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Research Article

SYNTHESIS, *IN SILICO* CHARACTERIZATION AND *EX VIVO* EVALUATION OF THE NOVEL ORGANIC NITRATE NDIBP AS A POTENTIAL VASORELAXANT AGENT

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ABSTRACT

Objective: This study aimed to describe the synthesis and biological/pharmacokinetic potential of the 1,3-diisobutoxypropan-2-yl nitrate (NDIBP) using *in silico* and *ex vivo* approaches.

Methods: The compound was characterized by Fourier-transform infrared spectroscopy and 1H and 13C- nuclear magnetic resonance spectra. NDIBP biological activity spectrum was obtained by Prediction of Activity Spectra for Substances (PASS). The pharmacological effect was validated in *ex vivo* studies using mesenteric artery. Drug-like properties and Absorption Distribution Metabolism Excretion and Toxicity (ADMET) studies were carried out by pkCSM (Predicting Small-Molecule Pharmacokinetic Properties Using Graph-Based Signatures) software.

Results: PASS prediction indicated NDIBP as nitric oxide (NO) donor with vasodilator effect. *Ex vivo* studies validated PASS analysis and showed the NDIBP vasorelaxant activity in mesenteric arteries. Physicochemical parameters and ADMET prediction suggested that NDIBP is a drug-like molecule with a good theoretical oral bioavailability, good absorption in the gastrointestinal tract, and a low distribution in the tissues.

Conclusion: All the data indicated that NDIBP possesses biological activities and drug-like properties to be considered as a vasorelaxant agent and a good candidate for further investigation in the treatment of arterial hypertension and drug development studies.

Keywords: Organic nitrate, Prediction of Activity Spectra for Substances, ADME, Hypertension, Mesenteric artery.

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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 synthetized 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 aiming 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 a 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 HNO_3 (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); ^{1}H NMR (200 MHz, CDCl3) δ 4.02 (p, 1H), 3.64 (d, 4H), 2.65 (s, 1H); ^{13}C 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), 2951, 2867, 1466 (C–H), 1109 (C–O); ^{1}H NMR (200 MHz, CDCl3) δ 4.03 – 3.83 (p, 1H), 3.52 – 3.38 (d, 4H,), 3.21 (d, 4H), 2.59 (s, 1H, O-H), 1.85 (m, 2H), 0.88 (d, 12H); ^{13}C 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 2956, 2872, 1469 (C–H), 1633, 1274, 850 (N–O), 1107 (C–O). ^{1}H 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

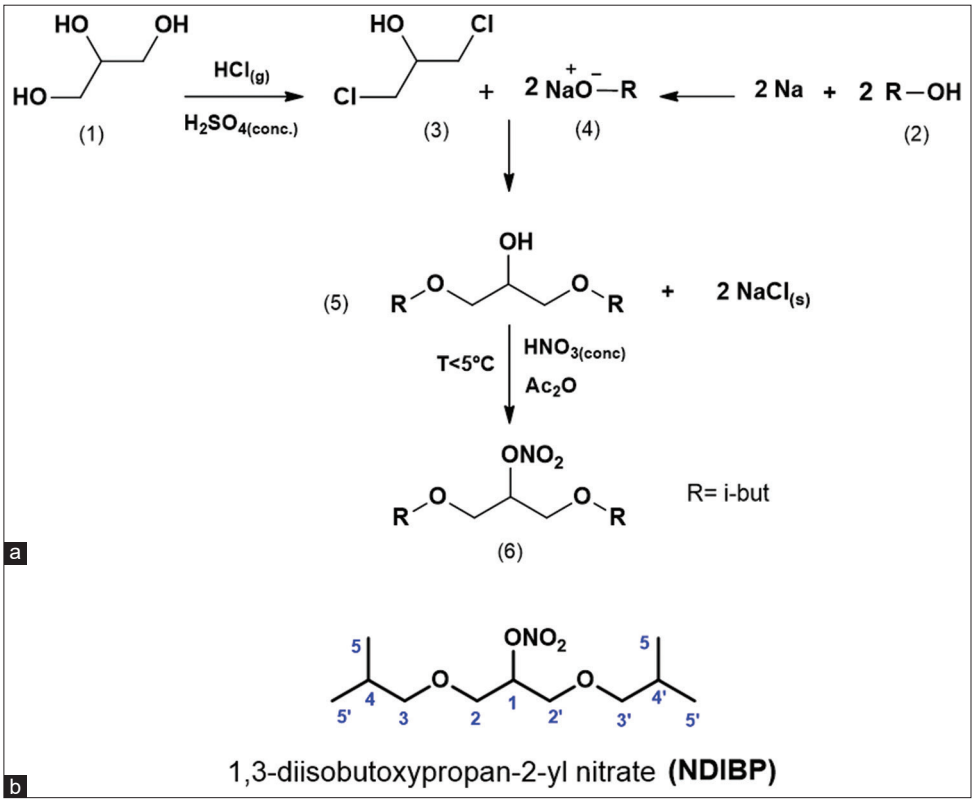

Fig. 1: Synthetic route and structural formulae for 1,3-diisobutoxypropan-2-yl nitrate. (a) Shows the synthetic reaction used to obtain NDIBP. The reaction yield was 85,7%. (1) Dry glycerin; (2) corresponding alcohol; (3) 1,3-dichloropropan-2-ol; (4) sodium alkoxide; (5) corresponding oxyalcohol; (6) organic nitrate. (b) The structural formulae for NDIBPs

(tt, J = 13.4, 6.9 Hz, 2H, H-4, H-4'), 0.90 (d, J = 6.7 Hz, 12H, H-5, H-5'). ^{13}C 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-diisobutoxypropan-2-yl nitrate) 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 activity 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 reflected 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 Δ-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% O_2 and 5% CO_2) 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 Paraíba 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 donors (nHBD), and rotable 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 RO5) [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 (logBB) and the volume of distribution at steady state (VDss). Metabolism property was evaluated based on the CYP models for substrate or inhibition (CYP1A2, CYP2C19, 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 I 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/HNO_3) 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 kinetic 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(hexyloxy) propan-2-yl (NDHP) confirming that this synthetic route is suitable for obtaining organic nitrates.

Regarding the chemical characteristics, NDIBP (Fig. 1b) presented $C_{11}H_{23}NO_5$ as molecular formula, molar mass value of 249.16 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 ^{1}H and ^{13}C 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 (NDBP), 1,3-bis (hexyloxy) propan-2-yl nitrate (NDHP), and 2-nitrate-1,3-di(octanoxy)propane [12,51,52] corroborating the idea that the use of low cost renewable natural sources, such as the glycerin, can be a promising alternative to obtain new substances to treat cardiovascular diseases.

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, antianginal, and vasodilator effect (0.973, 0.969, and 0.967, respectively). All these effects are in accordance with the effects 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 ventricular systolic wall tension; (iii) reduction in myocardial O_2 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, spasmolytic, 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.

To explain the pharmacological effects, 1333 of 4255 possible mechanisms of action with Pa > Pi were predicted, but according to our cutoff value, only 18 of 1333 were considered. The highest Pa values were associated with vasodilator, analgesic, and NO donor (Pa = 0.967, 0.944, and 0.923, respectively) activities (Table 2). In this study, we confirmed the NDIBP's ability to serve as NO donor, which is the main mechanism of action developed by the already known organic nitrates [2]. Endogenous NO is considered the most important biological signaling molecule involved in regulation of several cardiovascular functions, including the control of vascular tone, which directly influences in the systemic vascular resistance and the modulation of blood pressure [55-57]. Organic nitrates are used when the endothelium-dependent vasorelaxation is impaired acting as prodrugs able to release the free radical NO from its structure by enzymatic or nonenzymatic pathways [58]. Once released, it stimulates the soluble guanylyl cyclase (sGC) presented in the vascular smooth muscle cells (VSMCs) to induce the formation of cyclic guanosine monophosphate (cGMP). The increase in cGMP levels activates the protein kinase G (PKG), which promotes the reuptake of cytosolic calcium by sarcoplasmic-endoplasmic reticulum calcium pump (SERCA), expulsion of calcium out of the cell by membrane pumps and sodium/calcium exchanger, inhibition of voltage-dependent calcium channels (Ca_V) and opening of calcium-dependent potassium channels (BK_{Ca}). All of these events induce the reduction of intracellular calcium which impairs the myosin light chain kinase (MLCK) to phosphorylate the regulatory myosin light chain (rMLC) avoiding the contraction, resulting in VSMC relaxation and consequently the modulation of vascular resistance [2,54,59-61]. Our PASS analysis indicated NDIBP as a NO donor; therefore, it may be able to release NO from its structure and act in the vessels developing the vasodilator mechanism mentioned before.

Other important predicted mechanisms were spasmolytic, cGMP phosphodiesterase (PDE) inhibitor and platelet aggregation inhibitor (Pa=0.871, 0.850, and 0.846, respectively) (Table 2). We highlight the PDE inhibitor mechanism as another way to help in the predicted vasodilator effect induced by NDIBP. PDEs are a superfamily of enzymes that hydrolyze and inactivate the second messengers 3',5'-cyclic adenosine monophosphate (cAMP) and cGMP [62]. In VSMCs, the cGMP levels are controlled by the activity of PDE1 but mainly by PDE5 [63]. Normally, the increase in cGMP induced by NO can active the PDE5

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	0.967	0.001	Respiratory analeptic	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-opioid	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	Spasmolytic, urinary	0.761	0.004
Cardiotonic	0.874	0.004	Antiarthritic	0.751	0.009
Myocardial ischemia treatment	0.870	0.003	Analeptic	0.740	0.007
Spasmolytic	0.871	0.004	Anesthetic	0.729	0.004
Rheumatoid arthritis treatment	0.864	0.003	Vasoprotector	0.719	0.006
Antiischemic	0.863	0.004	Vasodilator, peripheral	0.708	0.007
Platelet aggregation inhibitor	0.846	0.004	Neurodegenerative treatment	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	Predicted mechanism of action	Pa	Pi
Vasodilator	0.967	0.001	Acrocylindropepsin inhibitor	0.852	0.007
Analgesic	0.944	0.004	Chymosin inhibitor	0.852	0.007
Nitric oxide donor	0.923	0.000	Platelet aggregation inhibitor	0.846	0.004
APOA1 expression enhancer	0.915	0.003	Cutinase inhibitor	0.820	0.004
Vasodilator, coronary	0.914	0.003	Miotic	0.774	0.004
Cardiotonic	0.874	0.004	Angiogenesis stimulant	0.767	0.003
Spasmolytic	0.871	0.004	Polyporopepsin inhibitor	0.777	0.015
Cyclic GMP PDE inhibitor	0.850	0.001	Fragilysin inhibitor	0.734	0.010
Saccharopepsin inhibitor	0.852	0.007	Vasodilator, peripheral	0.708	0.007

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

which initiates a negative feedback mechanism to limit the action of cGMP in the vasodilatory cascade [64-66]. In AH, the vascular changes can be closely linked to the increased in humoral factors, such as angiotensin II [67] and it has been reported that this peptide can raise PDE5A protein expression/activity in VSMCs [68].

The use of PDE inhibitors promotes an increase in the vascular signaling of NO/cGMP pathway giving support to a higher vasodilator effect [69,70]. Several studies demonstrated that inhibition of PDE5 increase endothelial function, improve baroreflex sensitivity, and decrease blood pressure in experimental models of hypertension [71-74]. In this study, NDIBP was also appointed as a PDE inhibitor suggesting that this drug can potentiate the vasodilatory cascade induced by the NO release through preventing the degradation of cGMP by PDE5. Our organic nitrate has the combination of these two mechanisms in one molecule and can represent an interesting alternative to treat hypertension due to the synergistic effects, which may enhance the hypotensive effects.

PASS software was also able to predict whether NDIBP can induce gene expression regulation. Thus, 65 of 96 possible gene expression regulation with Pa > Pi were detected, but only 3 of 65 can be considered (Pa > 0.7). NDIBP may be an apolipoprotein A1 (APOA1) and HMOX1 (Heme Oxygenase 1) expression enhancer (Pa = 0.915 and 0.755) and also a CASP3 (Caspase 3) expression inhibitor (Pa = 0.752) (Table 3).

Regarding cardiovascular diseases, the most interesting is the APOA1 expression induced by NDIBP. APOA1 is the main protein component in high-density lipoprotein (HDL) particles acting as a mediator in the transfer of cholesterol from cells to HDL, an important process for the reverse transport of cholesterol to the liver [75,76]. Thus, APOA1 is considered as atheroprotective and the risk to develop cardiovascular disease is inversely proportional to serum levels of HDL and APOA1 [75,77]. Lower levels in HDL due to APOA1 deficiency can reduce the atheroprotective potential and facilitate the development of atherosclerosis and endothelial dysfunction, important risk factors for hypertension [78]. Under our experimental conditions, NDIBP showed a predictive ability to enhance the APOA1 expression suggesting that this drug can also help to maintain the cardiovascular health by reducing the plasmatic cholesterol levels and preventing oxidation and aggregation of low-density lipoprotein particles in the vessel wall.

The last aspect of NDIBP biological spectra was related to toxic and adverse effects and was predicted 54 of 64 possibilities. Among those, only 27 were over Pa value cutoff (Table 4) and the most important actions involved skin and eyes irritative effects and hypotension. Other toxicity studies were done in the ADMET analysis using the pkCSM software. Therefore, NDIBP showed predicted pharmacological effects and mechanisms of action that support the investigation of its action on the cardiovascular function.

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 statistic 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 NDBP 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 [21,25]. Thus, the PK profile is crucial for the drug effectiveness. To understand this profile, NDIBP physicochemical properties of TPSA, LogP, nHBA, nHBD, and ROTB 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	0.962	0.002	Neurotoxic	0.855	0.010
Skin irritation, weak	0.946	0.002	Carcinogenic, rat, female	0.844	0.003
Eye irritation, weak	0.944	0.002	Carcinogenic, rat	0.842	0.004
Hypotension	0.927	0.007	Cardiotoxic	0.840	0.015
Skin irritation, high	0.921	0.003	Dermatitis	0.836	0.014
Irritation	0.904	0.005	Allergic reaction	0.810	0.016
Ocular toxicity	0.894	0.008	Carcinogenic	0.802	0.010
Nephrotoxic	0.889	0.008	Eye irritation, high	0.793	0.005
Hepatotoxic	0.881	0.012	Embryotoxic	0.786	0.012
Carcinogenic, rat, male	0.866	0.003	Teratogen	0.771	0.013
Anemia	0.870	0.008	Toxic	0.782	0.026
Endocrine disruptor	0.869	0.008	Mutagenic	0.749	0.009
Hematotoxic	0.870	0.014	Anaphylaxis	0.745	0.016
Reproductive dysfunction	0.865	0.013			

Pa: Probability of active, Pi: Probability of inactive

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

Compound	Physicochemical properties						
	MW (g/mol)	TPSA (Å^2)	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	6	1	1	0
ISDN	236.14	129	1.3	8	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 rotable bonds, RO5: Rule of five

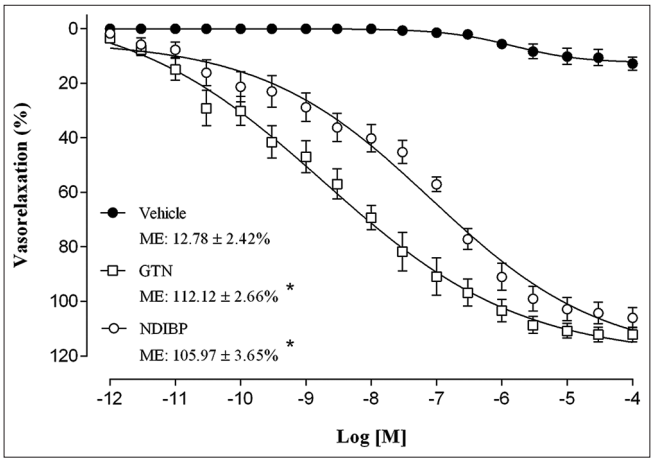

Fig. 2: Vasorelaxation effect of NDIBP, GTN, or vehicle. The panel shows the NDIBP, GTN, or vehicle cumulative concentration-response curves (10^{-12}–10^{-4} M) on mesenteric arteries isolated from rats (n=6) pre-contracted with phenylephrine (1 μM). Log [M] corresponds to the different concentrations of vehicle, NDIBP and GTN respectively. *p<0.05 statistically different in relation to the maximum effect of vehicle control. One-way analysis of variance followed by Tukey test

is, permeability, revealing aspects of membrane permeation rate, drug absorption, and bioavailability of the compound [82-88].

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 ROTB (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] (Table 5). 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 has 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 ADMET 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 (GTI) [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 VDss and BBB permeability (Table 6). VDss 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, VDss is considered low when it is <0.71 L/kg and high when >2.81 L/kg [25]. VDss 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 metabolization 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

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 L/kg)	Low: < 0.71; High: > 2.81
	Blood brain barrier permeability	-0.71	Numeric (logBB)	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 pkCSM (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 cardiotoxicity 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, pkCSM 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. S1: FTIR (ATR) spectrum of NDIBP; Fig. S2: ^{1}H NMR spectrum (400 MHZ, $CDCl_3$) of NDIBP; Fig. 3: ^{13}C NMR spectrum (50 MHZ, $CDCl_3$) of NDIBP.

AUTHOR CONTRIBUTIONS

Valdir Braga was responsible for the design of the study, funding, and supervision; Airlla Cavalcanti and Patrícia Rocha contributed with methodology, formal analysis, writing—original draft preparation; Isadora 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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