penetration and consequently facilitates its elimination. On the other hand, alpha tocopherol, another known antioxidant, is highly hydrophobic and accumulates in tissues, not reaching the pharmacological target.

Here we have tested 30 molecules derived from triazole and glucal in the DPPH oxidant test. It was possible to identify 8 molecules with radical scavenging activity, in different intensity. The compound **11m**, for example, caused the most intense activity −69.75% of antioxidant effect with 0.5 mM (Fig. 2A). For comparison, it was used 1 mM ascorbic acid, which caused 85.01% of radical scavenging activity.

The DPPH is a classic assay for determining antioxidant activity by radical scavenging, and it is appropriated for a screening, as it is fast and easy to conduct and analyze [12]. However, to determine an antioxidant activity, one complementary test with a different mechanism should be performed, and in this sense, we choose PFRAP, in order to evaluate the reduction of Fe^{3+} to Fe^{2+} in the presence of the antioxidant, condition that happens in the SCD [34].

Thus, 5 molecules that presented higher activity in the DPPH test were selected for PFRAP (also with 0.5 mM). It was observed that three of them caused relative reducing power effect (Fig. 2B), being two (**11g** and **11m**) with important activity in both antioxidant tests, which were

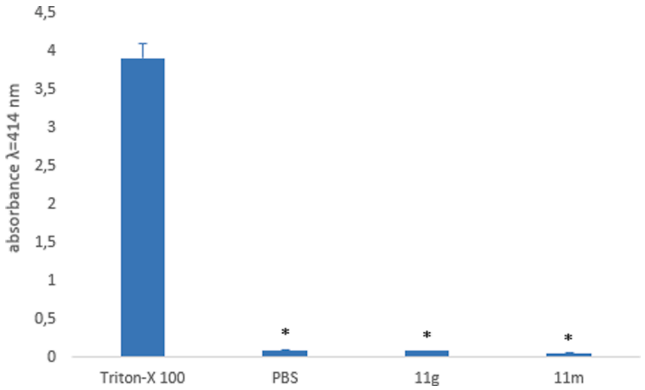


Fig. 4. Hemolytic activity of **11g** and **11m**. For comparison, positive control (0.1% Triton X-100) and negative control (PBS). *p < 0.05 in comparison to Triton-X 100 group.

selected for further studies.

In order to verify a concentration–response effect, **11g** and **11m** molecules were tested in DPPH and PFRAP assay with 0, 0.5, 1 and 2 mM. As shown in Fig. 3A and 3B, both molecules had their activity increased according to the concentration. Using the same concentration, (1 mM), **11m** had the same activity as ascorbic acid (85%) in the DPPH assay.

Hydroxyurea is the main drug currently used for SCD treatment that contributes for the reduction of ROS. However, other antioxidants are currently being studied for this disease. Quercetin is one example, and it is able to scavenge the free radicals through di-hydroxy orto structures, protecting the RBC from oxidative damages [35,18,42]. Kassa et al [23] showed that TD3 reduced 22% of ferryl heme caused by H_2O_2, which represents a reduction in the βCys93 oxidation.

3.3. Hemolytic activity

The **11g** and **11m** were submitted to a hemolysis test in order to check their cell toxicity. As shown in Fig. 4, the addition of Triton-X100 was able to disrupt the cell, causing hemolysis, as expected. On the other hand, the negative control, PBS incubation, caused no hemolytic activity. Both molecules, **11g** and **11m**, caused no hemolytic effect as well, as no hemoglobin release was observed, indicating low cytotoxicity *in vitro*.

In a study that used the SAR method to modify the resveratrol structure, a known natural antioxidant, 9 analogues were obtained aiming to reduce the cytotoxicity and induce the fetal hemoglobin (HbF) expression [52] – 3 of them could induce the HbF with low cell toxicity. In our study, two antioxidant molecules (**11g** and **11m**) did not cause cell toxicity. Thus, further experiments were performed to verify their activity on oxidative stress induced in such cell type.

3.4. Oxidative stress in RBC

The two molecules (**11g** and **11m**) were tested in RBC induced with oxidative stress in a prevention or treatment effect. This condition induced by hydrogen peroxide mimics the oxidative stress in RBC when the disease is installed and therefore it is being used as a SCD model, as it is simple and rapid way to verify the antioxidants effects [38,1].

Both molecules were effective in significantly reducing the hemolysis in the treatment effect, i.e. the incubation of samples after the oxidative stress induction – **11g** reduced 28.6% of hemolysis, and **11m** reduced 61% (Fig. 5A).

The use of **11m** molecule in a preventive effect, i.e. the incubation of molecules before the oxidative stress induction, could reduce 40% of hemolysis. The **11g** did not cause any preventive effect (Fig. 5B).

The active compounds, **11g** and **11m**, do not contain functional groups that favor the radical scavenging in the triazole group, thus this substitution could not increase the activity in a SAR study. On the other hand, the addition of glucal seemed to contribute for the activity, with the presence of NH2 and -O- probably responsible for the activity besides their contribution for the solubility proprieties.

This method allowed the conclusion that the molecules, especially **11m**, significantly reduced the hemolysis caused by the hydrogen peroxide by its radical scavenging capacity, besides preventing the hemolysis. This result confirms the antioxidant propriety of the molecule and its application in a cellular model, being a positive effect in the RBC damage caused by the ROS release.

Thus, as the 2 molecules were selected by DPPH and PFRAP tests, it is expected that the radical scavenging capacity of the molecules contributes to the stabilization of RBC structure and to the preservation of Fe ions oxidation, contributing to the hemoglobin maintenance and reduction of hemolysis.

Table 1
Pharmacokinetics and toxicity parameters predicted for the molecules **11g** and **11m** and their fits on the Lipinskís rules.

Parameter	Criterion	11 g	11m
Molecular weight	< 500 g/mol	550.53 g/mol	391.42 g/mol
Hydrogen-bond acceptors (HBA)	<10	11	6
Hydrogen-bond donors (HBD)	<5	2	3
LogP	2 a 5	3.75	2.98
TPSA	40 a 100 A^2	128.32A^2	100.63A^2
Gastrointestinal absorption	Yes	Yes	Yes
Blood-brain barrier permeant	No	No	No
Metabolism enzymes inhibitors	< 2	2	0
Acute oral toxicity	III or IV*	III	III
Carcinogenicity	No	No	No

* These criteria indicate LD50 values in a range of 500 mg/kg – 5000 mg/kg (category III) and greater than 5000 mg/kg (category IV).

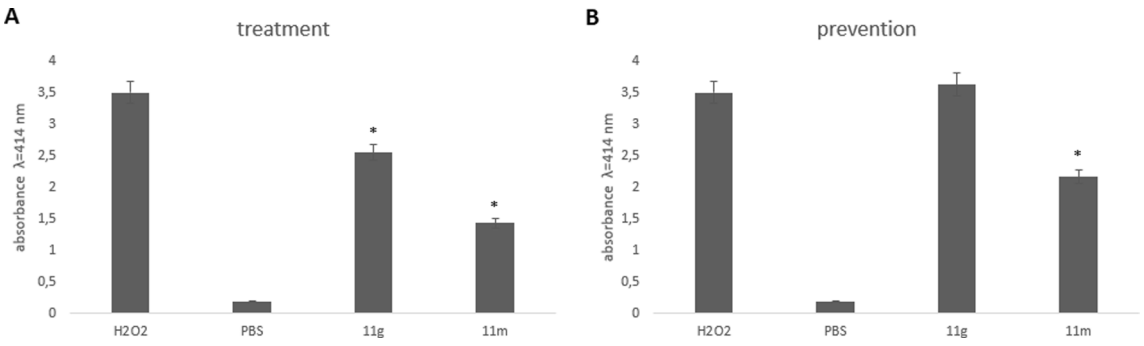


Fig. 5. Oxidative stress induction in RBC and treatment with **11g** and **11m** molecules. A – preventive effect. B – treatment effect.

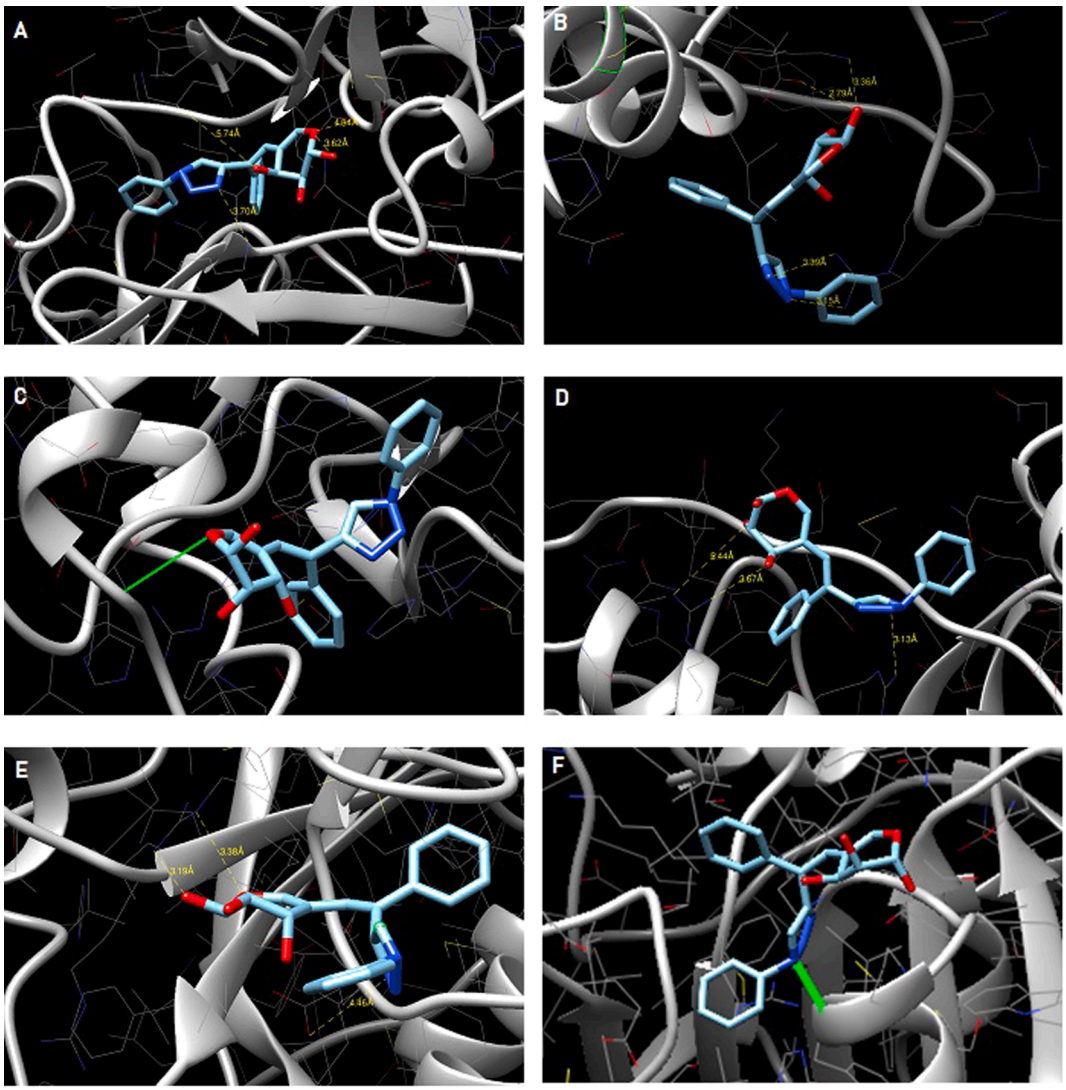


Fig. 6. Molecular docking interaction between the **11m** ligand and the antioxidant proteins. A – SOD; B – Catalase; C - Glutathione peroxidase 3; D - NADPH oxidase; E – Lipoxygenase; F - Peroxiredoxin-5.

3.5. *ADME in silico properties*

The two molecules, selected by their antioxidant activity, *in vitro* and by cellular effect, were analyzed by their pharmacokinetic properties. The physico-chemical parameters evaluated followed the Lipinski's rules, that estimate values through computational analysis for finding out the molecular mass, TPSA, LogP, hydrogen donor/acceptor, in order to give the molecule a status of "druggable". These parameters reflect the cell permeability, gastrointestinal (GI) absorption, blood–brain barrier (BBB) permeation and hepatic enzymes inhibition.

The values for each category were determined by the Lipinski's study, which are validated with several known compounds in high throughput screening analysis [30].

When both molecules were analyzed for ADME proprieties, the **11g** did not fit all requirements, being the **11m** the most relevant one (Table 1).

The **11m** molecule has a molecular mass of 391.42 g/mol, 6 hydrogen-bond acceptors (HBA), 3 hydrogen-bond donors (HBD), *n*-octanol/water partition coefficients (LogP) 2.98 and TPSA 100.63 A^2.

The molecular mass below 500 g/mol indicates that the molecule can cross passively plasmatic membranes, besides its ability to enter in the target pocket, which makes it possible to develop its pharmacological potential [33].

Moreover, the *n*-octanol amphipathic nature is considered an agent that mimics the phospholipidic membrane and it is considered a parameter to determine the druggability of a molecule [7]. The LogP between 2 and 5 reflects the lipophilicity of the molecule and also indicates its ability to cross membranes, in which the main component is a lipid layer. The value of LogP 2.98 for **11m** follows the Lipinski's rules and explain its good activity on RBC – the molecule could permeate the cell with short periods of time and exert its antioxidant activity in cells.

We also estimated the gastrointestinal absorption and blood–brain barrier permeation by the BOILED-Egg method (Brain or intestinal estimated permeation), as a predictive model of lipophilicity and polarity of small molecules, using LogP and TPSA parameters [8]. It was confirmed for both compounds good cell permeation and high GI absorption, which indicates that the molecules could be administered by oral route, a common, easy, and cheap way of drug administration, convenient for the patient.

Besides, the TPSA value (100.63 A^2) for **11m** reflects high polarity and low permeability to the BBB, although the LogP is in the adequate range. In this case, the inability of **11m** to penetrate the BBB indicates a peripherical effect, without affecting the central nervous system, which is positive for the systemic SCD.

HBA and HBD parameters reflect the interaction with ligands and determine the bioavailability for the N—H or O—H bind prediction in

Table 2
Protein targets and parameters of binding after molecular docking with the **11m** molecule.

Protein	PDB code	Binding energy (kcal/mol)	Full Fitness (kcal/mol)	Binding interaction (ΔG)	Bond length (Å)
Superoxide Dismutase (SOD)	1CB4 (chain A + B)	−9.14	−1369.18	LigOH-Asp11 LigN2-Lys9	3.62 3.70
Catalase	1DGB (chain A)	−8.56	−2144.19	LigNH-Arg431 LigOH-Asp157 LigOH-Lys349	3.15 2.79 3.36
Glutathione peroxidase 3	2R37 (chain A)	−8.90	−1049.03	LigNH-Thr165 LigO2-Arg168(NH) LigO2-Leu162(NH)	2.42 3.93 3.50
NADPH oxidase	2CDU (chain B)	−8.60	−2349.03	LigO2-Arg308(NH) LigO-Arg308 (NH2) LigN1-Arg305 (NH2)	3.67 5.44 3.13
Lipoxygenase	1N8Q (chain A)	−6.56	−3881.73	LigO-Arg621 (NH) LigO-Arg621-N LigN2-Glu625(O)	3.19 3.38 4.46
Peroxiredoxin-5	3MNG (chain A)	−8.59	−783.09	LigN-Gly46 (HN) Arg127 (NH2)	2.20 4.29

the intra or extracellular environment [17]. According to Lipinski's rules, both values are in accordance with the desirable one for a molecule being a drug. These binding predictions also help the verification of the interactive capacity of a molecule with the enzymes for the drug metabolism (CYP P450 superfamily). The **11m** does not have potential to inhibit any of the enzymes evaluated (CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP3A4), which indicates low hepatotoxicity and drug interaction. On the other hand, **11g** has potential to inhibit 2 CYPs, indicating possible drug interaction and effects on xenobiotics metabolism.

The acute oral toxicity of both molecules (**11g** and **11m**) was estimated as category III (Table 1). This parameter is important because indicates the possible adverse effects occurring within a short time after an oral administration of a substance and the category III is referred as "slightly toxic and slightly irritating" according to US EPA for chemicals [53].

In the classification stablished by OECD (Organization for Economic Co-operation and Development) [40], four categories are considered regarding lethal dose 50% (LD50) values: Category I contains compounds with LD50 values less than or equal to 50 mg/kg; Category II compounds with LD50 values >50 through 500 mg/kg; Category III compounds with LD50 values >500 through 5000 mg/kg; Category IV compounds with LD50 values greater than 5000 mg/kg.

The LD50 values for **11g** and **11m**, 500–5000 mg/kg predicted, are comparable to hydroxyurea, which was experimentally calculated as 5760 mg/kg after an oral administration, in rats. Considering that a chosen drug for sickle cell disease, our molecules have potential for a drug development program [37].

This feature, added to the one hydrogen-bond acceptors surplus and the molecular weight above 500 g/mol, made us eliminate **11g** for the study, being the **11m** the chosen one for the molecular docking evaluation.

3.6. Molecular docking studies

After identifying the activity and pharmacokinetics proprieties, **11m** was chosen for a virtual screening by molecular docking for predicting the interactions between a target protein and the molecule and elucidate its possible mechanism of action. Six protein targets were selected based on their participation on the antioxidant activity and simulated with **11m** (Fig. 6). The parameters resulting from the protein target and ligand interaction are shown in Table 2.

Although the lowest binding energy has been reached in the interaction with superoxide dismutase (−9.14 kcal/mol), the ligand was positioned out of the catalytic site, indicating that the ligand does not interfere with the enzyme antioxidant activity. The glutathione peroxidase 3, NADPH oxidase, catalase and lipoxygenase active site were not covered by **11m** as well, indicating that these proteins are not the target, even having low binding energy and/or full fitness.

On the other hand, **11m** interacted with the peroxiredoxin-5 catalytic pocket, with a strong hydrogen bond (−8.59 kcal/mol) identified in the side chain of the key residues Arg127, and Gly46 with low bond length (4.29 and 2.20 Å, respectively) [4]. Thus, we have an indication that peroxiredoxin-5 could be the molecular target for **11m**.

Peroxiredoxins are thiol-dependent peroxidases, able to reduce hydrogen peroxide, alkyl hydroperoxides, and peroxynitrite [19]. They are well conserved enzymes, mammalian, found in both cytosol and mitochondria, already identified in the liver and lung. These enzymes were related to several pathologies involving tendons, cartilages, nerves, carcinomas (ovarian, breast, colon, adrenocortical), thyroid, astrocytes, retina, and also blood cells [25].

The Peroxiredoxin-5 (PRDX5) expression was related to acute inflammatory effects, as it was upregulated in rats after the injection of lipopolysaccharides and during stress of endothelial cells and macrophages [24]. The increase in activity was also found in neutrophils and monocytes [27].

The molecular docking was performed using the PRDX5 because it is the most available crystalized protein isoform, the opposite of PRDX2, related to SCD. However, the catalytic site is conserved, which indicates that **11m** could interact with the PRDX2 as well.

In the SCD, there is a huge quantity of ROS being produced by the RBC, such as H_2O_2, and consequently the activities of antioxidant enzymes are increased, such as superoxide dismutase, catalase, glutathione peroxidase, and three isoforms of peroxiredoxin (I, II, and VI). So, it is expected that the PRDX5 activity would be increased, compatible to this radicaĺs production, trying to eliminate intracellular concentration of H_2O_2 [44].

Indeed, we have seen that the **11m** molecule reduced the hemolysis caused by H_2O_2, and besides the scavenging mechanism, it can be acting in the enzymatic activity of PRDX5 trying to remove the ROS.

Peroxiredoxins can be inactivated during catalysis because the cysteine residue, important for the active site, is oxidated to sulfinic acid (Cys-SO_2H), being their reactivation achieved by reduction of the sulfinic moiety [55]. Thus, by acting on the PRDX, the **11m** molecule could act coordinating the active site of the enzyme, increasing its catalytic efficiency, consequently increasing the H_2O_2 removal, as demonstrated here.

4. Conclusion

After evaluating 30 molecules, we could select one (**11m**) according to its antioxidant activity, evaluated by two different methods, which was able to reduce the hemolysis caused by reactive oxygen species in red blood cell, a model that mimics the sickle cell disease. The radical scavenging activity of **11m** can be added to the possible activation of peroxiredoxin-5, an antioxidant enzyme, which could remove H_2O_2 in both preventive and treatment action. Moreover, this molecule has adequate pharmacokinetics proprieties, hence making it a good candidate as a drug to treat sickle cell disease.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Grant received by Professor Stefani (FAPESP 2017/24821-4).

Declaration of Competing Interest

A competing interest statement is provided, even if the authors have no competing interests to declare.

References

[1] H. Al Balushi, A. Hannemann, D. Rees, J. Brewin, J.S. Gibson, The effect of antioxidants on the properties of red blood cells from patients with sickle cell anemia, Front Physiol. 10 (2019) 976.
[2] A.C.S. Alves, T. Munhoz, F.G.N. Soares, L. Rockenbach, B. Gauer; D.F. Kawano, G.L. V. Poser, S.C. Garcia, V.L.E. Lima, Synthesis and in vitro evaluation of 1,2,3-triazole-4-chloromethylcoumarins with antioxidant activity, Lett. Drug Design Discov., v. 15, n. 7, 2018.
[3] S. Azar, T.E. Wong, Sickle cell disease - a brief update, Med. Clin. N. Am. 101 (2017) 375–393.
[4] S. Barelier, D. Linard, J. Pons, A. Clippe, B. Knoops, J.M. Lancelin, I. Krimm, Discovery of fragment molecules that bind the human peroxiredoxin 5 active site, PLoS One. 5 (3) (2010), e9744.
[5] E. Bonandi, M.S. Christodoulou, G. Fumagalli, D. Perdicchia, G. Rastelli, D. Passarella, The 1,2,3-triazole ring as a bioisostere in medicinal chemistry. Drug Disc. Today, v. 22, n. 10, 2017.
[6] A. Cetin, I.H. Geçibesler, Evaluation as antioxidant agents of 1,2,4-triazole derivatives: effects of essential functional groups, J. App. Pharm. Sci. 5 (6) (2015) 120–126.
[7] A. Daina, O. Michielin, V. Zoete, iLOGP: a simple, robust, and efficient description of n-octanol/water partition coefficient for drug design using the GB/SA approach, J. Chem. Inf. Model. 54 (12) (2014) 3284–3301.
[8] A. Daina, V. Zoete, A BOILED-Egg to predict gastrointestinal absorption and brain penetration of small molecules, Chem. Med. Chem. 11 (2016) 1117–1121.
[9] A.L.A. Ferreira, L.S. Matsubara, Radicais livres: conceitos, doenças relacionadas, sistema de defesa e estresse oxidativo, Rev. Assoc. Méd. Bras. 43 (1) (1997) 61–68.
[10] S.B. Ferreira, A.C.R. Sodero, M.F.C. Cardoso, E.S. Lima, C.R. Kaiser, F.P. Silva, V. F. Ferreira, Synthesis, biological activity, and molecular modeling studies of 1H–1,2,3-Triazole derivatives of carbohydrates as α-glucosidases inhibitors, J. Med. Chem. 53 (6) (2010) 2364–2375.
[11] F.A. Ferrone, Sickle cell disease: Its molecular mechanism and the one drug that treats it, Int. J. Biol. Macromol. 93 (2016) 1168–1173.
[12] M.C. Foti, Use and abuse of the DPPH(•) radical, J. Agric. Food Chem. 14 (63) (2015) 8765–8776.
[13] M.T. Gladwin, J.H. Crawford, R.P. Patel, The biochemistry of nitric oxide, nitrite and hemoglobin: role in blood flow regulation, Free Radic. Biol. Med. 15 (2004) 707–717.
[14] I. Gülçin, Antioxidant and antiradical activities of L-carnitine, Life Sci. 78 (8) (2006) 803–811.
[15] Ö. Gürsoy-Kol, H. Yüksek, S. Manap, F. Tokali, Synthesis characterization, and antioxidant activities of novel 1-(morpholine-4-yl-methyl)-3-alkyl(aryl)-4-[4-(dimethylamino)-benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-ones, J. Turkish Chem. Soc., Section A Chem. 3 (3) (2016) 105–120.
[16] M. Hanif, M. Saleem, M.T. Hussein, N.H. Rama, S. Zaib, M.A.M. Aslam, P.G. Jones, J. Iqbal, Synthesis, urease inhibition, antioxidant and antibacterial studies of some 4-amino-5-aryl-3h-1,2,4-triazole-3-thiones and their 3,6-disubstituted 1,2,4-triazolo[3,4-b]1,3,4-thiadiazole derivatives, J. Braz. Chem. Soc. 23 (5) (2012) 854–860.
[17] C. Hansch, C. Selassie, Computer-assisted drug design: quantitative structure–activity relationship – a historical perspective and the future, in: J. B. Taylor, D.J. Triggle (Eds.), Comprehensive Medicinal Chemistry II, 2. ed., Elsevier Science, Nova York, 2007, pp. 43–63.
[18] R. Henneberg, M.F. Otuki, A.E.F. Furman, P. Hermann, A.J. Do Nascimento, M.S. S. Leonart, Protective effect of flavonoids against reactive oxygen species production in sickle cell anemia patients treated with hydroxyurea, Rev. Bras. Hematol. Hemoter. 35 (2013) 52–55.
[19] B. Hofmann, H.J. Hecht, L. Flohe, Peroxiredoxins, Biol. Chem., v. 383, p. 347–364, 2002.
[20] Y. Jia, P.W. Buehler, R.A. Boykins, R.M. Venable, A.I. Alayash, Structural basis of peroxide-mediated changes in human hemoglobin: a novel oxidative pathway, J. Biol. Chem. 282 (2007) 4894–4907.
[21] A. Kashyap, O. Silakari, Triazoles: Multidimensional 5-Membered Nucleus for Designing Multitargeting Agents. In: Key Heterocycle Cores for Designing Multitargeting Molecules. Ed. Om Silakari., p. 323-342, 2018.
[22] T. Kassa, M.B. Strader, A. Nakagawa, W.M. Zapol, I. Abdu, A.L. Alayash, Targeting βCys93 in hemoglobin S with an antisickling agent possessing dual allosteric and antioxidant effects, Metallomics. 9 (9) (2017) 1260–1270.
[23] T. Kassa, F. Wood, M.B. Strader, A.I. Alayash, Antisickling drugs targeting βCys93 reduce iron oxidation and oxidative changes in sickle cell hemoglobin, Front. Physiol. 10 (2019) 931.
[24] B. Knoops, A. Clippe, C. Bogard, K. Arsalane, R. Wattiez, C. Hermans, E. Duconseille, P. Falmagne, A. Bernard, Cloning and characterization of AOEB166, a novel mammalian antioxidant enzyme of the peroxiredoxin family, J. Biol. Chem. 274 (1999) 30451–30458.
[25] B. Knoops, J. Goemaere, V. van der Eecken, J.P. Declercq, Peroxiredoxin 5: structure, mechanism, and function of the mammalian atypical 2-Cys peroxiredoxin, Antioxid Redox Signal. 15 (3) (2011) 817–829.
[26] T.V. Kochikyan, M.A. Samvelyan, E.V. Arutyunyan, V.S. Arutyunyan, A. A. Avetisyan, M.G. Malakyan, L.A. Vardevanyan, S.A. Badzhinyan, Synthesis and antioxidant activity of new 1,2,4-triazole derivatives, Pharm. Chem. J. 44 (10) (2011) 525–527.
[27] R.I. Krutilina, A.V. Kropotov, C. Leutenegger, V.B. Serikov, Migrating leukocytes are the source of peroxiredoxin V during inflammation in the airways, J Inflamm., v. 3, n. 13, 2006.
[28] S.S. Kumar, H.P. Kavitha, Synthesis and biological applications of triazole derivatives – A review, Mini-Rev. Org. Chem. v.10, 2013.
[29] C. Lass-Flörl, Triazole antifungal agents in invasive fungal infections: a comparative review, Drugs 71 (18) (2011) 2405–3241.
[30] C.A.L. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Adv. Drug. Deliv. Rev. 1 (46) (2001) 3–26.
[31] Z. Liu, T. Zhou, A.C. Ziegler, P. Dimitrion, L. Zuo, Oxidative stress in neurodegenerative diseases: from molecular mechanisms to clinical applications., v. 2017 p.11, 2017.
[32] V. Lounnas, R. Ritschel, J. Kelder, R. McGuire, R.P. Bywater, N. Foloppe, Current progress in structure-based rational drug design marks a new mindset in drug discovery, Comput. Struct. Biotechnol. J. 5 (6) (2013) 2011–2020.
[33] R. Menichetti, K.H. Kanekal, T. Bereau, Drug-membrane permeability across chemical space, ACS Cent Sci. 5 (2) (2019) 290–298.
[34] H.A. Moharram, M.M. Youssef, Methods for determining the antioxidant activity: a review, Alexandria J. Food Sci. Tech. 11 (1) (2014) 31–42.
[35] M.Y. Moridani, J. Pourahmad, H. Bui, A. Siraki, P.J. O'Brien, Dietary flavonoid iron complexes as cytoprotective superoxide radical scavengers, Free Radic. Biol. Med. 34 (2003) 243–253.
[36] A. Nakagawa, M. Ferrari, G. Schleifer, M.K. Cooper, C. Liu, B. Yu, L. Berra, E. S. Klings, R.S. Safo, Q. Chen, F.N. Musayev, M.K. Safo, O. Abdulmalik, D.B. Bloch, W.M. Zapol, A triazole disulfide compound increases the affinity of hemoglobina for oxygen and reduces the sickling of human sickle cells, Mol. Pharm. 15 (2018) 1954–1963.
[37] NATIONAL TOXICOLOGY PROGRAM. Chemical Information Review Document for Hydroxyurea [CAS No. 127-07-1], 2011.
[38] Neil A. Alachant, Kouichi R. Tanaka, Antioxidants in sickle cell disease: the in vitro effects of ascorbic acid, Am. J. Med. Sci. 292 (1) (1986) 3–10.
[39] Y. Niihara, C.R. Zerez, D.S. Akiyama, K.R. Tanaka, Increased red cell glutamine availability in sickle cell anemia: demonstration of increased active transport, affinity, and increased glutamate level in intact red cells, J. Lab. Clin. Med. 130 (1997) 83–90.
[40] OECD GUIDELINE FOR TESTING OF CHEMICALS. Test No. 423: Acute Oral toxicity - Acute Toxic Class Method, 2001.
[41] C.M. Pompeo, A.I.Q. de Cardoso, M.C. da Souza, M.P. Ferraz, M.A. Ferreira Júnior, M.L. Ivo, Fatores de risco para mortalidade em pacientes com doença falciforme: uma revisão integrativa, Esc. Anna. Nery. 24 (2) (2020) 2019–2194.
[42] R.F. Queiroz, E.S. Lima, Oxidative stress in sickle cell disease, Rev. Bras. Hematol. Hemoter. 35 (2013) 3–17.
[43] D.C. Rees, T.N. Williams, M.T. Gladwin, Sickle-cell disease, Lancet 376 (2010) 2018–2031.
[44] T. Repka, R.P. Hebbel, Hydroxyl radical formation by sickle erythrocyte membranes: role of pathologic iron deposits and cytoplasmic reducing agents, Blood. 78 (1991) 2753–2758.
[45] C.A. Rice-Evans, V. Gopinathan, Oxygen toxicity, free radicals and antioxidants in human disease: Biochemical implications in atherosclerosis and the problems of premature neonates, Essays Biochem. 29 (1995) 39–63.
[46] A. Shamim, C.S. Barbeiro, B. Ali, H.A. Stefani, Synthesis of stannyl-substituted D-glucal derivatives via palladium-catalyzed regioselective hydrostannation and their synthetic applications, Chem. Select 1 (2016) 5653–5659.
[47] A. Shamim, F.B. Souza, G.H.G. Trossini, F.M. Gatti, H.A. Stefani, Synthesis of C-glycosyl-bis-1,2,3-triazole derivatives from 3,4,6-tri-O-acetyl-D-glucal, Mol. Divers. 19 (2015) 423–434.
[48] A. Shamim, F.B. Souza, S.N.S. Vasconcelos, H.A. Stefani, Synthesis of a library of glucal-derived triazoles via copper-catalyzed azide–alkyne cyclization, Tetrah. Let. 58 (2017) 884–888.
[49] A. Shamim, S.N.S. Vasconcelos, B. Ali, L.S. Madureira, J. Zukerman-Schpector, H. A. Stefani, Tetrahedron Letters. 56 (2015) 5836–5842.
[50] A. Shamim, S.N.S. Vasconcelos, I.M. Oliveira, J.S. Reis, D.C. Pimenta, J. Zukerman-Schpector, H.A. Stefani, Synthesis of glycosyl azides and their applications using CuAAC Click chemistry to generate bis- and tris(triazolyl)glycosyl derivatives, Synthesis 49 (2017) 5183–5196.
[51] H.A. Stefani, N.C.S. Silva, F. Manarin, D.S. Lüdtke, J. Zukerman-Schpector, L. S. Madureira, E.R.T. Tiekink, Synthesis of 1,2,3-triazolylpyranosides through click chemistry reaction, Tetrahedron Lett. 53 (2012) 1742–1747.
[52] A. Theodorou, M. Phylactides, L. Forti, M.R. Cramarossa, P. Spyrou, R. Gambari, S. L. Thein, M. Kleanthous, The investigation of resveratrol, Blood Cells, Mol. Dis. 58 (2016) 6–12.
[53] UNITED STATES ENVIRONMENTAL PROTECTION AGENCY. Health Effects Test Guidelines OPPTS 870.1000 Acute Toxicity Testing—Background; 2002.

[54] H. Yang, C. Lou, L. Sun, J. Li, Y. Cai, Z. Wang, W. Li, G. Liu, Y. Tang, AdmetSAR 2.0: web-service for prediction and optimization of chemical ADMET properties, Bioinformatics 35 (6) (2018) 1067–1069.
[55] H.A. Woo, W. Jeong, T.S. Chang, K.J. Park, S.J. Park, J.S. Yang, S.G. Rhee, Reduction of cysteine sulfinic acid by sulfiredoxin is specific to 2-cys peroxiredoxins, J. Biol. Chem. 280 (2005) 3125–3128.
[56] D. Wright, M. Reid, R. Stennett, Antioxidant intake in sickle cell disease, Int. J. Clin. Nutr. 2 (3) (2014) 53–59.
[57] H. Zhu, T.M. Martin, L. Ye, A. Sedykh, D.M. Young, A. Tropsha, Quantitative structure-activity relationship modeling of rat acute toxicity by oral exposure, Chem. Res. Toxicol. 22 (12) (2009) 1913–1921.
[58] J. Zukerman-Schpector, I. Caracelli, H.A. Stefani, A. Shamim, E.R.T. Tiekink, Acta Crystallogr. E71 (2015) o53–o54.
[59] T. Kassa, S. Jana, F. Meng, A.I. Alayash, Differential heme release from various hemoglobin redox states and the upregulation of cellular heme oxygenase-1, FEBS Open Bio. 6 (9) (2016) 876–884, https://doi.org/10.1002/2211-5463.12103.