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ORIGINAL RESEARCH

Antiplatelet activity and TNF-α release inhibition of phthalimide derivatives useful to treat sickle cell anemia

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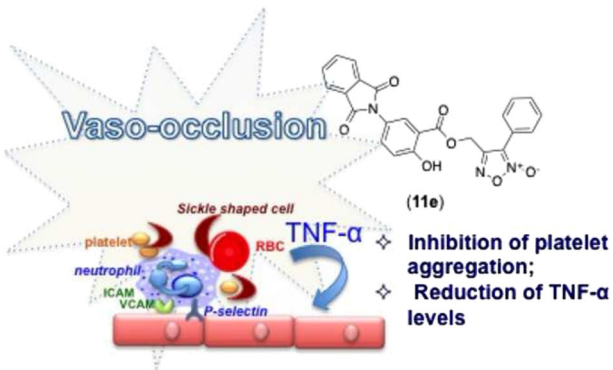
Abstract

Sickle Cell Anemia (SCA) is one of the most prevalent hereditary hematological diseases worldwide. The disease is characterized by chronic inflammation, hypercoagulable state, and pro-thrombotic profile, which lead the vaso-occlusive process. In this work, we described the antiplatelet activity and the ability to reduce tumor necrosis factor-alpha (TNF-α) levels of phthalimide derivatives. All compounds inhibited platelet aggregation induced by collagen and adenosine diphosphate, at levels ranging from 26.0 to 74.2% and 30.7 to 79.6%, respectively. The compounds exhibited reduced bleeding time compared to acetylsalicylic acid (ASA). Moreover, compounds **4c** and **10c** inhibited TNF-α levels at 73.5% and 65.0%, respectively. These findings suggest that phthalimide derivatives **4c** and **10c** are promising lead compounds useful to prevent vaso-occlusion and inflammation associated with the sickle cell anemia.

Graphical Abstract



Keywords Sickle cell disease · Vaso-occlusion · Phthalimide · Platelet aggregation inhibition · TNF-α inhibition.

Introduction

Sickle Cell Anemia (SCA) is a chronic hereditary hematological disease caused by a single mutation in the HBB gene, which encodes hemoglobin subunit beta. This mutation causes the replacement of glutamic acid (Glu) to valine (Val) on the surface of the variant β-globin chain (βs-globin) of hemoglobin S (HbS) (Ingram 1957). Interactions among β_s-globin chains, at low oxygen tensions, lead to polymers formation inside HbS and alter the red blood cell

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(RBC) cytoskeletal structure to sickle-shaped cells (Kato et al. 2018). RBC containing HbS are prone to suffer hemolysis, releasing hemoglobin and arginase into the plasma. Arginase reduces the levels of arginine, a substrate for endothelial nitric oxide synthase, and decreases the levels of nitric oxide (NO). In addition, the released hemoglobin kidnaps NO produced by endothelium conferring a vaso-constrictor character to SCA patients. The reduced bioavailability of NO also results in activation of endothelial cells (increasing the expression of adhesion molecules) and platelets (increasing the expression of P-selectin and αIIbβ3 integrin; Kato et al. 2017).

Platelets have an important role in the vaso-occlusion process of SCA (Davila et al. 2015). Several markers of activated platelet are increased such as β-thromboglobulin, thrombospondin and chemokine (C-X-C motif) ligand 4 (Davila et al. 2015; Sugiharam et al. 1992). Platelets are also found in circulation as heterocellular aggregates and contribute to the innate immune system through cytokines releasing (Davila et al. 2015; Zhang et al. 2016). Therapies containing antiplatelet drugs should be an alternative to prevent cardiovascular diseases in SCA patients, once these patients exhibit abnormal activation of the fibrinolytic system, increased tissue factor expression and high risks of thrombotic complications (Noubouossie et al. 2016). However, despite the efficacy of antiplatelet therapies to reduce platelet aggregation in SCA patients, clinical trials have failed to demonstrate the ability of these drugs to prevent vaso-occlusion complications (Heeney et al. 2016). This lack of effectiveness may be result from the complexity of the disease, which involves other phenomenons such as ischemia-reperfusion injury, chronic inflammation, hemolysis, vaso-occlusion and NO deficiency (Noubouossie et al. 2016).

Therefore, compounds designed to act through multiple targets are promising strategies to find out an alternative to hydroxyurea and glutamine – the only approved drugs to treat SCA. Our research group has previously described that compound 3-(1,3-dioxoisoindolin-2-yl)benzyl nitrate (**4c**) exhibits multiple effects such as in vivo analgesic/anti-inflammatory activities and NO-donor properties. Transgenic sickle mice treated with this compound for three weeks have reduced priapism, reversed PDE5 protein expression and reduced reactive-oxygen species markers in the penises of SCA mice (Silva et al. 2016). In addition, compound (**4c**) increased ~53% the fetal hemoglobin (HbF) levels when compared to the vehicle, using a transgenic SCA mouse model. This effect was superior to that of hydroxyurea in the same assay (Lanaro et al. 2017).

In this study, we described the antiplatelet activity and tumor necrosis factor-alpha (TNF-α) release from a culture of mice monocytes of several phthalimide derivatives containing a NO-donor subunit. Two different NO-donors represented by organic nitrate esters and furoxan (1,2,5-oxadiazole-2-*N*-oxide) were selected. In addition to antiplatelet and vasodilatory effects, NO also contributes to increase the levels of HbF. It has been reported that NO/cGMP pathway plays an important role in γ-globin expression by activating transcription factors, including c-Jun and c-Fos (Perrine et al. 2014).

The phthalimide subunit, presented in the drug thalidomide, was selected due to its ability to reduce the levels of TNF-α in culture of monocytes. The structure-activity relationship shows that phthalimide subunit is the primary pharmacophore responsible for reducing TNF-α levels, being, therefore, selected in our studies (Miyachi et al. 1997; Muller et al. 1999). It was demonstrated that SCA patients exhibit high levels of TNF-α in circulation aggravating painful vaso-occlusive crisis and inflammatory episodes (Dworkis et al. 2011). Beyond the anti-inflammatory effect, thalidomide also increases γ-globin mRNA expression through activation of p38 mitogen-activated protein kinase (MAPK) signaling pathway along with histone H4 hyperacetylation (Aerbajinai et al. 2007).

Material and methods

General synthesis information

Reagents and solvents were purchased from commercial suppliers at reagent purity grade and were used without any further purification. Dry solvents used in the reactions were obtained by distillation of technical grade materials over appropriate dehydrating agents. Melting points were measured with an electrothermal melting-point apparatus Stuart™ melting point apparatus SMP3 (Bibby Stuart Scientific, Cole-Parmer, Staffordshire, UK) in open capillary tubes and are uncorrected. Infrared spectra (range of 400–4000 cm^{-1}) were obtained using KBr pellets in the FTIR-8300 infrared spectrophotometer (Shimadzu Seisakusho Co. Ltd., Kyoto, Japan). One-dimensional nuclear magnetic resonance spectra of ^{1}H and ^{13}C were obtained using the Fourier 300 spectrometer with Dual probe $^{1}H/^{13}C$ (Bruker Corporation, Billerica, Massachusetts, EUA). Chemical shifts are reported in parts per million (ppm - δ) relative to tetramethylsilane, and the coupling constants (*J*) are shown in Hertz (Hz). HPLC analysis was performed on an LC-10AD chromatograph equipped with a model SPD-10A UV–Vis detector (Shimadzu Seisakusho Co., Ltd., Kyoto, Japan). All compounds were analyzed by HPLC, and their purity was confirmed to be >98.5%. The mass spectra were obtained using an LCMS-8045 spectrometer (Shimadzu Corporation, Kyoto, Japan), with triple quadrupole detector and electrospray ionization (ESI). Compounds were separated on a reversed phase C18 column (5 µm

particle, 250 mm × 4.6 mm I.D.) Shim-pack CLC-ODS(M)® C18 (M). HPLC-grade solvents (acetonitrile, methanol, acetic acid, and toluene) were used in the analyses. Thin layer chromatography (TLC) analyses were performed on TLC Silica gel 60 FSigma®, to monitor the reactions and purifications of the synthesized compounds. Substances were visualized in ultraviolet light (254 and 365 nm), and/or by exposure to powdered iodine and/or functional group developer when necessary. Merck silica gel (70–230 mesh) was used for preparative column chromatography. The final compounds **4a-e**, **6a-b**, and **10a-c** and the intermediate compounds **3a-e**, **7** and **9a-c** were previously obtained in our laboratory (Dos Santos et al. 2011; Dos Santos et al. 2012) however, the synthetic methodology adopted in this work to obtain **6b** was altered and is shown below. Compound **8** is unprecedented and its synthetic procedure and structural characterization are demonstrated in this work and in the supplemental material.

Synthetic procedures

General procedure for the synthesis of 4-(2-(1,3-dioxoisoindolin-2-yl) ethoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (**6b**)

The synthesis of compound (**6b**) involves two steps. First, it was synthesized the furoxan derivative 3-(phenylsulfonyl)-4-(2-(tosyloxy)ethoxy)-1,2,5-oxadiazole 2-*N*-oxide. A mixture of 4-(2-hydroxyethoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-*N*-oxide (1.5 mmol) and triethylamine (4.5 mmol) was added in dry dichloromethane (10.0 mL) at 0 °C. Then, using addition funnel, *p*-toluenesulfonyl chloride (2.5 mmol) previously solubilized in dry dichloromethane (10.0 mL) was added slowly to the medium. The reaction was stirring at room temperature under nitrogen atmosphere for 3 h. The solvent was dried under reduced pressure to provide a crude, which was purified by column chromatography (5.0 cm diameter column; stationary phase silica: 60 Å pore size; 40–60 µm particle size; mobile phase: hexane: ethyl acetate (80:20 → 60:40, v/v) in gradient mode).

In the second step for the synthesis of compound (**6b**), a mixture of 3-(phenylsulfonyl)-4-(2-(tosyloxy)ethoxy)-1,2,5-oxadiazole 2-*N*-oxide (7.5 mmol) and potassium phthalimide (**5**) (7.5 mmol) was added in *N*,*N*-dimethylformamide (DMF) (15.0 mL). The reaction was stirring at room temperature under nitrogen atmosphere for 24 h. After, ice-cold water (10.0 mL) was added to precipitate the product, which was filtrated and washed with distilled water.

3-(phenylsulfonyl)-4-(2-(tosyloxy)ethoxy)-1,2,5-oxadiazole 2-*N*-oxide: white powder, yield 78%; mp 117-120 °C. ^{1}H-NMR (300 MHz, $CDCl_3$), 7.77-7.75 (1 H; m; Ar-H); 7.83-7.80 (2 H; m; Ar-H); 8.08-8.05 (2 H; m; Ar-H); 4.62-4.59 (2 H; m; O-CH_2-CH_2-O); 4.43-4.40 (2 H; m; O-CH_2-CH_2-O); 7.67-7.62 (2 H; m; Ar-H); 7.36 (2 H; d; J_{ortho} = 8,0 Hz; Ar-H); 2.45 (3 H; s; Ar-CH_3).

4-(2-(1,3-dioxoisoindolin-2-yl) ethoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-*N*-oxide (**6b**): white powder, yield 73%; mp 150-155 °C. IR Vmax (cm^{-1}; KBr pellets): 1780.3 and 1718.5 (C = O imide); 1620.2 and 1550.7 (C = N); 1363.6 and 1168.8 (O = S = O sulfone). ^{1}H-NMR (300 MHz, $CDCl_3$), 7.78-7.76 (2 H; m; Ar-H); δ 7.91-7.89 (2 H; m; Ar-H); 4.20 (2 H; t; N-CH_2-CH_2-O); 4.69 (2 H; t; N-CH_2-CH_2-O); 8.04-8.01 (2 H; m; Ar-H); 7.60-7.54 (2 H; m; Ar-H); 7.74-7.69 (1 H; m; Ar-H). ^{13}C-NMR (75 MHz, $CDCl_3$), 134.4 (CH, C-23, C-24); 123.7 (CH, C-22, C-25); 132.0 (C, C-21, C-26); 168.0 (C = O, C-20, C-18); 36.2 (N-CH_2-CH_2-O, C-17); 68.0 (N-CH_2-CH_2-O, C-16); 158.6 (C, C-4); 110.5 (C, C-3); 138.1 (SO_2-C, C-9); 128.80 (CH, C-10, C-14); 129.7 (CH, C-11, C-13); 135.6 (CH, C-14). MS-ESI (*m/z*): $C_{18}H_{13}N_3O_7S$ $[M + H]^+$ 415.55.

General procedure for the synthesis of 4-(3-((4-(1,3-dioxoisoindolin-2-yl)benzoyl)oxy) propoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-*N*-oxide (8)

N-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) (2.7 mmol) and 4-dimethylaminopyridine (DMAP) (0.2 mmol) was added in a solution containing 4-(1,3-dioxoisoindolin-2-yl) benzoic acid (**7**) (1.5 mmol) in dichloromethane (15.0 mL) at 0 °C. Then, it was slowly added the intermediate 4-(3-hydroxypropoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-*N*-oxide (**11**) (1.5 mmol) (Cena et al. 2001). The reaction was stirring at room temperature under nitrogen atmosphere for 4 h. The crude was diluted with dichloromethane (50.0 mL) and it was washed using brine and water (3 × 15.0 mL). The organic phase was dried with sodium sulfate and dried under reduced pressure. The crude was purified by column chromatography using silica gel (60 Å pore size; 40–60 µm particle size) and mobile phase hexane: ethyl acetate (60:40; v/v) in isocratic mode.

4-(3-((4-(1,3-dioxoisoindolin-2-yl)benzoyl)oxy)propoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-*N*-oxide (**8**): White powder, yield 41%; mp 176-180 °C. IR Vmax (cm^{-1}; KBr pellets): 1.716 (C = O imide); 1.650 (C = O ester); 1.384 (O = S = O sulfone). ^{1}H-NMR (300 MHz, $CDCl_3$); 7.84-7.81 (m; 2 H; Ar-H); 7.99-7.96 (m; 2 H; Ar-H); 7.65-7.60 (m; 4 H; Ar-H); 8.18 (dt; J_{ortho} = 8.8 Hz/J_{meta} = 2.2 Hz; 2 H; Ar-H); 4.61 (t; 2 H; CH_2); 2.38 (q; 2 H; CH_2); 4.55 (t; 2 H; CH_2); 8.08-8.05 (m; 2 H; Ar-H); 7.75 (tt; J_{ortho} = 7.5 Hz/J_{meta} = 1.3 Hz; 1 H; Ar-H). ^{13}C-NMR (75 MHz, CDCl3), 134.8 (CH, C-33, C-34); 124.1 (CH, C-32, C-35); 131.6 (C, C-31, C-36); 166.9 (C = O, C-28, C-30); 136.3 (N-C, C-24); 126.2 (CH, C-23, C-25); 130.6 (CH, C-22, C-26); 129.0 (C-C = O, C-21); 165.6 (C = O, C-20); 61.1 (O-CH_2-CH_2-CH_2-O, C-18); 28.2 (O-CH_2-CH_2-CH_2-O, C-17); 68.1 (O-CH_2-CH_2-CH_2-O, C-16); 159.0 (C, C-4); 110.6 (C, C-3); 138.1 (SO_2-C, C-9); 128.6 (CH, C-10, C-14); 129.8

(CH, C-11, C-13); 135.8 (CH, C-14). MS-ESI (*m/z*): $C_{26}H_{19}N_3O_9S$ $[M + H]^+$ 550.20.

Animals

Adult males of *Mus muscules* (Swiss albino), weighing ~25–30 g, were obtained from the central laboratory of São Paulo State University (UNESP). The animals were maintained under controlled conditions of temperature (22 ± 2 °C), humidity and light (dark cycle/light 12 h/12 h). The animals had access to food and water ad libitum. The experiment was previously approved by the Institutional Ethics Committee of the Faculty of Pharmaceutical Sciences (FCFAr) as described below in the section "compliance with ethical standards".

Campus Araraquara (CEUA / FCFAr) under No. 37/2013, 38/2013 and 09/2014. All experiments were performed in accordance with current guidelines for laboratory animal care and ethical guidelines for the investigation of experimental pain in conscious animals.

Antiplatelet activity assay

The antiplatelet activity assay was performed according to the method described previously (Born and Cross, 1963). Blood from mice was collected and added to 3.8% trisodium citrate (9:1 v/v). The platelet-rich plasma (PRP) was obtained by centrifugation of whole blood (200 × *g*) at room temperature for 15 min. Washing buffer (140.0 mM NaCl, 0.5 mM KCl, 12.0 mM trisodium citrate, 10.0 mM glucose and 12.5 mM sucrose, pH 6.0) in the ratio 7:5 (buffer/plasma) was added to PRP and centrifuged for 13 minutes (800 × *g*), twice. Platelets were suspended in Krebs-Ringer solution devoid of calcium ions and the platelet count was adjusted to 1.2 × 10-8 platelets/mL by manual counting using the Neubauer chamber. Calcium chloride ($CaCl_2$) was added to the platelet suspension to a final concentration of 1.0 mM.

The platelet solution (400.0 µL) was transferred to the aggregation cuvette and taken to the two-channel aggregometer (Chrono-log Lumi-Aggregometer model 560-Ca, Harvertown, PA, USA). Platelets were incubated in presence of compounds (**4a-e**; **6a-b**; **8** and **10a-c**) at concentration of 150 µM, dissolved in 0.1% DMSO, for 3 minutes prior to the addition of ADP (10.0 µM) or collagen (5.0 µg/mL). The aggregation was monitored for 10 minutes. Acetylsalicylic acid (ASA) at 150.0 µM was used as positive control.

Statistical analysis was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, California, USA), by analysis of variance (ANOVA) with the determination of significance levels as *p*-value <0.05 ($p < 0.05$) from Tukey's multiple comparisons test. The results were presented as mean ± standard error of the mean (SEM).

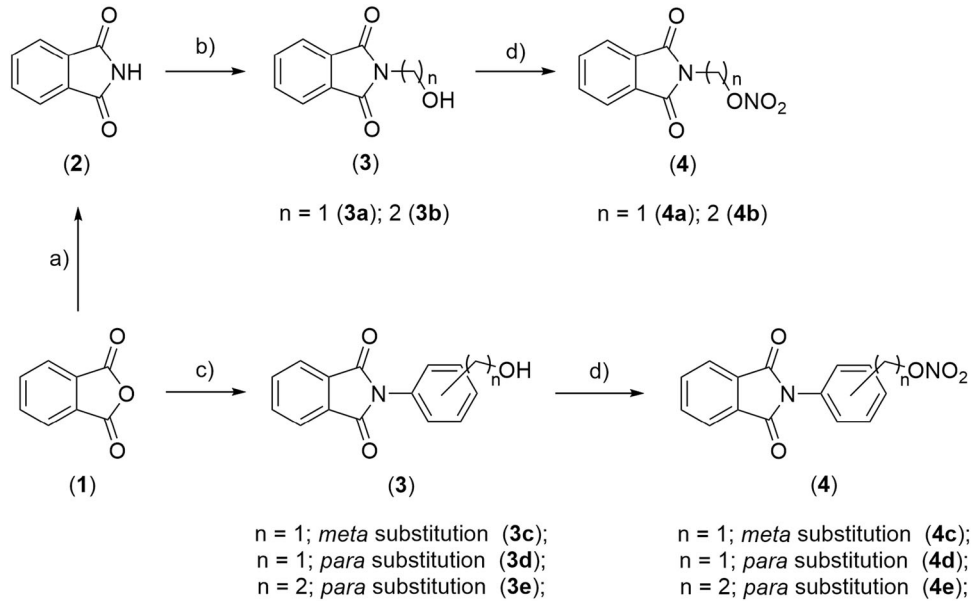
Bleeding time assay

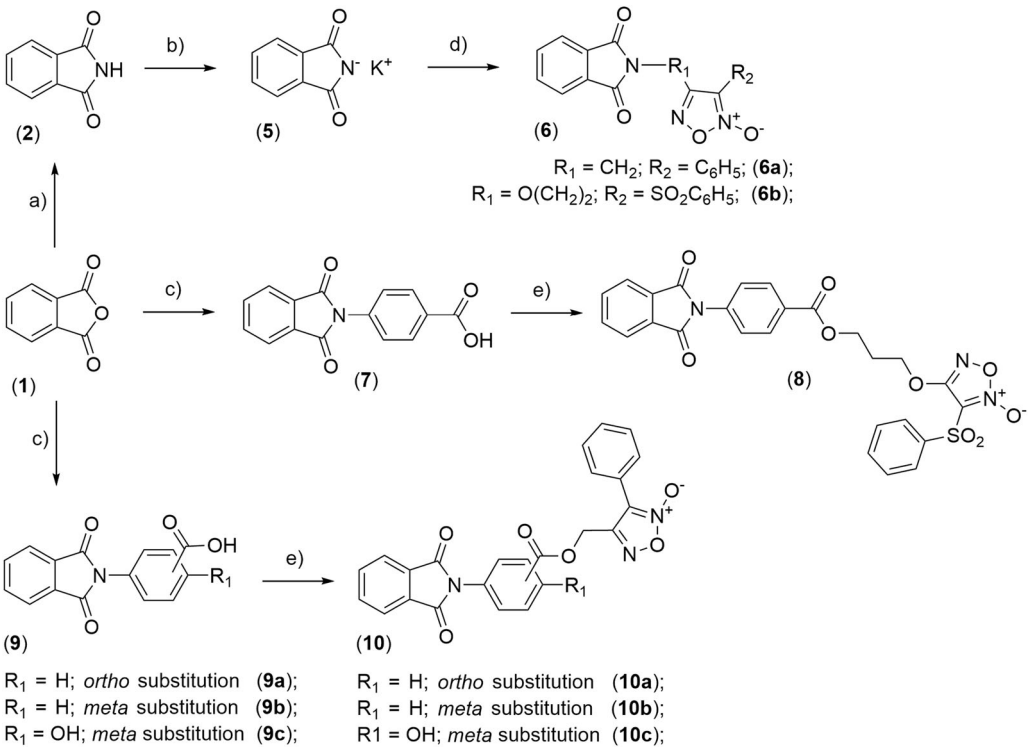
The bleeding time assay was performed as described previously (Dejana et al. 1979). *Mus musculus* mice (Swiss albino) ($n = 6$) received the compounds (**4a-e**; **6a-b**; **8** and **10a-c**), ASA (positive control) at concentration of 100 µM or carboxymethylcellulose (CMC) 0.5% (negative control) orally. After 60 min, the animals were anesthetized with ketamine hydrochloride (100 mg/Kg) by intraperitoneal (IP) route. An incision of 2.0 mm counted from the end of the tail was performed and the bleeding time registered from that moment. It was determinate 15 min as the maximum time if bleeding remains continuously. Statistical analysis was performed using the statistical program GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, California, USA) applying ANOVA with a significance level as a *p*-value <0.05 ($p < 0.05$), followed by Tukey's multiple comparisons test. The results were expressed as mean ± SEM.

TNF-α cytokine inhibition assay

The determination of cell viability and the detection of TNF-α of the compounds (**4a-e**; **6a-b**; **8** and **10a-c**) were performed using *Mus musculus* mice (Swiss albino). The animals were previously inoculated by IP route with 3.0 mL of a 3% sodium thioglycolate solution. After 72 h of stimulation, the animals were euthanized in a CO_2 chamber. The peritoneum was exposed in laminar flow for removal of the peritoneal exudate. Cells removed from the peritoneal exudate were washed three times with 5.0 mL of phosphate buffered saline (PBS) (pH 7.2; 4 °C) and centrifuged (400 × *g*/5 min) at room temperature. The pelleted cells were then suspended in complete RPMI-1640-C culture medium. The number of cells was determined using Neubauer's chamber, and trypan blue. The cell concentration was then diluted to 5×10^6 cell/mL. Cells were plated and incubated at 37 °C for 60 min in an atmosphere containing 5% of CO_2 for adherence of macrophages.

The cell viability was determinate by a colorimetric method in 96-well microplate using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Mosmann 1983). The cell and all compounds (**4a-e**; **6a-b**; **8** and **10a-c**) were incubated for 24 h at 37 °C in an atmosphere containing 5% CO_2, in presence of lipopolysaccharide LPS 1.0 µg/mL. After the contents of the plates were discarded and 100.0 µL of MTT solution (1.0 g/mL) was added to each well. The plate was incubated for 3 hours at 37 °C. After this time, the contents of the plate were discarded once again and 100.0 µL of isopropyl alcohol was added to





Scheme 1 The synthesis scheme of phthalimides containing organic nitrate ester subunit (**4a-e**)

Scheme 2 The synthesis scheme of phthalimides containing furoxan subunit (**6a-b**; **8** and **10a-c**)

each well. Absorbance reading was performed using UV/Vis Spectrophotometer BioTeck® plate reader with wavelength (λ) of 540 and 620 nm. The results of the absorbance values were converted into a percentage of cell viability, where the negative control corresponded to 100% of viability. For quantification of the TNF-α levels, concentrations with cell viability >70% were considered.

The levels of cytokine TNF-α were quantified in the culture supernatants by enzyme-linked immunosorbent assay ELISA (OptEIA; BD Biosciences, San Diego, CA) according to the manufacturer's instructions. The cytokine concentrations were calculated from a standard curve of known cytokine concentrations and expressed in pg/mL. In all of the experiments, the results were expressed as the

Table 1 Inhibition of platelet aggregation induced by collagen (5 μg/mL), ADP (10 μM) and bleeding time for phthalimide derivatives

Compounds	Inhibition of platelet aggregation (%)		Bleeding time (sec)
	Collagen (5 μg/mL)	ADP (10 μM)	
Negative control	27.2 ± 5.4[b]	26.9 ± 6.6[b]	369.0 ± 23.0[d]
ASA	64.4 ± 4.8[a]	26.6 ± 11.4	828.0 ± 100.3[c]
4a	39.5 ± 9.4	58.3 ± 7.3	448.2 ± 37.3[d]
4b	26.0 ± 6.4	41.3 ± 8.3	344.6 ± 24.0[d]
4c	58.5 ± 13.9	52.3 ± 5.9	276.0 ± 16.3[d]
4d	74.3 ± 12.6[a]	48.0 ± 7.1	–
4e	72.5 ± 14.8[a]	79.7 ± 3.3[ab]	258.0 ± 28.4[d]
6a	38.6 ± 7.8	58.2 ± 4.7[ab]	998.8 ± 183.4[c]
6b	48.5 ± 5.6	52.3 ± 6.9	288.5 ± 91.9[d]
8	61.6 ± 5.8	30.8 ± 5.6	276.0 ± 45.9[d]
10a	48.8 ± 1.3	40.4 ± 9.6	678,5 ± 56.8
10b	59.4 ± 6.1	47.5 ± 7.0	263.0 ± 40.2[d]
10c	45.8 ± 6.4	55.0 ± 8.0	398.6 ± 48.0[d]

Results are expressed as the mean ± SEM. All compounds were evaluated at 150 μM. The statistical analysis used was one-way ANOVA followed by Tukey's multiple comparisons test

[a] $p < 0.05$ phthalimide derivatives vs. Negative control group

[b] $p < 0.05$ phthalimide derivatives vs. ASA group

[c] $p < 0.05$ phthalimide derivatives vs. Negative control group (bleeding time)

[d] $p < 0.05$ phthalimide derivatives vs. ASA group (bleeding time)

mean ± SEM. Each experiment was performed in triplicate at least three times. One-way ANOVA with Tukey's Multiple Comparison Test as a post hoc test was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, California, USA). The statistical significance was defined as p-value <0.05.

Results and discussion

Synthesis

Compounds (**4a-e**) were prepared in three followed steps (Scheme 1). Intermediates (**3a-e**) were obtained by condensation of phthalic anhydride and phthalimide with functionalized amines in yields ranging from 93 to 65%. The last step, involving the nucleophilic substitution of alcohol function to organic nitrate esters (**4a-e**), in reaction medium containing acetic acid and nitric acid (99.5%), with yields of 90-74% (Dos Santos et al. 2011).

The furoxan derivatives (**10a-c**) and the synthetic intermediates (**7** and **9a-c**) were prepared according to procedures previously described (Cena et al. 2001; Dos Santos et al. 2012), compound (**8**) is novel and its synthetic procedure is described in the previous section. All furoxan derivatives were coupled with phthalimides (**7** and **9a-c**), using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and dimethylaminopiridine (DMAP) as coupling reactive, to obtain the final compounds (**8** and **10a-c**) at yields ranging from 39 to 55% (Scheme 2). The alkyl chloride or tosyl containing furoxan derivatives were prepared through a reaction involving with the respective alcohol (Cena et al. 2001; Dos Santos et al. 2012) with thionyl chloride or p-toluenesulfonyl chloride. After, compounds (**6a-b**) were obtained by nucleophilic substitution using potassium phthalimide (**2**), with yields of 73–91%, respectively (Scheme 2). The structures of all compounds were established by infrared spectroscopy, and ^{1}H and ^{13}C nuclear magnetic resonance (^{1}H and ^{13}C NMR). All compounds were analyzed by high-performance liquid chromatography and their purities were >98.5%.

Antiplatelet activity

The inhibition of platelet aggregation for all compounds was evaluated in vitro using platelet-rich plasma in the presence of ADP (10 μM) and collagen (5 μg/mL; Table 1) (Born and Cross, 1963). All compounds, tested at 150 μM, were able to inhibit the platelet aggregation induced by both of these agonists. For collagen-platelets stimulated, the percentage of inhibition ranged from 26.0 to 74.2%. The most active compounds found were **4d** and **4e**, which inhibited the aggregation platelet at 72.5 and 74.2%, respectively. For ADP-platelet stimulated, the percentage of inhibition ranged from 30.7 to 79.6%. The most active compound found was **4e**, which inhibited 79.6% of platelet aggregation induced by ADP. Despite the differences of NO-release levels among organic nitrate esters and furoxans (Dos Santos et al. 2011; Dos Santos et al. 2012), it was not found differences among these NO-donors to inhibit platelet aggregation induced by both agonists. Only compound **4e** demonstrated activity superior to acetylsalicylic acid (ASA) (Table 1), one of the most used antiplatelet drug in cardiovascular diseases.

Bleeding time

Bleeding is a serious adverse effect and one of the current limitations of antiplatelet therapy. Assays aiming to characterize the bleeding time are useful to evaluate platelet function and the ability of the body to form clots. In this work, we found for all compounds values of bleeding time ranging from 258.0–998.7 s. Except for compound **6a**, all derivatives demonstrated reduced bleeding time compared to ASA, which exhibits bleeding time of 828.0 s (Table 1).

Table 2 Reduction of TNF-α levels in murine monocytes cultures stimulated with LPS induced by phthalimide derivatives

Compounds	Reduction of TNF-α levels (%)						
	100.0 μM	50.0 μM	25.0 μM	12.5 μM	6.2 μM	3.1 μM	1.5 μM
Thalidomide	–	28.4 ± 2.3	21.1 ± 4.4	14.9 ± 3.7	–	–	–
4a	–	–	–	–	–	–	–
4b	7.0 ± 4.5	6.1 ± 3.1	14.0 ± 6.2	–	–	–	–
4c	73.5 ± 5.9[a]	65.2 ± 2.9[a]	38.3 ± 8.4	–	–	–	–
4d	21.2 ± 11.6	3.2 ± 1.6	7.6 ± 4.4	–	–	–	–
4e	0.3 ± 0.1	4.3 ± 2.5	6.6 ± 4.8	–	–	–	–
6a	–	–	–	–	33.1 ± 5.3	19.4 ± 5.1	14.0 ± 5.8
6b	19.6 ± 5.4	23.8 ± 7.1	13.8 ± 5.8	–	–	–	–
8	30.7 ± 12.0	20.3 ± 8.0	22.1 ± 10.6	–	–	–	–
10a	–	1.4 ± 0.7	5.7 ± 3.9	7.8 ± 4	–	–	–
10b	–	23.2 ± 5.9	17.0 ± 6.7	29.0 ± 9.5	–	–	–
10c	65.0 ± 9.3[a]	53.3 ± 8.8	37.5 ± 10.8	–	–	–	–

Results are expressed as the mean ± SEM of Inhibition of TNF-α (%). The statistical analysis used was one-way ANOVA followed by Tukey's multiple comparisons test

[a] $p < 0.05$ vs. thalidomide (50.0 μM) group

Quantification of TNF-α levels in the macrophage supernatant

The ability of all compounds to decrease TNF-α was characterized in murine monocytes cultures stimulated with lipopolysaccharide (LPS). Initially, the cellular viability was determined for all compounds in order to obtain the suitable dose that could be used in the assay to quantify TNF-α levels (Mosmann 1983). For this experiment, we have considered only those concentrations with cellular viability equal to or >95%. The results were expressed as a percentage of inhibition, comparing the values obtained with those of positive control - thalidomide. Thalidomide was selected with control once it has well-described TNF-α inhibitory activity (Sampaio et al. 1991; Moreira et al. 1993). Also due to the fact that the structural planning for the molecules evaluated in this work contains the phthalimide subunit present in thalidomide and responsible for its inhibitory action of TNF-α. The reduction of TNF-α levels ranged from 0.3 to 73.5%. The most active organic nitrate ester and furoxan were **4c** and **10c**. At 100 μM, these compounds inhibited TNF-α levels at 73.5 and 65.0%, respectively (Table 2). At 50 μM, compounds **4c** and **10c** were more active than thalidomide, inhibiting TNF-α at 65.2 and 53.3%, respectively.

Conclusions

In summary, we synthesized phthalimide derivatives (**4a-e**; **6a-b**; **8** and **10a-c**) containing different NO-donor subunit represented by organic nitrate ester and furoxan. Our findings demonstrated that all compounds exhibit antiplatelet effect. In addition, the compounds were able to reduce TNF-α levels in murine monocytes cultures stimulated with LPS. Compounds **4c** and **10c** were the most potent to reduce TNF-α levels. Although compounds **4c** and **10c** were not the most active antiplatelet compounds, it was found for these compounds an appropriate balance between all investigated effects. Moreover, compounds **4c** and **10c** have shown reduced bleeding time compared to ASA. Considering the current limitations for SCA treatment, compounds **4c** and **10c** have emerged as promising lead compounds useful to prevent vaso-occlusion and inflammation associated with the disease.

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Compliance with ethical standards

Ethical approval The experiment was previously approved by the Institutional Ethics Committee of the Faculty of Pharmaceutical Sciences (FCFAr), Campus Araraquara (CEUA/FCFAr) under No. 37/2013, 38/2013, and 09/2014. All experiments were performed in accordance with current guidelines for laboratory animal care and ethical guidelines for the investigation of experimental pain in conscious animals.

Conflict of interest The authors declare that they have no conflict of interest.

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