INTRODUCTION

Arterial hypertension (AH) has become the most prevalent chronic disease in the world [1] and is considered the main risk factor for the development of a range of other cardiovascular diseases, such as coronary diseases, stroke, and renal failure [2]. The endothelium-dependent vasorelaxation is normally reduced in hypertension due to alterations in the homeostatic regulation of vascular tone known as endothelial dysfunction [3,4]. This process is mainly attributed to impairment on nitric oxide (NO) production and bioavailability in the vascular wall and increased NO degradation due to the rise in oxidative stress leading to increase in vascular resistance [5-7].

Therapy with organic nitrates such as nitroglycerine (GTN) and isosorbide dinitrate (ISDN) has been used for many years in the treatment of cardiovascular disorders including AH [8]. These drugs release NO from its structures and replace the NO deficiency leading to improvement of the endothelial function and modulation of the vascular tone, reducing AH and its comorbidities [2,9-12]. Despite the benefits, these NO donors have limitations that include high reactivity, short half-life, and induction of the tolerance phenomenon [13,14], limiting their efficient clinical use [15]. Therefore, the search for new promising organic nitrates with the absence of those undesirable effects and presenting desirable pharmacological characteristics to treat AH is still a scientific challenge. To obtain a new compound, we synthesized the novel NO donor 1,3-diisobutoxypropan-2-yl nitrate (NDIBP). Once this molecule is a novel organic nitrate and its biological and pharmacokinetics characteristics are unknown, a virtual screening of NDIBP is a good approach to determine its initial profile and verify the chance to be used in cardiovascular field.

Computational techniques such as Prediction of Activity Spectra for Substances (PASS) and pkCSM (Predicting Small-Molecule Pharmacokinetic Properties Using Graph-Based Signatures) have become important tools in medicinal chemistry to predict the biological activities and pharmacokinetics (PK) properties of a specific compound based exclusively in its structure and physicochemical properties [16-18]. The in silico assessment of the biological aspects determines the most correct direction for pharmacological studies of the new substance, reducing both cost and time required to perform in vitro screenings [19-23]. In addition, these new approach methodologies (NAMs) could help to justify the ethical principles of 3Rs (reduce, refine, and replace) by avoiding unnecessary animal studies, reducing the number of animals used in the research, and refining the protocol to a minimum level of animal pain, distress, or suffering [24].

Although the pharmacological properties are important factors for drug discovery, the PK aspects are the main responsible to predict if the drug will advance its effectiveness and safety aiding the therapeutic success [25]. The in silico studies to predict Absorption Distribution
Metabolism, Excretion and Toxicity (ADMET) properties help in the analysis of novel substances to avoid spending time on candidates that would be toxic or metabolized by body enzymes into an inactive or unable form to cross cell membranes and thus work with only promising compounds [26,27].

Therefore, using in silico techniques and validation of the predicted potential by ex vivo experiments, this study aimed to explore the pharmacological and PK aspects of NDIBP as a candidate for a new NO donor. Our study showed that NDIBP has the biological and predicted pharmacokinetic characteristics to be a promising drug candidate acting as a vasorelaxant agent and becoming a useful drug in the treatment of hypertension.

METHODS

Synthesis of NDIBP

NDIBP was obtained from glycerin by organic synthesis at the Department of Chemistry at the Federal University of Paraíba. The reaction is illustrated in Fig. 1a. Briefly, an aliquot of dry glycerin (1) was transferred to a three-neck round bottom flask, heated at 100–110°C for 12 h to remove humidity and then hydrochloric acid was bubbled in the flask through a tubular system to obtain the 1,3-dichloropropan-2-ol (3). To produce the gaseous HCl, 100 mL of sulfuric acid (12 N) was dropped over slurry of sodium chloride (100 g) and hydrochloric acid (36.5%, 2 mol). The reaction finished when the absorption of HCl (g) by glycerin not occurred anymore (usually at the end of absorption occurs a 25% increase in the initial volume of glycerin). Afterward, 1,3-dichloropropan-2-ol (3) was purified using fractional distillation at 174–176°C obtaining 70% yield. In second step, sodium alkoxide (4) was obtained by mixing sodium metal (2 mol, finely cut) in a flask containing 1 mol of the corresponding alcohol (2) under constant stirring up to the total sodium added consumption. Sodium alkoxide (4; 2 mol) was placed in a round bottom flask and 1 mol of 1,3-dichloropropan-2-ol (3) was added dropwise under continuous stirring for 6 h to synthesize the corresponding oxyalcohol: 1,3-diisobutoxypropan-2-ol (5). This compound was purified using a fractional distillation under vacuum at 185–190°C with a 91% reaction yield and then thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR) were used to evaluate the purity. Finally, to obtain the organic nitrate (6) a reaction using an aliquot of 0.5 mol of compound (5) and 0.6 mol of acetic anhydride was performed in a round bottom flask and fuming HNO₃ (0.6 mol) was added dropwise to obtain the NDIBP (6) (Fig. 1b). This mixture was kept under constant stirring and in an ice bath for temperature control (5°C). The reaction was interrupted with the addition of 100 mL of ice-cold distilled water resulting in the formation of a biphasic system. Aqueous phase was separated through separation funnel and neutralized by adding sodium bicarbonate while organic phase containing the nitrate was solubilized in chloroform and dried with anhydrous sodium sulfate to remove humidity [28,29]. Chloroform was subsequently removed by rotoevaporation. The organic nitrate (6) was storage in the darkness at 5°C and after 45 days TLC, Fourier-transform infrared (FTIR) and NMR confirmed that the purity remains stable.

1,3-dichloropropan-2-ol (3): yield: 70%; IR (ATR) ν/cm⁻¹ 3460 (O-H), 2992, 1438 (C – H), 1270 (C – O), 851, 733 (C – Cl); ¹H NMR (200 MHz, CDCl₃) δ 4.05 (p, 1H), 3.65 (d, 4H), 2.65 (s, 1H); ¹³C NMR (50 MHz, CDCl₃) 70.6, 45.5.

1,3-diisobutoxypropan-2-ol (5): yield: 91%; RMN ¹H e ¹³C. IR (ATR) ν/cm⁻¹ 3466 (O-H), 2992, 1438 (C – H), 1270 (C – O); ¹H NMR (200 MHz, CDCl₃) δ 4.06 (p, 1H), 3.64 (d, 4H), 2.65 (s, 1H); ¹³C NMR (50 MHz, CDCl₃) 77.6, 71.2, 66.7, 27.6, 18.5.

1,3-diisobutoxypropan-2-yl nitrate (6; NDIBP): yield: 85.7%; IR (ATR) ν/cm⁻¹ 3466 (O-H), 2956, 2872, 1469 (C – H), 1363, 1274, 850 (N – O), 1107 (C – O); ¹H NMR (200 MHz, CDCl₃) δ 3.95 (dt, J = 10.9, 5.4 Hz, 1H, H-1), 3.47 (dd, J = 5.4, 2.7 Hz, 4H, H-3, H-3'), 3.23 (d, J = 6.7 Hz, 4H, H-2, H-2'), 1.87 (dd, J = 5.4, 2.7 Hz, 4H, H-3, H-3'), 1.87 (dd, J = 5.4, 2.7 Hz, 4H, H-3, H-3'), 1.32 (d, J = 6.7 Hz, 4H, H-2, H-2'), 1.32 (d, J = 6.7 Hz, 4H, H-2, H-2'), 1.08 (d, J = 5.4 Hz, 1H, H-1), 1.08 (d, J = 5.4 Hz, 1H, H-1), 0.88 (d, 1H, H-1).
(tt = 13.4, 6.9 Hz, 2H, H-4, H-4), 0.90 (d, J = 6.7 Hz, 12H, H-5, H-5). 13C NMR (50 MHz, CDCl3): δ 84.4 (C-1), 76.9 (C-3), 71.9 (C-2), 25.1 (C-4), 22.7 (C-5).

Prediction of NDIBP (1,3-diiosubutoxypropen-2-yl nitr)ate biological activity spectrum by PASS analysis

Prediction of NDIBP biological activity was obtained through PASS analysis. This program was designed to access the overall biological potential of a molecule [18] and uses the 2D structural formula of the compound as the basis for structure description [30-32]. This in silico tool can estimate simultaneously over 7000 kind of probable pharmacological effects, mechanisms of action, and specific toxicities including carcinogenicity, mutagenicity, teratogenicity, and embryotoxicity, adverse effects, interaction with metabolic enzymes and the influence on gene expression [30,31,33-36]. The set of all these characteristics predicted by PASS is termed "biological activity spectrum," an intrinsic characteristic of the compound based exclusively in its structure and physicochemical properties [16,17]. PASS approach involved the SAR analysis of the training set containing about one million of drugs, drug-candidates, leads, and toxic compounds that were collected from several data sources including publications, patents, chemical databases, and private communications [32,37]. In the training set the "active" compounds are those with quantitative characteristics of a Pa > Pi value better than 10^-4 M and the compounds less active or with unknown activity are considered as "inactive" [32]. Construction of the NDIBP chemical structure and SAR models with the compounds from the training set were based on original descriptors of multilevel neighborhoods of atoms (MNA). These descriptors are a linear notation of atom-centered fragments in the structure of an organic molecule and not specify the bond types presented in the compound but includes hydrogen atoms according to the partial charge and valence of the atoms [38,39]. The algorithm to construct SAR models using the compounds from the training set and predict the activities was based on the Bayesian estimates [40,41]. PASS software predicted qualitatively the biological activity spectrum of NDIBP in terms of probability of being active (Pa) and the probability of being inactive (Pi) [40]. Values of Pa and Pi can vary between 0 and 1 and in general Pa + Pi < 1 when the probabilities are calculated independently [16,30]. Most probable activities are characterized by Pa values close to 1, and Pi values close to 0.16 Interpretation of the results is flexible and depends on the purpose of the study. By default, only activities with Pa>Pi value are used as a threshold for possible activities of a new compound which provides the mean accuracy of about 95% [34,42,43]. The Pa index just interpreted the similarity of the studied compound with the structure of active molecules presented in the corresponding subset of training set and is not related to quantitative activity characteristics [31,32].

Effects of NDIBP on isolated mesenteric arteries contracted with phenylephrine

Male Wistar rats (250–300 g) were used for this protocol. They were housed in cages under controlled conditions of temperature (21±1°C), a 12 h light-dark cycle and were allowed food and water ad libitum. After euthanasia, the cranial mesenteric artery of the normotensive animals was collected, dissected, and sectioned to obtain arterial rings (2–3 mm). These preparations were mounted on two 3-shaped stainless-steel wires attached to a tension transducer (PowerLab™, ADInstruments, MA, EUA) to assess changes in isometric tone. All the rings were kept in 10 mL tissue chambers filled with Tyrode's solution, gassed with carbogenic mixture (95% O2 and 5% CO2) and maintained at 37°C. Each ring was stabilized under 0.75 g resting tension for 60 min. After stabilization period, tissue viability was verified by a contraction to phenylephrine (10 μM) added to the bath and the presence of functional endothelium was assessed by the response of relaxation induced by Ach (10 μM). Mesenteric rings with vasorelaxation percentage less than 10% represent an absence of functional endothelium [12]. After the initial procedures, mesenteric artery rings (n=6) were pre-contracted using phenylephrine (1 μM). When contraction plateau was reached, cumulative concentrations of vehicle or NDIBP (10^-12–10^-4 M) were added to the organ bath to build a concentration-response curve.

NDIBP was previously emulsified with Cremophor® and then mixed with distilled water. Initial solution was subsequently diluted to obtain the desired concentrations. Final concentration of Cremophor® never exceeded 0.01%. The effect was expressed as percentage of relaxation in relation to the phenylephrine contraction. Maximum effect (ME) was calculated using GraphPad Prism v. 5.01. ME reflects the efficacy of the drug [44,45]. This experimental study was approved by the Federal University of Paraiba Animal Care and Use Committee (CEUA-UFPB, Protocol ID: 094/2017 – 10/05/2017) in João Pessoa, Brazil, and conducted in accordance with the standards and ethical principles of experimentation established by the National Council of Animal Experimentation Control (CONCEA).

Computational assessment of the drug-like properties of NDIBP
NDIBP physicochemical properties, PK aspects, and toxicity profile were determined using an ADMET descriptors algorithm protocol of pkCSM that uses the concept of graph-based structural signatures to predict and optimize ADMET aspects [25]. This software has been used as evidence to train accurate molecular predictors of important physicochemical parameters such as molecular weight (MW), topological polar surface area (TPSA), partition coefficient (octanol-water) – LogP, number of hydrogen bond acceptor (nHBA), number of hydrogen bond donor (nHBD) and rotatable bonds (ROTB). Analysis of these aspects was used to verify the drug-likeness properties of NDIBP based on the guidelines of the Lipinski’s Rule of Five (Lipinski’s R05) [46]. Absorption properties were analyzed based on membrane permeability (indicated by colon cancer cell line (Caco-2) permeability), human intestinal absorption (HIA), skin permeability, and the categorical classification of NDIBP as a P-glycoprotein efflux substrate or nonsubstrate. Distribution of the drug was predicted according the blood-brain barrier permeability (logP) and the volume of distribution at steady state (VDss). Metabolism property was evaluated based on the CYP models for substrate or inhibition (CYP1A2, CYP2C9, CYP2C9, CYP2D6, and CYP3A4). Excretion aspect was predicted by the total clearance model and the categorical classification of NDIBP as a renal OCT2 substrate. Toxicity of NDIBP was obtained based on Ames toxicity, hERG inhibition, hepatotoxicity, and skin sensitization. After the results, all the PK parameters were calculated and checked for compliance with their standard ranges [47].

Statistical analysis
Data were expressed as the mean±standard error of the mean. Statistical analysis was performed using analysis of variance followed by the recommended post hoc analysis that was carried out using the GraphPad Prisms version 5.01 (GraphPad Software Inc., San Diego, CA, USA). Values were considered significantly different when p<0.05.

RESULTS AND DISCUSSION
NDIBP synthesis
The natural source used to obtain NDIBP was glycerin, one of the most important co-products of the biodiesel production process and an excellent source of C-C to be used in the reaction. NDIBP was synthesized from the esterification reaction of the corresponding alcohol using concentrated nitric acid in the presence of acetic anhydride (Fig. 1a).

One of the advantages of this route is the reduced water content in the system since both concentrated nitric acid and acetic anhydride have a very low water amount in their composition and according to Shen et al. [48] the greater is the content of water in reaction medium lower is the conversion rate. Therefore, the chance of obtaining a higher conversion to organic nitrate was increased. Suppes and Dasari [49] also recommend this synthetic route once the acetic anhydride use instead of sulfuric acid is better due to its higher selectivity, reduced oxidation, and ability to make the reaction at higher temperatures.

An excess of 20% in the number of moles of nitrating solution (Ac2O/HNO3) in relation to the stoichiometric amount of the compound to be nitrated was used to obtain a higher yield of the new organic nitrate. Nitrating solution addition time did not exceed 20 min due
to kinetical factors and the system temperature did not exceed 5°C due to the exothermic nature of the reaction. After all the steps, the yield obtained for NDIBP was 85.7%, a satisfactory amount considering the selected synthetic route. This result was similar to the obtained by Zhuge et al. [50] who synthesized the organic nitrate 1,3-bis(hexylxox) propan-2-yl (NDIBP) confirming that this synthetic route is suitable for obtaining organic nitrates.

Regarding the chemical characteristics, NDIBP (Fig. 1b) presented C29H48N12O12 as molecular formula, molar mass value of 2491.6 g/mol and the following elemental analysis: C: 52.99%; H: 9.30%; N: 5.62% and O: 32.09%. NDIBP was considered a molecule with simple structure, and then its purity and structure were completely confirmed using FTIR spectroscopy and 1H and 13C NMR (supporting information). Furthermore, it was a viscous liquid with slightly yellow color and thermal stability up to 60°C, soluble in chloroform, dichloromethane and, ethyl ether and poorly soluble in water and ethanol. Besides NDIBP, our group has previous experience with other organic nitrates obtained from glycerin with confirmed pharmacological activity such as 2-nitrate-1,3-dibuthoxypropan (NDPB), 1,3-bis (hexylxox) propan-2-yl nitrate (NDHP), and 2-nitrate-1,3-di(octanoxy)propan [12,51,52].

PASS prediction indicates NDIBP as a NO donor with vasodilator effect

After NDIBP synthesis, PASS was used to predict the spectrum of NDIBP biological activities. For this study, we considered only predicted activities with probability (Pa) > 0.7. PASS analysis showed 1656 of 5050 possible biological activities. The prediction of pharmacological effects demonstrated that NDIBP presented 167 of 504 possible effects (Pa > Pi). However, according to cutoff value of Pa > 0.7, NDIBP showed only 29 of 167 predicted pharmacological effects (Table 1).

Among the selected effects, we highlight the highest values of Pa for antihypertensive, antiangiologial, and vasodilator effect (0.973, 0.969, and 0.967, respectively). All these effects are in accordance with the changes presented by the organic nitrates used in the clinics such as GTN, isosorbide mononitrate (ISMN) and ISDN. Those compounds develop several hemodynamic actions that are induced by the effect of vasodilation of capacitance veins and conductance arteries leading to: (i) A reduction in vascular resistance; (ii) decrease in ventricular pre-load and left ventricle systolic wall tension; (iii) reduction in myocardial O2 consumption, and (iv) increase in subendocardial myocardial blood flow which improve the symptoms of several cardiovascular disorders such as acute and chronic congestive heart failure, angina pectoris, coronary artery disease, and hypertension [53,54]. Therefore, the in silico data showed that predicted effects of NDIBP are desirable for a drug candidate for the treatment of cardiovascular disorders, including hypertension.

Furthermore, other pharmacological effects were predicted for NDIBP (Table 1), including analgesic, spasmylotic, and platelet aggregation inhibitor (Pa = 0.944, 0.871, and 0.846, respectively). These findings give information and provide support to stimulate the assessment of unknown potential effects of NDIBP that can be promising.

Table 1: Prediction of NDIBP pharmacological effects by PASS software

| Predicted pharmacological effect | Pa  | Pi  | Predicted Pharmacological Effect | Pa  | Pi  |
|---------------------------------|-----|-----|---------------------------------|-----|-----|
| Antihypertensive                 | 0.973 | 0.003 | Alzheimer's disease treatment | 0.821 | 0.004 |
| Antianginal                      | 0.969 | 0.001 | Antidote, cyanide               | 0.811 | 0.004 |
| Vasodilator                     | 0.907 | 0.001 | Respiratory spasmylotic         | 0.810 | 0.006 |
| Myocardial infarction treatment  | 0.948 | 0.002 | Reproductive disfunction treatment | 0.784 | 0.003 |
| Analgesic                       | 0.944 | 0.004 | Miotic                          | 0.774 | 0.004 |
| Analgesic, non-opoid            | 0.943 | 0.004 | Erectile dysfunction treatment  | 0.768 | 0.003 |
| Vasodilator, coronary           | 0.914 | 0.003 | Angiogenesis stimulant         | 0.767 | 0.003 |
| Osteoarthritis treatment        | 0.878 | 0.001 | Spasmylotic, urinary          | 0.761 | 0.004 |
| Cardiotonic                     | 0.874 | 0.004 | Antiarthritic                  | 0.751 | 0.009 |
| Myocardial ischemia treatment   | 0.870 | 0.003 | Analgesic                      | 0.740 | 0.007 |
| Spasmylotic                     | 0.871 | 0.004 | Anesthetic                    | 0.729 | 0.004 |
| Rheumatoid arthritis treatment  | 0.864 | 0.003 | Vasoconstrictor               | 0.719 | 0.006 |
| Antischemic                     | 0.863 | 0.004 | Vasoconstrictor, peripheral    | 0.704 | 0.007 |
| Platelet aggregation inhibitor  | 0.846 | 0.004 | Neurodegenerative              | 0.707 | 0.008 |
| Heart failure treatment         | 0.830 | 0.003 |                                |      |     |

Pa: Probability of active, Pi: Probability of inactive
Table 2: NDIBP’s mechanisms of action predicted by PASS software

| Predicted mechanism of action | Pa  | Pi  |
|-------------------------------|-----|-----|
| Vasodilator                   | 0.967| 0.001|
| Analgesic                     | 0.944| 0.004|
| Nitric oxide donor            | 0.923| 0.000|
| APOA1 expression enhancer     | 0.915| 0.003|
| Vasodilator, coronary         | 0.914| 0.003|
| Cardiotonic                   | 0.874| 0.004|
| Spasmolytic                   | 0.871| 0.004|
| Cyclic GMP PDE inhibitor      | 0.850| 0.001|
| Saccharopepsin inhibitor      | 0.852| 0.007|

| Predicted mechanism of action | Pa  | Pi  |
|-------------------------------|-----|-----|
| Acrocyllindropepsin inhibitor | 0.852| 0.007|
| Chymosin inhibitor            | 0.852| 0.007|
| Platelet aggregation inhibitor | 0.846| 0.004|
| Cutinase inhibitor            | 0.820| 0.004|
| Miotic                        | 0.774| 0.004|
| Angiogenesis stimulant        | 0.767| 0.003|
| Polypopotepsin inhibitor      | 0.777| 0.015|
| Fragilysin inhibitor          | 0.734| 0.010|
| Vasodilator, peripheral       | 0.708| 0.007|

Pa: Probability of active; Pi: Probability of inactive; APOA1: Apolipoprotein A1; GMP: Guanosine monophosphate; PDE: Phosphodiesterase

NDIBP induces vasorelaxant effect in superior mesenteric artery

An ex vivo approach using mesenteric arteries rings without functional endothelium was used to experimentally confirm the vasodilator effect predicted by PASS. The removal of vascular endothelium avoided the influence of the endothelium-derived relaxing factors (EDRF).

Table 3: Prediction of NDIBP gene expression regulation by PASS software

| Predicted gene expression regulation | Pa  | Pi  |
|-------------------------------------|-----|-----|
| APOA1 expression enhancer           | 0.915| 0.003|
| HMOX1 expression enhancer           | 0.755| 0.012|
| CASP3 expression inhibitor          | 0.752| 0.013|

Pa: Probability of active; Pi: Probability of inactive; APOA1: Apolipoprotein A1; HMOX1: Heme oxygenase 1; CASP3: Caspase3

Cumulative administration of the glycerin-derived organic nitrate NDIBP (10⁻¹²–10⁻⁴ M) induced a concentration-dependent vasorelaxant effect in phenylephrine pre-contracted mesenteric artery rings (Fig. 2).

GTN was used as a positive control once it is a classical organic nitrate and as expected, its cumulative addition (10⁻¹²–10⁻⁴ M) induced a concentration-dependent relaxant effect with 112.12±2.66% of ME. Although NDIBP presents only one nitrate group in the structure its concentration-response curve obtained an expressive ME of 105.97±3.65% with no statistically significant differences in comparison to GTN, which exhibits three groups in its molecule. Any effect was not shown in the vessel preparations when just the vehicle was used, demonstrating that the cumulative administration of the vehicle was unable to induce any significant vasorelaxation. The ME obtained by the vehicle in the concentration-response curve was 12.78±2.42%.

According to this, NDIBP response proves that the theoretical predictions are in agreement with the experimental results, validating PASS analysis and demonstrating that NDIBP is a vasorelaxant agent.

Other organic nitrates derived from glycerin such as NDDBP and NDHP also produced vasorelaxant effect in ex vivo experiments using mesenteric arteries rings [12,50,52]. They were considered as NO donors developing vasorelaxation through NO release and activation of NO-sGC-cGMP-PKG pathway with the participation of some K⁺ channels [12,50,52]. In this study, we suggested by PASS analysis (Table 2) that NDIBP may be a NO donor and based on the EDRF independent vasorelaxant effect we suggest that NDIBP response would occur following the same mechanism of action of the organic nitrates mentioned above.

ADMET prediction indicates that NDIBP is a drug-like molecule

One drug is considered promising when it has a fine balance between low toxicity, good pharmacological effects (potency and efficacy) and ADMET properties [2,12,5]. Thus, the PK profile is crucial for the drug effectiveness. To understand this profile, NDIBP physicochemical properties of TPSA, LogP, nHBA, nHBD, and ROBT were predicted with the help of pkCSM software.

The physicochemical parameters obtained in this study were used to understand and predict NDIBP aspects of ADMET and its drug-like nature. Theoretical physicochemical characteristics of NDIBP are shown in Table 5 and can be compared with other organic nitrates described in the literature [79-81]. All of these pharmacological descriptors are related to the passive transport across membranes, that
Table 4: Prediction of NDIBP toxic and adverse effects by PASS software

| Predicted toxic/adverse effects | Pa | Pi | Predicted toxic/adverse effects | Pa | Pi |
|--------------------------------|----|----|---------------------------------|----|----|
| Skin irritative effect         | 0.96 | 0.02 | Neurotoxic                      | 0.85 | 0.01 |
| Skin irritation, weak          | 0.94 | 0.02 | Carcinogenic, rat, female       | 0.84 | 0.00 |
| Eye irritation, weak           | 0.94 | 0.02 | Carcinogenic, rat               | 0.84 | 0.00 |
| Hypotension                    | 0.86 | 0.00 | Cardiotoxic                     | 0.84 | 0.01 |
| Skin irritation, high          | 0.89 | 0.01 | Dermatitis                      | 0.85 | 0.01 |
| Irritation                     | 0.90 | 0.01 | Allergic reaction               | 0.81 | 0.01 |
| Ocular toxicity                | 0.89 | 0.01 | Carcinogenic                    | 0.82 | 0.01 |
| Nephrotoxic                    | 0.88 | 0.00 | Eye irritation, high            | 0.79 | 0.00 |
| Hepatotoxic                    | 0.86 | 0.00 | Embryotoxic                     | 0.79 | 0.01 |
| Carcinogenic, rat, male        | 0.87 | 0.00 | Teratogen                       | 0.77 | 0.01 |
| Anemia                         | 0.87 | 0.00 | Toxic                           | 0.78 | 0.02 |
| Endocrine disruptor            | 0.86 | 0.00 | Mutagenic                       | 0.74 | 0.00 |
| Hematotoxic                    | 0.87 | 0.01 | Anaphylaxis                     | 0.74 | 0.01 |
| Reproductive dysfunction       | 0.65 | 0.13 |                                |     |    |

Table 5: Theoretical analysis of NDIBP physicochemical properties obtained by pkCSM software

| Compound | Physicochemical properties |
|----------|---------------------------|
|          | MW (g/mol) | TPSA (Å²) | LogP | nHBA | nHBD | nROTB | RO5 violations |
| NDIBP    | 249.16     | 102       | 2    | 5    | 0    | 10    | 0             |
| GTN      | 227.09     | 165       | 1.6  | 9    | 0    | 5     | 0             |
| ISMN     | 191.14     | 93.7      | -0.4 | 8    | 0    | 1     | 0             |
| ISDN     | 236.14     | 1.29      | 1.3  | 2    | 0    | 2     | 0             |

**MW:** Molecular weight, **TPSA:** Topological polar surface area, **LogP:** Partition coefficient, **nHBA:** Number of hydrogen bond acceptor, **nHBD:** Number of hydrogen bond donors, **nROTB:** Number of rotatable bonds, **RO5:** Rule of five

According to the prediction, NDIBP presented the lowest TPSA value when compared to other organic nitrates used in the clinic such as GTN and ISDN suggesting a probable greater facility to cross cell membranes (Table 5). In general, a higher logP value indicates a high lipophilicity and consequently a good drug permeability across cell membranes, but compounds with logP > 5 are poorly absorbed [46,88]. According to PASS analysis, NDIBP was within the recommended range of the optimum region of lipophilicity with logP between -2 and 5 [89,90] and also presented the highest logP value in comparison to GTN, ISMN, and ISDN, indicating probable higher lipophilicity (Table 5). The higher is the number of RO5 (nROTB) the greater is the molecule flexibility, leading to negative effects on the compound permeability [82]. NDIBP showed the highest predicted value of nROTB compared to the other mentioned organic nitrates (Table 5). Although NDIBP presented a high nROTB, it is still within the range established by Verber et al. [82] (nROTB ≤ 10) to develop a good permeation and oral bioavailability. Thus, according to pkCSM predictions and when all the physicochemical parameters are associated and compared, NDIBP probably results in a better permeation across cell membranes and molecule flexibility than the other mentioned organic nitrates, which can influence to it develop a better drug absorption and bioavailability.

Next, NDIBP molecular descriptors were analyzed through the Lipinski’s RO5, which can estimate the likeliness of the molecule to act as a drug [46]. This analysis is important for theoretical prediction of oral bioavailability profile and its based on five conditions: (1) nHBA less than or equal to 10; (2) nHBD less than or equal to 5; (3) MW less than or equal to 500 g/mol; (4) logP less than or equal to 5, and (5) TPSA less than or equal to 140 Å² [46,91,92]. When more than one of these rules are violated it means that the molecule may have problems with absorption or permeability and consequently with bioavailability [37,93].

The data obtained in this study (Table 5) showed that NDIBP satisfied all the rules established by Lipinski suggesting that it may have a high tendency to penetrate into cell membrane and develop a good theoretical oral bioavailability. In addition, according to Veber et al. [82], compounds with nROTB less than or equal to 10 present a high probability of good oral bioavailability. As shown previously, the value of nROTB obtained for NDIBP is within the threshold established and corresponds to one more aspect to suggest the high probability of being used orally. As shown, NDIBP presents all the eligible aspects to meet the drug-likeness criteria.

It is important to highlight that the appreciation of ADME properties throughout the drug discovery process has become relevant to unravel compounds with poor ADME aspects at the earlier stage of
the drug development and thus reduce the number of compounds that fail in clinical trials [94-96]. Predicted NDIBP ADMET aspects are demonstrated in Table 6.

The absorption of drugs proposed to be used orally depends on their ability to cross the walls of gastrointestinal tract (GIT) [97]. Due to this, NDIBP absorption profile was based on Caco-2 permeability and HIA. For this predictive model, a compound may have a high Caco-2 permeability when Papp > 0.90 and a good intestinal absorbance when the value is higher than 30% [25]. According to the results, NDIBP presented both a high Caco-2 permeability and high intestinal absorption (Table 6). Caco-2 cells are extracted from the human epithelial colorectal adenocarcinoma being widely used once these cells can mimic the gastrointestinal epithelium representing a validated assay system for oral absorption studies [88,98,99] and HIA is the sum of bioavailability and absorption evaluated from the cumulative excretion in bile, urine, and feces [100]. Therefore, the data suggested that NDIBP may probably cross the membrane of GTI and develop a good oral absorption corroborating the analysis based on Lipinski’s RO5.

Skin permeability was also predicted and NDIBP demonstrated a relatively low skin permeability as indicated by the value of logKP ~2.5 [25]. This parameter suggested that a topical administration could not be a good alternative for NDIBP absorption. The drug absorption can also be influenced by the presence of efflux proteins in cell membrane. One of this protein is Pgp an ATP-binding cassette transporter that pumps drug out from the intestinal cell [101-104]. The predicted result showed that NDIBP is a non-substrate as well as a non-inhibitor of Pgp (Table 6). Due to not being a substrate, it means that Pgp may not recognize the organic nitrate and may not cause its cell efflux and being a non-inhibitor the NDIBP shall not make interactions with Pgp in any way, so its function to promote xenobiotics efflux will be not blocked.

The distribution of NDIBP from the systemic circulation to extravascular tissues was assessed based on the VDs and BBB permeability (Table 6). VDs represent the volume of body fluid that a total dose of a drug needs to be distributed to obtain the same concentration presented in blood plasma [105], being an important indicator to determine dosage prescription of a compound [27]. According pkCSM predictive model, VDs is considered low when it is <0.71 L/kg and high when >2.81 L/kg [25]. VDs value of NDIBP was below 0.71 reflecting a low distribution to extravascular tissues. Most of the pharmacological targets of the drugs are not presented at the vasculature and the access to them relies on organ distribution [106]. However, most of the activities predicted for organic nitrates are related to vascular action, justifying the low value of distribution of NDIBP.

Another important aspect is the BBB permeability since this physiological barrier composed by endothelial cells regulates the passage of compounds from the blood to the central nervous system (CNS), developing a protective property [97]. For the pkCSM model, a compound with logBB > 0.3 can readily cross the BBB while compounds with logBB < -1 have difficulty to be distributed to the brain [25]. NDIBP presented an intermediate value of BBB permeability suggesting that it may have some distribution into the brain. The liposolubility of compound may contribute to cross this barrier and probably develop actions at central nervous system level.

Evaluation of first pass metabolism in the liver characteristics depends on interaction with the several microsomal enzymes known as cytochrome P450 (CYP450) [97]. These enzymes are mostly located in the liver and responsible for the majority of drug first-pass metabolism highlighting CYP3A4 that performs almost 50% of the metabolism of xenobiotics in humans [107,108]. ADMET prediction showed that NDIBP is a non-substrate of CYP2D6 and CYP3A4 isoforms as well as a non-inhibitor of CYP2C9, CYP2C19, CYP2D6, and CYP3A4 isoforms (Table 6). The data suggested that NDIBP may not be metabolized by the selected CYP isoforms being chemically inert once it is not able to active the enzymes as a substrate and at the same time it may not promote the loss of function of CYP isoforms and interferes in the metabolism of other drugs because it was not considered an inhibitor.

Molecules with high levels of metabolism by cytochrome P450 have reduced oral bioavailability and plasma half-life [109]. Based on this, NDIBP probably presents a good bioavailability and length of time in the plasma once this nitrate does not pass through first-pass metabolism by CYP isoforms.

Excretion is another important PK parameter that describes the process to remove intact drug molecules or its metabolites from the body determining the period of time the drug remains in the organism as well the volume of distribution [95,106,107]. This process was analyzed based on total clearance and a categorical classification of NDIBP as a renal OCT2 substrate (Table 6). There is not a limited range of total clearance, the higher the value, the faster will be the excretion process [25]. The NDIBP value of total clearance was just an estimative and with this, the rate of excretion can be predicted. PASS prediction also showed that NDIBP may not be an OCT2 substrate. OCT2 is a renal transporter.

Table 6: Theoretical analysis of NDIBP ADMET properties obtained by pkCSM software

| ADMET properties | Model name | Predicted result | Unit | Interpretation |
|------------------|------------|------------------|------|----------------|
| GI Absorption    | Caco-2 permeability | 0.92 | Numeric (log Papp in 10^-6 cm/s) | High: > 0.90 |
|                  | Human intestinal absorption | 95.02 | Numeric (% Absorbed) | Poor absorbed: < 30% |
|                  | Skin permeability | -2.28 | Numeric (log Kp) | Low permeability: > -2.5 |
|                  | P-glycoprotein substrate | No | Categorical (Yes/No) | Yes/No |
|                  | P-glycoprotein I inhibitor | No | Categorical (Yes/No) | Yes/No |
|                  | P-glycoprotein II inhibitor | No | Categorical (Yes/No) | Yes/No |
| Distribution     | Volume of distribution | -0.28 | Numeric (log Vd/L) | Low: < 0.71; High: > 2.81 |
|                  | Blood brain barrier permeability | -0.71 | Numeric (log BB) | Low: < -1; High: > 0.30 |
| Metabolism       | Substrate CYP2D6 | No | Categorical (Yes/No) | Yes/No |
|                  | Substrate CYP3A4 | No | Categorical (Yes/No) | Yes/No |
|                  | Inhibitor CYP2C9 | No | Categorical (Yes/No) | Yes/No |
|                  | Inhibitor CYP2C19 | No | Categorical (Yes/No) | Yes/No |
|                  | Inhibitor CYP2D6 | No | Categorical (Yes/No) | Yes/No |
|                  | Inhibitor CYP3A4 | No | Categorical (Yes/No) | Yes/No |
| Excretion        | Total clearance | 0.67 | Numeric (log ml/min/kg) | Log ml/min/kg |
|                  | Renal OCT2 Substrate | No | Categorical (Yes/No) | Yes/No |
| Toxicity         | Ames toxicity | No | Categorical (Yes/No) | Yes/No |
|                  | Pred hERG | No | Categorical (Yes/No) | Yes/No |
|                  | Hepatotoxicity | No | Categorical (Yes/No) | Yes/No |
|                  | Pred-skin | Yes | Categorical (Yes/No) | Yes/No |

OCT2: Organic cation transporter 2, hERG: Human ether-a-go-go-related gene, Pred-skin: Skin sensitization prediction.
uptake transporter responsible for renal clearance of the drugs [110].

The ability of the compound to binding to this protein is an indication of its clearance which is an important aspect to determine the dosing rate to achieve a steady-state on plasma [111]. According to the results, NDIBP is unable to interact and bind to this transporter and may not be excreted by this way.

Drug toxicity is the most important causes of impairment of the process of drug discovery and development [112]. Due to this, NDIBP-induced toxicity was also evaluated by pCKSM (Table 6). The genetic toxicity screening to identify NDIBP potential to be mutagenic or non-mutagenic was assessed by the AMES mutagenic test. Compounds that present a positive result in AMES test may cause mutagenicity [25,113].

According to the analysis, NDIBP showed a negative result and probably is not a mutagenic compound and therefore may not act as a carcinogen. The cardiototoxicity of NDIBP was evaluated by testing whether this organic nitrate could be a hERG I and II inhibitor; hERG channels play an important role in the cardiac repolarization [114-116] and the hERG current inhibition is the most likely mechanism involved in the drug-induced QT interval prolongation and severe cardiac arrhythmias being an important reason of drug failure in preclinical studies [25,117].

NDIBP did not show any positive results for inhibition of neither hERG I nor hERG II, reflecting the cardioprotective nature of this organic nitrate.

Another important concern in drug development is hepatotoxicity, one of the main reasons to remove medications post-market [118]. Drug-induced liver injury can lead to acute liver failure and even death [119,120]. In silico analysis showed that NDIBP was not able to cause disruption in the normal liver function and therefore, may not be considered as a hepatotoxic compound (Table 6). However, NDIBP developed a predicted skin sensitization that was also showed in PASS analysis. Skin sensitization is a potential adverse effect for drugs that are applied dermally [25]. The NDIBP information about its skin permeability in addition to skin sensitization corroborated the idea that the topical administration of this organic nitrate could be an inadequate way to obtain the pharmacological effect due to its poor absorption at the skin and the development of this undesirable adverse effect.

CONCLUSION

In summary, our study described NDIBP as a drug candidate with vasorelaxant activity. In silico analysis provided relevant data about its biological activities and PK aspects. PASS software was successfully applied to predict the organic nitrate biological spectra directing the study to the most correct experimental protocol to test the pharmacological effect related to cardiovascular aspects, avoiding waste of time and cost of chemicals. This study emphasizes that vasodilator effect was not only predicted but also validated in ex vivo experiments that allowed to propose the NDIBP as a potential vasorelaxant that could be investigated in the treatment of hypertension. Furthermore, pCKSM analysis suggested that NDIBP possess good oral absorption and bioavailability, suggesting that this organic nitrate can be a promising hit. All these computational data qualify the NDIBP for further in vitro and in vivo studies to understand the PK aspects more deeply and to uncover the therapeutic importance of NDIBP as an effective agent to treat AH.

SUPPLEMENTARY MATERIALS

Additional figures illustrating the FTIR spectroscopy and 1H and 13C NMR spectra of NDIBP. Fig. 51: FTIR (ATR) spectrum of NDIBP; Fig. 52: 1H NMR spectrum (400 MHz, CDCl3) of NDIBP; Fig. 53: 13C NMR spectrum (50 MHz, CDCl3) of NDIBP.

AUTHOR CONTRIBUTIONS

Valdir Braga was responsible for the design of the study, funding, and supervision; Airla Cavalcanti and Patrícia Rocha contributed with methodology, formal analysis, writing—original draft preparation; Isadona Luna carried out the in silico methodology; Maria Cláudia Brandão and Emmely Trindade performed the synthesis of the compound; Geovani Pereira contributed with the writing—review and editing; Petrônio Athayde-Filho, Eugene Muratov, Barkat Khan, and Marcus Scotti provided to the study the access to crucial research components (reagents, equipment, and computational software), expertise, and feedback. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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