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Design, Synthesis, and Pharmacological Evaluation of Novel Hybrid Compounds to Treat Sickle Cell Disease Symptoms. Part II: Furoxan Derivatives

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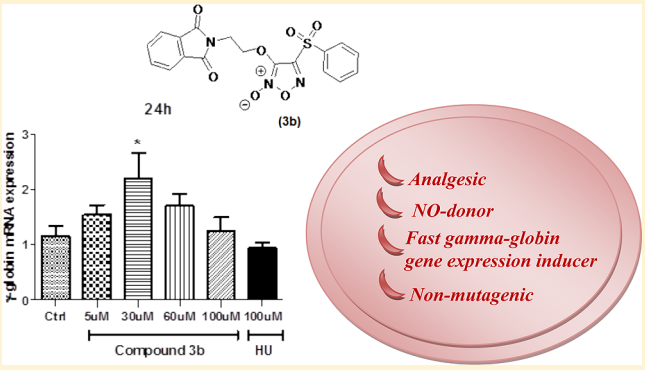
ABSTRACT: Phthalimide derivatives containing furoxanyl subunits as nitric oxide (NO)-donors (**3a**−**g**) were designed, synthesized, and evaluated in vitro and in vivo for their potential uses in the oral treatment of sickle cell disease symptoms. All compounds (**3a**−**g**) demonstrated NO-donor properties at different levels. Moreover, compounds **3b** and **3c** demonstrated analgesic activity. Compound **3b** was determined to be a promising drug candidate for the aforementioned uses, and it was further evaluated in K562 culture cells to determine its ability to increase levels of γ-globin expression. After 96 h at 5 μM, compound **3b** was able to induce γ-globin expression by nearly three times. Mutagenic studies using micronucleus tests in peripheral blood cells of mice demonstrated that compound **3b** reduces the mutagenic profile as compared with hydroxyurea. Compound **3b** has emerged as a new leading drug candidate with multiple beneficial effects for the treatment of sickle cell disease symptoms and provides an alternative to hydroxyurea treatment.



INTRODUCTION

Sickle cell disease (SCD) is the most prevalent hereditary chronic hemolytic anemia worldwide. It has been hypothesized that the disease appeared as a form of resistance to malaria in endemic regions of Africa.[1] This autosomal recessive disease is characterized by a punctual mutation (GTG to GAG) at the sixth codon of the β-globin gene that leads to the substitution of glutamic acid to valine residue in the gene for β-globin chain. At low oxygen tensions, hydrophobic interactions of β-chains induce hemoglobin polymers form in sickle cell human hemoglobin (HbS). The formation of these polymers, aggravated by the constant process of HbS oxygenation and deoxygenation, initiates by homogeneous nucleation which progress to a heterogeneous nucleation characterized by the formation of crystals and tactoids. These polymers can change the structures of red blood cells (RBCs) into rigid irregularly shaped cells, causing intravascular hemolysis and releasing of hemoglobin and arginase into the plasma.[2] The arginase is the enzyme that converts arginine into ornithine and urea, reducing the plasma concentration of arginine that acts as a substrate for endothelial nitric oxide synthase to produce nitric oxide (NO). The released hemoglobin also kidnaps endothelial nitric oxide (NO), contributing to vasoconstriction in affected patients.[3]

Vaso-occlusion is a common phenomenon involved in major clinical symptoms of SCD including: ischemia, painful crisis, acute chest syndrome, and strokes. The process initiates by an increased RBC adhesion to venular endothelium due interactions between $\alpha 4\beta 1$/vascular cell adhesion molecule 1 (VCAM-1). Studies have demonstrated that RBC containing HbS present higher adhesion to venular endothelium than RBC from healthy individuals.[4] In addition, leukocytes−endothelium interactions contribute to the heterocelullar aggregation (white blood cells and sickle cells), resulting in obstruction and local hypoxia. White blood cells contribute to endothelial inflammation in microvasculature. Chronic inflammatory character is maintained by

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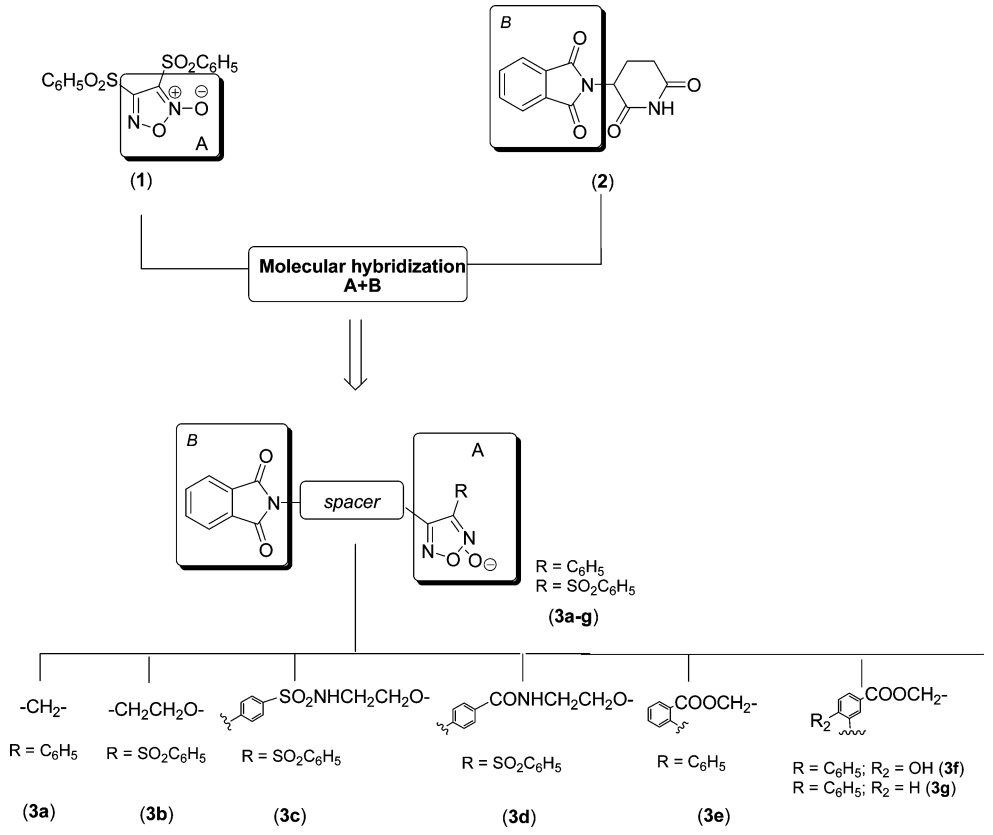
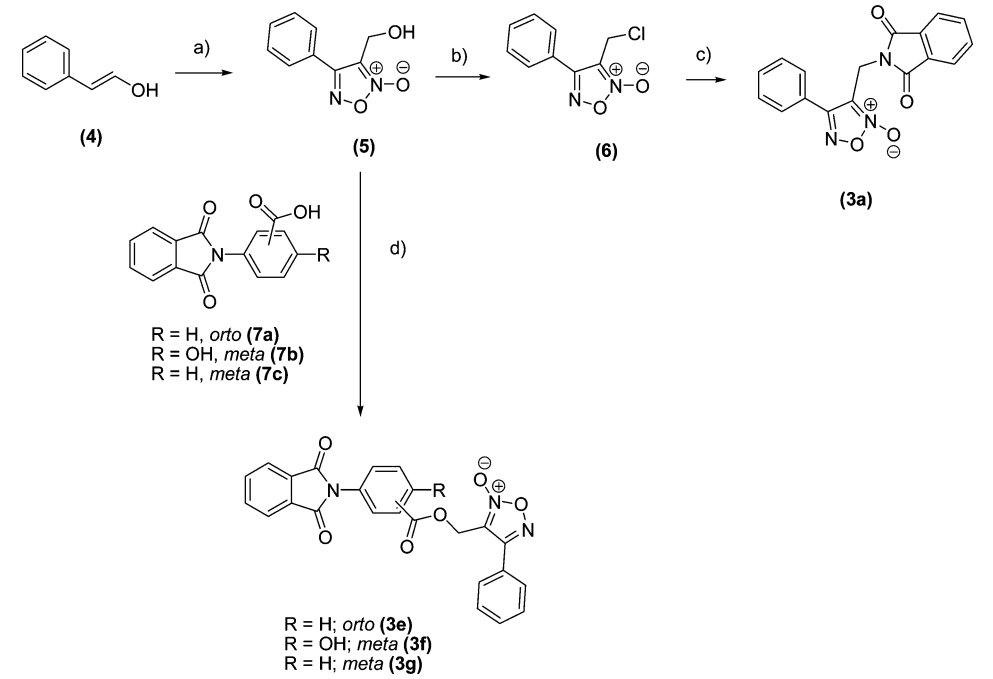
Scheme 1. Structural Planning Using Molecular Hybridization



Scheme 2[a]



[a]Reagents and conditions: (a) $NaNO_2$, CH_3COOH, 0 °C, 24 h, 90%; (b) $SOCl_2$, 25 °C, 24 h, 45%; (c) potassium phthalimide, dimethylformamide, 90 °C, 24 h, 80% d) CH_2Cl_2, dicyclohexylcarbodiimide, 0 °C, 5−6 h, 42−50%.

constant injury caused by sickle cell aggregates to the vascular endothelial wall.[5−7] All of these factors contribute to vaso-occlusive phenomena. Strategies which increase γ-globin expression and consequently HbF can inhibit HbS polymerization. An inverse relationship between HbF increased and mortality reduction of SCD patients has been described.[5]

Pain is the main complication reported by SCD patients;[8] acute pain is the main cause of morbidity and the common cause

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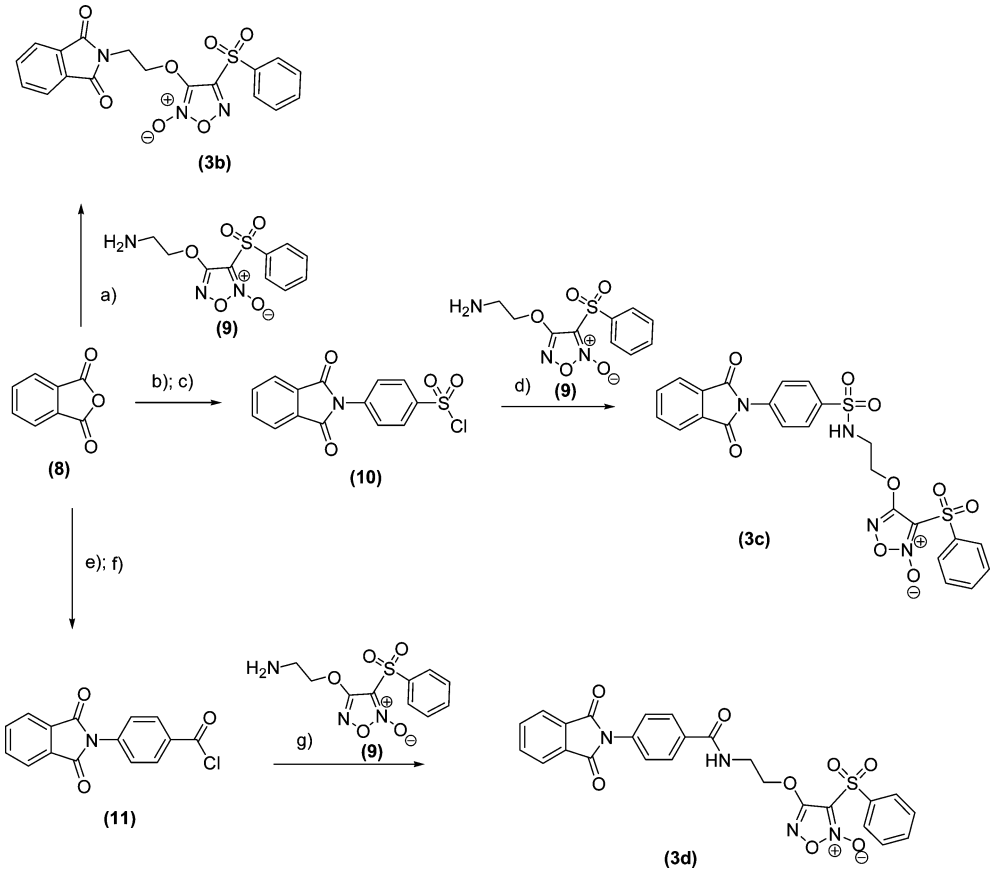
Scheme 3[a]



[a]Reagents and conditions: (a) CH_3COOH, 130 °C, 3 h, 24%; (b) aniline, 130 °C, 3 h, 90%; (c) $HOSO_2Cl$, PCl_3, 50 °C, 45 min, 80%; (d) CH_2Cl_2, triethylamine, rt, 2 h, 46%; (e) *p*-aminobenzoic acid, CH_3COOH, 130 °C, 3h, 91%; (f) $SOCl_2$, dimethylformamide$_{\text{catalytic amount}}$, 60 °C, 4h, 90%; (g) CH_2Cl_2, triethylamine, rt, 2 h, 40%.

of hospitalization.[9,10] High levels of pro-inflammatory cytokines presented in SCD patients contribute to pain associated with vaso-occlusive processes.[11,12]

Currently, the only drug approved by U.S. Food and Drug Administration (FDA) for treatment of SCD is hydroxyurea (HU). The beneficial effects of HU are attributed to its ability to increase the expression of γ chains in HbF.[13] After metabolism, HU is converted to NO, which induces γ-globin gene expression and fetal hemoglobin production, promoting vasodilatation and the inhibition of platelet aggregation.[14,15] However, despite these beneficial effects, the drug can cause myelosuppression and stimulates the production of pro-inflammatory gene expression and levels of cytokines such as TNFα, IL1A, IL1B, IL6, and IL8.[16,17] Hydroxyurea therapy also fails to treat some individuals, which justifies the search for new compounds to treat SCD symptoms.[18]

Recently, it has been reported that thalidomide (Thal) and some of its derivatives, such as pomalidomide and lenalidomide, induce γ-globin mRNA expression and HbF synthesis in vitro and in vivo in transgenic sickle mice.[19−21] Furthermore, these compounds possess analgesic activity related to inhibition of pro-inflammatory cytokines.[22,23] We reported that thalidomide derivatives containing organic nitrate esters, as a NO-donor subunit, demonstrated anti-inflammatory and analgesic activity, decreasing the levels of pro-inflammatory cytokines such as TNFα.[24]

In a continuing effort to develop new drug candidates with improved pharmacokinetic profiles for treatment of SCD symptoms, we report here on the design, synthesis, and pharmacological evaluation of phthalimide derivatives (**3a−g**), obtained by molecular hybridization of the prototypes furoxan (**1**) and thalidomide (**2**) (Scheme 1). The furoxan subunit was selected because of its ability to generate different levels of NO.[25,26] This approach aims to combine enhanced analgesic activity and NO-donor capacity in the same molecule to act in multiple SCD pathways.

RESULTS

Chemistry. The synthetic preparation of phthalimide derivatives (**3a−g**) is summarized in Schemes 2 and 3. Compound **3a** was obtained in three synthetic steps, with an overall yield of 32.4%.[27] Cinnamic alcohol (**4**) reacted with sodium nitrite in acetic acid medium to give 3-(hydroxymethyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (**5**); this compound was converted to alkyl chloride (**6**), which in the last step reacted with potassium phthalimide to give compound **3a** (Scheme 2).

Phthalimide derivatives (**7a−c**) were prepared by using coupling reactions of phthalic anhydride with functionalized amino derivatives, with variable yields of 68%−91%. The last step required to obtain compounds **3e**, **3f**, and **3g** involved coupling reactions of phthalimide derivatives with compound (**5**), with variable overall yields of 28.5−39% (Scheme 2).

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The compound 4-(2-aminoethoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (**9**) (Scheme 3) was synthesized according to procedures described by Sorba and co-workers.[28] Compound **3b** was prepared through a condensation reaction of phthalic anhydride (**8**) and compound **9** in acetic acid at 130 °C, with a 24% yield after purification (Scheme 3). In this reaction, as previously described by Gasco, the furoxan ring suffers a thermal isomerization process, yielding the 4-phenylsulfonyl isomer (**3b**) (Scheme 3), according to the analyses of HMBC experiments.[29−31]

Compounds **3c** and **3d** were obtained through condensation of phthalimide derivatives (**10** and **11**) with furoxan (**9**), using coupling reactions in dichloromethane, with global yields of, respectively, 34.6% and 21.8% (Scheme 3). The structures of all compounds were established by mass spectroscopy, elemental analysis, IR spectroscopy, and ^{1}H and ^{13}C NMR. All compounds were analyzed by HPLC, and their purities were confirmed to be over 98.5%.

Detection of Nitrite (Griess Reaction). The nitrite produced by all compounds (**3a−g**) from the oxidative reaction of nitric oxide, oxygen, and water was measured using the Griess reaction procedure.[28,32] The extent of thiol-induced NO generation was determined after incubation for 1 h in the presence of a large excess of L-cysteine (1:50). The results, expressed as percentage nitrite (NO_2^-; mol/mol), are summarized in Table 1.

Table 1. NO Release Data

compd	% NO_2^- (mol/mol)[a,b] L-Cys 50 × 10^{-4} M
DNS[c]	11.6 ± 0.4
3a	14.8 ± 0.2
3b	9.5 ± 0.9
3c	28.1 ± 0.1
3d	28.2 ± 0.2
3e	9.7 ± 0.5
3f	10.6 ± 0.1
3g	13.2 ± 0.8

[a]All values are mean ± SEM. [b]Determined by Griess reaction, after incubation for 1 h at 37 °C in pH 7.4 buffered water, in the presence of 1:50 molar excess of L-cysteine. [c]DNS: isosorbide dinitrate (DNS possesses two ONO_2 groups that may release NO).

All compounds (**3a−g**) were capable of inducing nitrite formation at levels of 9.5−28.2%. Isosorbide dinitrate (DNS), used as the control, induced 11.6% nitrite formation. From a structural point of view, we observed a clear relationship between furoxan substitutions and NO-release capability. Derivatives with electron-withdrawing substituents at the 3-position, i.e., 3-phenylsulfonyl-substituted derivatives **3c** and **3d**, released the most NO while the 3-alkyl-substituted furoxans, i.e., **3a−b** and **3e−g**, released the least NO in these experimental conditions.

Biology. *Antinociceptive Activity.* The antinociceptive profiles of compounds **3a−g** were evaluated using acetic acid-induced abdominal constrictions in mice.[33] All compounds were administered orally at a dosage of 100 μmol/kg (Figure 1). Compound **3b** was the most active antinociceptive compound, reducing the acetic acid-induced abdominal constrictions by 43%. Compound **3c** showed antinociceptive activity similar to that of the control, dypirone (Dyp), which was administered at the same dose as that of the phthalimide derivatives. Hydroxyurea and saline, used as control, did not demonstrate analgesic activity (data not shown). Compounds **3a** and **3d−g** demonstrated low protection of acetic acid-induced abdominal constrictions (Figure 1) as compared with the effects of compounds **3b** and **3c**.

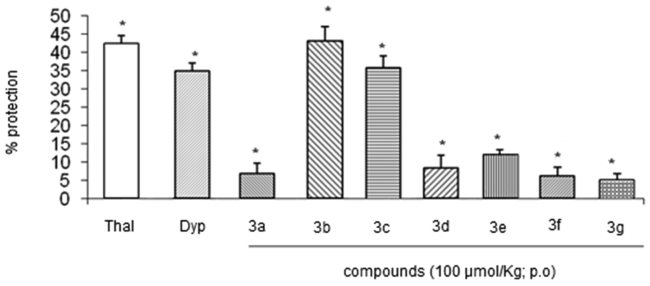

Figure 1. Antinociceptive effect of Thal, Dyp, and studied compounds (**3a−g**) (all in doses of 100 μmol/kg, po) in the 0.6% acetic acid (AcOH)-induced abdominal constrictions observed for 20 min after the administration of acetic acid. Data are expressed as the inhibition percentage of total writhings calculated from eight animals. *$P < 0.01$ (ANOVA followed by Dunnett's test).

Quantitative Real Time Polymerase Chain Reaction and Gene Expression Analysis. γ-Globin mRNA levels in K562 cells induced by compound **3b** were measured at different times (24, 48, 72, and 96 h) and concentrations (5, 30, 60, and 100 μM) (Figure 2). After 24 h, compound **3b** was able to induce γ-globin gene expression at a concentration of 5 μM. The compound also was able to induce nearly a 3-fold (2.8×) increase in γ-globin gene expression after 96 h at 5 μM as compared with levels in control cells (Ctrl; Figure 2). K562 cells were incubated with HU (5 and 100 μM) for different durations (24, 48, 72, and 96 h); HU was able to induce a nearly 2-fold (1.77×) γ-globin gene expression after 96 h of treatment at a concentration of 100 μM as compared with K562 control cells (Ctrl-HU); HU did not increase the γ-globin gene expression at concentrations of 5 μM (data not shown).

The β-soluble guanilate cyclase (sGC) subunit gene expression (RT-PCR) induced by compound **3b** in K562 cells occurred only at times in which γ-globin also increased during the treatment. We analyzed the data collected at different times (24, 48, 72, and 96 h) and with different concentrations of compound **3b** (5 and 30 μM) and found that compound **3b** was not able to induce the expression of β-sGC gene relative to levels in K562 control cells (Ctrl) under any combination of tested conditions (Figure 3).

Evaluation of Mutagenicity Using a Micronucleus Test in Peripheral Blood Cells of Mice. Mutagenicity induced by HU and compound **3b** after oral administration at different concentrations (25, 50, and 100 mg/kg) were evaluated in vivo using a micronucleus test in the peripheral blood cells of mice.[34] At 25 mg/kg, the average frequencies of micronucleated reticulocytes (MNRETs) induced by HU and compound **3b** were, respectively, 9.9 ± 2.47 and 4.0 ± 1.4; at 50 mg/kg, the average MNRET frequencies were, respectively, 15.0 ± 7.9 and 5.5 ± 0.54; at 100 mg/kg, HU showed an average MNRET frequency of 33.7 ± 10.7, while that of compound **3b** was 6.7 ± 1.8 (Figure 4), indicating that compound **3b** is less mutagenic than HU.

DISCUSSION

Despite efforts to develop new drugs for treating SCD symptoms, HU still remains as the only drug approved by the regulatory agency U.S. FDA. Beneficial effects of HU include: (a) increase γ-globin expression and fetal hemoglobin concentration, (b) decreased platelet and white cells counts, and (c) changed

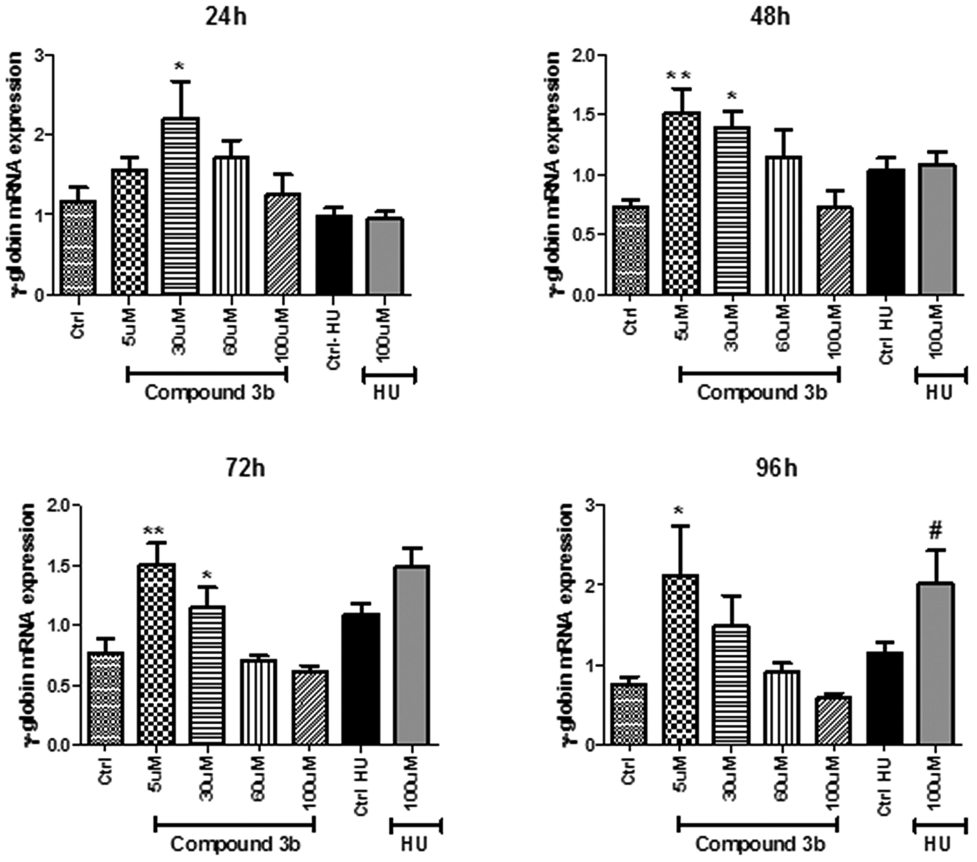

Figure 2. γ-Globin gene expression (RT-PCR) using K562 cells induced by compound **3b** at different times (24, 48, 72, and 96 h) and different concentrations (5, 30, 60, and 100 μM). The concentration of HU used was 100 μM at different times of treatment. Result is expressed as mRNA levels of γ-globin gene, normalized according β-actin and GAPDH expressions. Values represent mean ± SEM (n = 5) and are representative of five independent experiments. ** $P < 0.01$ and *$p < 0.05$ compared with Ctrl (vehicle: DMSO). # $p < 0.05$ compared with Ctrl-HU (vehicle: water). (ANOVA followed by Dunnett's test).

expression of adhesion molecules.[35,36] However, some adverse effects of HU, such as myelosuppression and genotoxicity, can limit its use in long-term therapy.[37,38] In addition, many patients fail to respond to HU therapy, thus justifying the search for new compounds which are able to induce fetal hemoglobin in SCD patients.[39]

Metabolic activity bioconverts HU to NO, which has several beneficial roles in the treatment of SCD, including increased γ-globin gene expression, inhibition of platelet aggregation, and vasodilatation. On the basis of the discovery that individuals with SCD present reduced plasmatic levels of NO, it was proposed that NO-supplementation therapies might ameliorate some SCD symptoms.[40−42] The severity and duration of crisis by NO inhalation is still controversial in SCD.[43,44] An increase in NO levels can also reverse the adhesive properties of neutrophils, thus decreasing vaso-occlusive phenomena in SCD patients.[45] Furthermore, NO donors, such as organic nitrate esters, *S*-nitrosocysteine, and deta-NONOate, can increase γ-globin mRNA in K562 cells.[46] We have previously reported that organic nitrate esters with NO-donor capacity could represent a new approach to SCD treatment. However, we observed no differences in the levels of NO donation among all thalidomide derivatives.[24] To improve NO-donor capacity, we designed and synthesized a series of phthalimide analogues containing the 1,2,5-oxadiazole-2-oxide (furoxan) NO-donor subunit (Scheme 1). The NO-donation properties of furoxan derivatives are well established in the literature.[26] The compounds **3a−g** demonstrated different levels of NO-donor ability, as measured by the generation of nitrite in the medium using the Griess reaction. Compounds **3c** and **3d**, containing the 3-phenylsulfonylfuroxan subunit, were the most potent inductors of nitrite formation, yielding 28.1% and 28.2% NO_2^- (mol/mol), respectively. Compounds **3a−b** and **3e−g** were capable of inducing nitrite formation at concentrations of 9.5−14.8% NO_2^- (mol/mol).

From a patient's perspective, acute pain is the most common complication of SCD, with a lifetime prevalence of greater than 95%.[47] An important relationship exists between inflammation and pain: patients with SCD present overexpression of pro-inflammatory cytokines, which activates the vascular endothelium, compromises vascular integrity, and increases the expression of adhesion molecules by activated leukocytes. Although the exact relationship between pain and vaso-occlusion is still unclear, it has been shown that cytokines such as TNFα play an important role in this process. The inhibition of TNFα by Thal was demonstrated to be responsible for the antinociceptive effect in a writhing-response model using mice.[48] Indeed, our previous results demonstrate that phthalimide derivatives containing NO-donor subunits are able to reduce TNFα levels in adult knockout−transgenic sickle cell mice at levels comparable to dexamethasone, which was used as a control.[24] Paradoxically, HU induced the formation of the pro-inflammatory cytokine TNFα in vivo. Laurence and co-workers have described HU-induced production of gene expression and of pro-inflammatory cytokines such as TNFα, IL-1, and IL-8.[16] To

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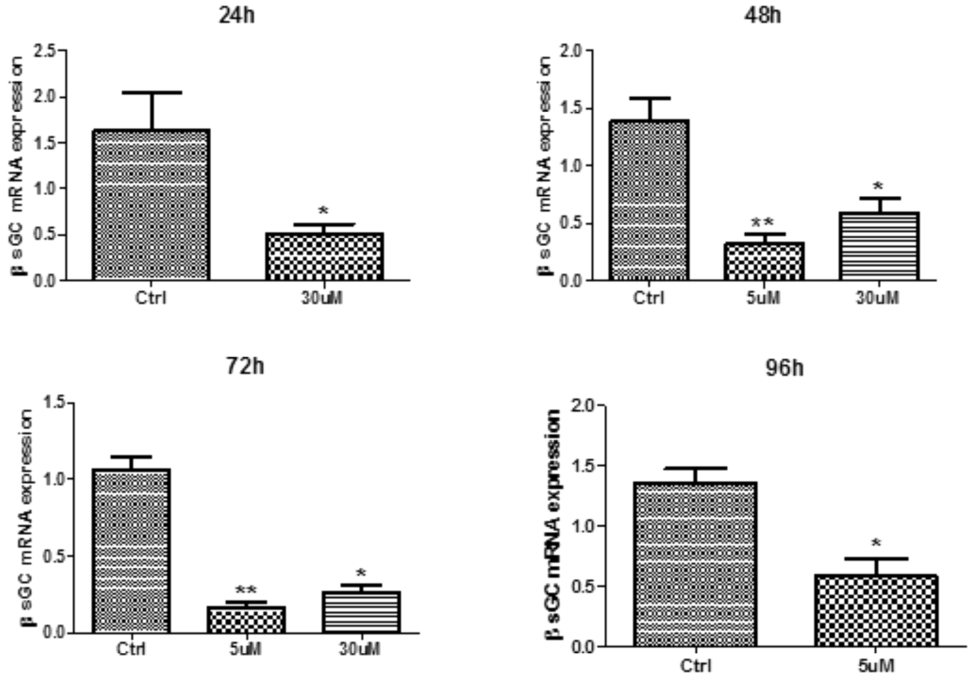

Figure 3. β-sGC subunit gene expression (RT-PCR) was evaluated in K562 cells induced by compound **3b** at different times (24, 48, 72, and 96 h) and different concentrations (5, 30 μM). We analyzed these times and concentrations because it was where γ-globin gene expression was increased compared with K562 control (Ctrl). Result is expressed as mRNA levels of β-GCs gene, normalized according β-actin and GAPDH expressions. Values represent mean ± SEM (n = 5) and are representative of five independent experiments. ** $p < 0.01$ and *$p < 0.05$ (Mann−Whitney) compared with Ctrl.

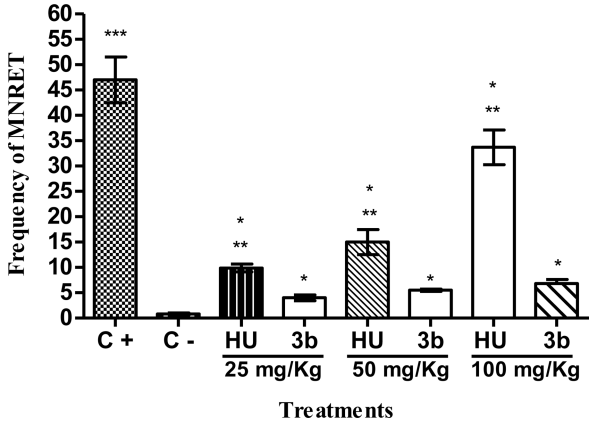

Figure 4. Average frequency of micronucleated reticulocytes (MNRET) and standard deviation of 1000 cells obtained from mice treated with the positive control cyclophosphamide (50 mg/kg), CMC/Tween (negative control), hydroxyurea (HU), and compound **3b** (25, 50, and 100 mg/kg). *$p < 0.05$ (compared to negative control), **$p < 0.05$ (compared to the compound 3b), ***$p < 0.05$ (compared to the negative control, hydroxyurea, and compound **3b**).

characterize the analgesic activity of compounds **3a−g**, we evaluated their effects after oral administration (100 μmol/kg)by using a mouse model of acetic acid-induced abdominal constriction.[33] The most active compounds, **3b** and **3c**, reduced acetic acid constrictions by 43% and 36%, respectively. This effect seems to be related to TNFα inhibition by these compounds (unpublished results).

Considering these results, we selected compound **3b** for further evaluation of its ability to induce γ-globin expression in K562 cells. γ-Globin and α-globin constitute part of the fetal hemoglobin tetramer present during fetal development. After the postnatal period, HbF is gradually replaced by HbA ($\alpha_2\beta_2$) in healthy individuals. The beneficial effects of HU are associated with its capacity to increase γ-globin gene expression and consequently fetal hemoglobin. Activation of soluble guanylate cyclase (sGC) by NO up-regulates γ-globin gene transcription in erythroid cell lines and primary erythroblasts. The increase in cGMP levels by NO in erythroid cells is one proposed mechanism for HbF induction.[49] We observed in our experiments with K562 cells that HU increases γ-globin gene expression only after 96 h of treatment at 100 μM (Figure 2). Compound **3b** was able to induce γ-globin gene expression after treatments of 24 h at 30 μM. At 96 h at 5 μM, compound **3b** was able to induce nearly a three-time γ-globin gene expression as compared with controls. We determined gene-expression levels of human sGC α and β subunits by qRT in K562 cells which had received compound **3b** treatments. However, the α subunit showed no gene expression in our K562 cells, although a positive control of gene expression by the α subunit in PC-3 cells (ATCC no. CRL-1435) was used to confirm the primer's integrity. The β subunit was expressed at lower levels during treatment with compound **3b** as compared with K562 controls at every treatment time. Conran and co-workers showed that sGC mRNA expression was not significantly higher in reticulocytes of sickle cell anemia patients than in the reticulocytes of control individuals; however, they found that treatment of red blood cells with NO-donor sodium nitroprusside (SNP) resulted in enhanced cGMP production by endogenous sGC in both normal and sickle red blood cells.[49] Thus, new experiments will be necessary to evaluate whether compound **3b** alters the NO/sGC pathway. Curiously, Aerbajinai and co-workers have demonstrated that Thal also induces γ-globin expression in a dose-dependent manner by increasing the concentration of reactive oxygen species (ROS); they have proposed that the mechanism for increased ROS is related to the p38 mitogen-activated protein kinase (MAPK) signaling pathway and histone

H4 acetylation.[19] Further studies using the Thal derivatives pomalidomide and lenalidomide demonstrated that these drugs also induce HbF expression by modulating erythroid differentiation in $CD34^+$ progenitor cells. The HbF synthesis induction seems to be related to modification of the chromatin structure that results from histone H3 acetylation.[20]

We also evaluated the mutagenicity induced by compound **3b** using a micronucleus test in the peripheral blood cells of mice. Compound **3b** demonstrated a reduced ability to induce mutagenicity as compared with HU. Although exposure to NO has been reported to cause mutagenic and carcinogenic effects because of the formation of *N*-nitroso compounds and the formation of oxidative species such as peroxynitrite and peroxide anions, the few reports existing in the literature show that furoxan derivatives with NO-donor ability demonstrate reduced induction of mutagenicity.[50−53]

Taken together, these results show that hybrid furoxan derivatives combining NO-donor ability, analgesic activity, and the capacity to induce γ-globin expression with reduced mutagenicity could provide an alternative to HU therapy, thus representing a new approach to the development of molecules to treat SCD symptoms.

CONCLUSIONS

A novel series of phthalimide derivatives (**3a−g**), containing a furoxanyl-moiety as NO-donor subunit, was synthesized and characterized by elemental analysis, IR spectroscopy, and ^{1}H and ^{13}C NMR. All compounds demonstrated NO-donor properties. Compounds **3b** and **3c** were the most active analgesic molecules developed in the study, reducing acetic acid-induced abdominal constrictions by 43% and 36%, respectively. Compound **3b** was able to increase γ-globin gene expression by nearly three times after 96 h at 5 μM, while HU did not demonstrate any activity at these conditions. In addition, at doses of 100 mg/kg, compound **3b** demonstrated reduced mutagenic activity as compared with HU, as determined by a micronucleus test in the peripheral blood cells of mice. Compound **3b**, which contains a furoxan subunit, has emerged as a novel and leading drug candidate for the treatment of SCD symptoms.

EXPERIMENTAL SECTION

General. Melting points were measured with an electrothermal melting-point apparatus (SMP3, Bibby Stuart Scientific) in open capillary tubes and are uncorrected. Infrared spectra (KBr disk) were produced on an FTIR-8300 Shimadzu and the frequencies expressed in cm^{-1}. ^{1}H NMR and ^{13}C NMR spectra were scanned on a Bruker DRX-400 (400 MHz) NMR spectrometer using DMSO-d_6 or acetone-d as the solvent. Chemical shifts were expressed in parts per million (ppm) relative to tetramethylsilane. Elemental analyses (C, H, and N) were performed on a Perkin-Elmer model 240C analyzer, and the data were within ±0.4% of the theoretical values. HPLC analysis was performed on a Shimadzu LC-10AD chromatograph equipped with a model SPD-10A UV−Vis detector (Shimadzu). All compounds were analyzed by HPLC, and their purity was confirmed to be greater than 98.5%. The compounds were separated on a reversed phase C18 column (5 μm particle, 250 mm × 4.6 mm I.D.) Shimadzu Shim-pack CLC-ODS (M). HPLC-grade solvents (acetonitrile, methanol, acetic acid, and toluene) were used in the analyses and were bought from a local supplier. The progress of all reactions was monitored by TLC, which was performed on 2.0 × 6.0 cm^2 aluminum sheets precoated with silica gel 60 (HF-254, Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under UV light (265 nm) and treated with iodine vapor. Merck silica gel (70−230 mesh) was used for preparative column chromatography. Reagents and solvents were purchased from commercial suppliers and used as received.

Compounds **3a**, **5**, **7a−c**, **9**, **10**, and **11** were synthesized according to a previously described methodology.[54−57] Compound **8** was purchased commercially.

*Preparation of Compound (**3b**).* A mixture of 4-(2-aminoethoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (**9**) (0.23 g, 0.6 mmol), phthalic anhydride (**8**) (0.09 g, 0.6 mmol), and 7 mL of acetic acid was stirred under nitrogen at 140 °C for 2 h. The compound (**3b**) was isolated by the addition of 4 mL of ice−water. The pH was adjusted to 7.0 using sodium hydroxide solution 20%. Then, 20 mL of ethyl acetate was added. The aqueous phase was washed twice with 20 mL of ethyl acetate. The organic phase was dried with sodium sulfate or magnesium sulfate. After filtration, the organic phase was concentrated under reduced pressure to produce a brown powder.

If necessary, the samples were further purified with silica gel column chromatography, using hexane:ethyl acetate (6:4) as the mobile phase. After, this compound was purificated by silica gel column chromatography, using hexane:ethyl acetate (60:40) as the mobile phase to give the compound (**3b**) as a white powder.

*4-[2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)ethoxy]-3-(phenylsulfonyl)-2-N-1,2,5-oxadiazole (**3b**).* Yield 24%; mp 136−139 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3059 (C−H aromatic), 2933 (C−H), 1778 and 1720 (C═O imide), 1620 and 1548 (C═N furoxan), 1600 and 1460 (C═C aromatic), 1358 (N−O). NMR ^{1}H (400 MHz, acetone-d_6) δ: 8.01 (2H; dd, J_{ortho} = 7.4 Hz and J_{meta} = 1.3 Hz), 7.92 (4H; m), 7.82 (1H; ddd, J_{ortho} = 7.4 Hz and J_{meta} = 1.3 Hz), 7.66 (2H; dd, J_{ortho} = 7.4 Hz and J_{meta} = 1.3 Hz), 4.77 (2H; t, J = 5.41 Hz), 4.22 (2H; t, J = 5.41 Hz) ppm. ^{13}C NMR (100 MHz, acetone-d_6) δ: 168.41, 156.48, 136.36, 135.00, 132.87, 130.37, 129.17, 123.77, 95.44, 68.76, 36.78 ppm. Calculated for $C_{18}H_{13}N_3O_7S$: C, 52.05; H, 3.15; N, 10.12. Found: C, 52.3; H, 3.19; N, 10.5.

*General Procedure for the Synthesis of Compounds (**3c−d**).* A mixture of 4-(2-aminoethoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (**9**) (0,1 g, 0.35 mmol), 48 μL of triethylamine (0.35 mmol), and 5 mL of anhydrous dichloromethane was stirred at room temperature under nitrogen for 10 min. Further, sulfonyl chloride (**10**) (0.1 g, 0.31 mmol) or acyl chloride (**11**) was slowly added. The reaction, under nitrogen, was stirred for more 2 h. The solvent was evaporated at reduced pressure, and the compounds were purified by silica gel column chromatography, using hexane:ethyl acetate (60:40) as the mobile phase to give the compounds (**3c** and **3d**).

*4-[2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl-N-phenylbenzenosulfonamide)ethoxy]-3-(phenylsulfonyl)-2-N-1,2,5-oxadiazole (**3c**).* Yield 46%; mp 144−148 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3300 (N−H aromatic), 3059 (C−H aromatic), 2925 and 2854 (C−H), 1745 and 1718 (C═O imide), 1629 (C═N furoxan), 1560 and 1464 (C═C aromatic), 1389 (C−N−C), 1363 (N−O). ^{1}H NMR (400 MHz, acetone-d_6) δ: 8.13 (2H; m), 8.08 (2H; m), 8.30−7.95 (4H; m), 7.89 (1H; ddd), 7.82 (2H; dd), 7.76 (2H; ddd), 4.6 (2H; t), 3.53 (2H; q) ppm. ^{13}C NMR (100 MHz, acetone-d_6) δ: 167.39, 159.96, 136.70, 135.64, 132.76, 130.72, 129.51, 128.37, 128.09, 124.37, 100.83, 70.81, 42.41 ppm. Calculated for $C_{24}H_{18}N_4O9S2$: C, 50.52; H, 3.18; N, 9.82; O, 25.24; S, 11.24. Found: C, 50.73; H, 3.33; N, 10.03.

*4-[2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl-N-phenylbenzenocarboxamide)ethoxy]-3-(phenylsulfonil)-2-N-1,2,5-oxadiazole (**3d**).* Yield 40%; mp 137−140 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3303 (N−H imide), 3059 (C−H aromatic), 2923 and 2852 (C−H), 1784 and 1718 (C═O imide), 1560 (C═N furoxan), 1606, 1560, and 1464 (C═C aromatic), 1380.9 (N−O). ^{1}H NMR (400 MHz, acetone-d_6) δ: 8.16−8.21 (2H; m), 8.04−8.09 (2H; m), 7.94−8.00 (4H; m), 7.81 (1H; ddd), 7.67−7.72 (2H; ddd), 7.59−7.63 (2H; ddd), 4.71 (2H; t), 3.95 (2H; q) ppm. ^{13}C NMR (100 MHz, acetone-d_6) δ: 167.4, 160.0, 136.80, 135.32, 131.8, 132.8, 130.5, 129.7, 128.66, 128.13, 124.44, 100.9, 70.77, 42.38 ppm. Calculated for $C_{25}H_{18}N_4O_8S$: C, 56.18; H, 3.39; N, 10.48. Found: C, 56.44; H, 3.42; N, 10.62.

*General Procedure for the Synthesis of Compounds (**3e−g**).* A mixture of previously selected phthalimide derivatives (**7a−c**) (1.3 mmol), 3-(hydroxymethyl)-4-phenyl-1,2,5-oxadiazole 2-oxide (**5**) (1.5 mmol), dicyclohexylcarbodiimide (1.3 mmol), dimethylaminopyridine (0.13 mmol), and 15 mL of anhydrous dichloromethane was stirred under nitrogen at 0 °C for 5 h. After, the solvent was evaporated at

reduced pressure and the compounds were purified by silica gel column chromatography, using hexane:ethyl acetate (60:40) as the mobile phase to give the compounds (**3e**−**g**) with variable yields (42−50%).

*3-({[2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)benzoyl]oxy}-methyl)-2-hydroxy-4-phenyl-)-2-N-oxide-1,2,5-oxadiazole (**3e**).* . Yield 42%. IR ν_{max} (cm^{-1}; KBr pellets): 3084 (C−H aromatic), 2933 and 2861 (C−H), 1786−1718 (C═O imide), 1730 (C═O), 1598 (C═N furoxan), 1560−1450 (C═C aromatic), 1318 (C−N−C), 1356 (N−O). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.05 (1H; dd, J_{ortho} = 7.7 Hz and J_{meta} = 1.4 Hz), 7.95 (2H; H16), 7.91−7.81 (7H; m, H1, H2, H17 and H18), 7.7 (1H; H7), 7.63 (1H; H8), 7.56 (1H; H6), 5.2 (1H; s, H12) ppm. ^{13}C NMR (100 MHz, acetone-d_6) δ: 167.9, 165.23, 157.99, 135.7, 134.84, 134.18, 133.03, 132.54, 131.77, 130.35, 130.17, 130.10, 128.73, 128.60, 124.35, 112.22, 56.06 ppm. Calculated for $C_{24}H_{15}N_3O_6$: C, 65.31; H, 3.43; N, 9.52. Found: C, 65.5; H, 3.47; N, 9.71.

*3-({[5-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-2-hydroxybenzoyl]oxy}methyl)-2-N-oxide-4-phenyl-1,2,5-oxadiazole (**3f**).* . Yield 50.4%; mp 153−157 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3058 (C−H aromatic), 2925 and 2837 (C−H), 1786 and 1715 (C═O imide), 1724 (C═O), 1598 (C═N furoxan), 1494 and 1450 (C═C aromatic), 1381 (C−N−C), 1356 (N−O). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.1 (2H; m, H1 and H2), 7.97 (1H; dd, H16), 7.9 (1H; dd, H10), 7.8 (2H; m, H17 and H18), 7.4 (1H; dd, H7), 5.1 (1H; s, H12) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ: 168.00, 158.00, 134.5, 132.31, 130.4, 130.00, 128.92, 128.03, 123.5, 115.00, 55.00 ppm. Calculated for $C_{24}H_{15}N_3O_7$: C, 63.02; H, 3.31; N, 9.19. Found: C, 63.4; H, 3.34; N, 9.16.

*3-({[3-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)benzoyl]oxy}-methyl)-2-N-oxido-4-phenyl-1,2,5-oxadiazole (**3g**).* . Yield 48%; mp 150−153 °C. IR ν_{max} (cm^{-1}; KBr pellets): 3084 (C−H aromatic), 2927 and 2850 (C−H), 1786 and 1717 (C═O imide), 1724 (C═O), 1573 (C═N furoxan), 1560 and 1450 (C═C aromatic), 1381 (C−N−C), 1356 (N−O). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 7.87 and 8.1 (2H; m, H1 and H2), 7.2 and 7.8 (7H; m, H6, H7, H8, H10, H16, H17 and H18), 5.4 (1H; s, H12) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 168.00, 158.00, 134.5, 123.5, 115.0, 55.0 ppm. Calculated for $C_{24}H_{15}N_3O_6$: C, 65.31; H, 3.43; N, 9.52. Found: C, 65.6; H, 3.6; N, 9.81.

Detection of Nitrite (Griess Reaction). A solution of the appropriate compound (20 μL) in DMSO was added to 2 mL of a mixture of 50 mM phosphate buffer (pH 7.4) and methanol (1:1, v:v) containing 5 mM of L-cysteine. The final concentration of the compound was 10^{-4} M. After 1 h at 37 °C, 1 mL of the reaction mixture was treated with 250 μL of Griess reagent (4 g of sulfanilamide, 0.2 g of *N*-naphthylethylenediamine dihydrochloride, 85% phosphoric acid [10 mL] in distilled water [final volume, 100 mL]). After 10 min at room temperature, the absorbance was measured at 540 nm using a Shimadzu UV-2501PC spectrophotometer. Standard sodium nitrite solutions (10−80 nmol/mL) were used to construct the calibration curve. The yields of nitrite are expressed as % NO_2^- (mol/mol). No production of nitrite was observed in the absence of L-cysteine.[28,32]

Pharmacology. *Drugs and Reagents.* The agents used here were acetic acid and, arabic gum, thalidomide, and dipyrone (Sigma Chemical). The compounds and standards were used as suspensions in arabic gum in all experiments.

Animals. Adult male Swiss albino mice (20−35 g) were used in the experiments. They were housed in single-sex cages under a 12 h light:12 h dark cycle in a controlled-temperature room (22 ± 2 °C). The mice had free access to food and water. The experiments were performed after the protocol was approved by the local Institutional Ethics Committee (CEP/FCFAR/no. 05/2010 and CEP/FCFAR/no. 25/2011). All experiments were performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals.

Antinociceptive Activity. Analgesic activity was determined in vivo with the acetic acid-induced (0.6%, 0.1 mL/10 g) abdominal constriction test in mice.[33] Groups of eight Swiss mice of both sexes (18−23 g) were used in each test group and the control animals. The compounds were administered orally (100 μmol/kg) as a suspension in 5% arabic gum in saline (vehicle). Dypirone (100 μmol/kg) was used as the standard drug, administered under the same conditions. Acetic acid solution was administered ip 1 h after the administration of the compounds. Ten after the ip acetic acid injection, the number of constrictions per animal was recorded for 20 min. The control animals received an equal volume of vehicle. Antinociceptive activity was expressed as percentage inhibition of the constrictions compared with those in the vehicle-treated control group. The results are expressed as means ± SEM of 10 animals per group. The data were analyzed statistically with Student's *t* test at a significance level of $P < 0.05$.

K562 Cell Culture. Human K562 erythroleukemia cells (ATCC, Philadelphia, PA, USA) were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen) in a humidified 5% CO_2 atm at 37 °C. The cells were cultivated in a density of 1×10^7 cells/100 mL and treated with the concentrations 5, 30, 60, and 100 μM of compound **3b** and 100 μM of HU (Sigma). The samples were collected after 24, 48, 72, and 96 h of treatment.

Quantitative Real-Time Polymerase Chain Reaction and Gene Expression Analysis. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol for cell cultures. cDNA was synthesized from total RNA extracts with Super Script III, following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Synthetic oligonucleotide primers were designed to amplify cDNA for γ-globin, α-sGC, β-sGC, β-actin (BAC), and glyceraldehyde phosphate dehydrogenase (GAPDH) genes (Primer Express; Applied Biosystems, Foster City, CA, USA). The α-sGC and β subunits were evaluated only at points that showed increased gene expression of γ-globin. For primer sequences and concentrations, see Table 2. Primers were synthesized by Invitrogen. All samples were assayed in a 12 μL volume containing 1 ng of cDNA (3.0 μL), 6.0 μL of SYBR Green Master Mix PCR (Applied Biosystems), and 3.0 μL of specific primers in a MicroAmp Optical 96-well reaction plate (Applied Biosystems) using the StepOne Plus (Applied Biosystems), as previously described. BAC, GAPDH genes were used as endogenous control. Gene expression was quantified using the Gnorm program.[58] Two replicas were run on the plate for each sample. Results are expressed as mRNA levels of γ-globin and β-sGC genes, normalized according to β-actin and GAPDH expressions. Data were analyzed statistically using the INSTAT software version 3.0. Results were compared before and after the treatment with ANOVA followed by Dunnett's test. To compare β-sGC gene expression between control and treated cells was used Mann−Whitney. *P* value of less than 0.05 was considered to be statistically significant.

Table 2. Primers Sequences

genes	forward and reverse sequences
γ-globin	F−5′: TGTGGAAGATGCTGGAGGAGA−3′
	R−5′: CAAAGAACCTCTGGGTCCATG−3′
α-sGC	F−5′: AAATCAATGTCAGCCCAACA−3′
	R−5′: AAACACGAAACCAGGACAGTC−3′
β-sGC	F−5′: GCCAGGTTCAAGTAGATGGTG−3′
	R−5′: GGCATCCGCTGTCCTATG−3′
BAC	F−5′: AGGCCAACCGCGAGAAG−3′
	R−5′: ACAGCCTGGATAGCAACGTACA−3′
GAPDH	F−5′: GCACCGTCAAGGCTGAGAAC−3′
	R−5′: CCACTTGATTTTGGAGGGATCT−3′

Evaluation of Mutagenicity Using a Micronucleus Test in Peripheral Blood Cells of Mice. The doses of HU and synthesized compounds **3b** were evaluated (25, 50, and 100 mg/kg) after they were administered to the animals by gavage. Groups of 10 Swiss mice of both sexes (25−30 g) were used in each test group and the control animals. The positive control group receive ip cyclophosphamide (50 mg/kg). The negative control group received 0.3 mL of 1% carboxymethylcellulose (CMC) suspension and 0.2% Tween by gavage. A group in which the animals received only water was also used. The laminas were prestained with acridine orange. After 30 h, the animals were killed to collect their blood. We counted 1000 reticulocytes per animal and recorded the frequencies of micronucleated cells. After the cytological

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analysis of the laminas containing samples of peripheral blood from the mice treated with the various drugs, we calculated the average frequencies of cell micronuclei (and the standard deviations) for each treatment group. These results were tested with analysis of variance (ANOVA). When $P < 0.05$, the average values for the treatments were compared using the Tukey method, calculating the minimum significant difference at $\alpha = 0.05$.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

BAC, β-actin; cGMP, cyclic guanosine monophosphate; Ctrl, control; DNS, isosorbide dinitrate; Dyp, dypirone; GAPDH, glyceraldehyde phosphate dehydrogenase; HbA, hemoglobin A; HbF, fetal hemoglobin; HbS, hemoglobin S; HU, hydroxyurea; IL, interleukin; MAPK, mitogen-activated protein kinase; MNRET, micronucleated reticulocytes; NO, nitric oxide; NO_2^-, nitrite; qRT, quantitative real time; RBC, red blood cells; ROS, reactive oxygen species; RT-PCR, real time polymerase chain reaction; SCD, sickle cell disease; sGC, soluble guanilate ciclase; SNP, sodium nitroprusside; Thal, thalidomide; TNFα, tumor necrosis factor α

REFERENCES

(1) Cyrklaff, M.; Sanchez, C. P.; Kilian, N.; Bisseye, C.; Simpore, J.; Frischknecht, F.; Lanzer, M. Hemoglobins S and C interfere with action remodeling in *Plasmodium falciparum*-infected erythrocytes. *Science* **2011**, *334* (6060), 1283−1286.

(2) Stuart, M. J.; Nagel, R. L. Sickle-cell disease. *Lancet* **2004**, *364*, 1343−1360.

(3) Hoppe, C. C. Novel therapies targeting the endothelium in sickle cell disease. *Hemoglobin* **2011**, *35* (5−6), 530−546.

(4) Hebbel, R. P.; Yamada, O.; Moldow, C. F.; Jacob, H. S.; White, J. G.; Eaton, J. W. Abnormal adherence of sickle erythrocytes to cultured vascular endothelium: possible merchanism for microvascular occlusion in sickle cell disease. *J. Clin. Invest.* **1980**, *65*, 154−160.

(5) Dos Santos, J. L.; Chin, C. M. Recent insights on the medicinal chemistry of sickle cell disease. *Curr. Med. Chem.* **2011**, *18* (15), 2339−2358.

(6) Lanaro, C.; Franco-Penteado, C. F.; Albuqueque, D. M.; Saad, S. T.; Conran, N.; Costa, F. F. Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. *J. Leukocyte Biol.* **2009**, *85* (2), 253−242.

(7) Okpala, I. Leukocyte adhesion and the pathophysiology of sickle cell disease. *Curr. Opin. Hematol.* **2006**, *13*, 40−44.

(8) Miller, S. T.; Kim, H. Y.; Weiner, D.; Wager, C. G.; Gallagher, D.; Styles, L.; Dampier, C. D. Investigators of the Sickle Cell Disease Clinical Research Network (SCDCRN). Inpatient management of sickle cell pain: a "snapshot" of current practice. *Am. J. Hematol.* **2012**, *87* (3), 333−336.

(9) Platt, O. S.; Thorington, B. D.; Brambilla, D. J.; Milner, P. F.; Rosse, W. F.; Vichinsky, E.; Kinney, T. R. Pain in sickle cell disease. Rates and risk factors. *N. Engl. J. Med.* **1991**, *325* (1), 11−16.

(10) Yusuf, H. R.; Atrash, H. K.; Grosse, S. D.; Parker, C. S.; Grant, A. M. Emergency department visits made by patients with sickle cell disease: a descriptive study, 1999−2007. *Am. J. Prev. Med.* **2010**, *38* (4), S536−S541.

(11) Graido-Gonzalez, E.; Doherty, J. C.; Bergreen, E. W.; Telfer, M.; McMillen, M. A. Plasma endothelin-1, cytokine and prostaglandin E2 levels in sickle cell disease and acute vaso-occlusive sickle crisis. *Blood* **1998**, *92* (7), 2551−2555.

(12) Tomer, A.; Harker, L. A.; Kasey, S.; Eckman, J. R. Thrombogenesis in sickle cell disease. *J. Lab. Clin. Med.* **2001**, *137* (6), 398−407.

(13) Charache, S.; Barton, F. B.; Moore, R. D.; Terrin, M. L.; Steinberg, M. H.; Dover, G. J.; Ballas, S. K.; McMahon, R. P.; Castro, O.; Orringer, E. P. Hydroxyurea and sickle cell anemia: clinical utility of a myelosuppressive "switching" agent: the multicenter study of hydroxyurea in sickle cell anemia. *Medicine* **1996**, *75* (6), 300−325.

(14) Hankins, J.; Aygun, B. Pharmacotherapy in sickle cell disease-state of the art and future prospects. *Br. J. Hamaetol.* **2009**, *145* (3), 296−308.

(15) Santos, J. L.; Lanaro, C.; Chin, C. M. Advances in sickle cell disease treatment: from drug discovery until the patient monitoring. *Cardiovasc. Hematol. Agents Med. Chem.* **2011**, *9* (2), 113−127.

(16) Laurance, S.; Pellay, F. X.; Dossou-Yovo, O. P.; Verger, E.; Krishnamoorthy, R.; Lapoumeroulie, C.; Benecke, A.; Elion, J. Hydroxycarbamide stimulates the production of proinflammatory cytokines by endothelial cells: relevance to sickle cell disease. *Pharmacogenet. Genomics* **2010**, *20* (4), 257−268.

(17) Santos, J. L.; Bosquesi, P. L.; Almeida, A. E.; Chung, M. C.; Varanda, E. A. Mutagenic and Genotoxic Effect of Hydroxyurea. *Int. J. Biomed. Sci.* **2011**, *16*, 2982−2989.

(18) Valfar, H.; Valfar, F.; Darvill, A.; Albersheim, P.; Kutlar, A.; Woods, K. F.; Hardin, J. Predicting the effectiveness of hydroxyurea in individual sickle cell anemia patients. *Artif. Intell. Med.* **2000**, *18* (2), 133−148.

(19) Aerbajinai, W.; Zhu, J.; Gao, Z.; Chin, K.; Rodgers, G. P. Thalidomide induces gamma-globin gene expression through increased reactive oxygen species-mediated p38 MAPK signaling and histone H4 acetylation in adult erythropoiesis. *Blood* **2007**, *110*, 2864−2871.

(20) Moutouh-de-Parseval, L. A.; Verhelle, D.; Glezer, E.; Jensen-Pergakes, K.; Ferguson, G. D.; Corral, L. G.; Morris, C. L.; Muller, G.; Brady, H.; Chan, K. Pomalidomide and lenalidomide regulated erythropoiesis and fetal hemoglobin production in human CD34+ cells. *J. Clin. Invest.* **2008**, *118*, 248−258.

(21) Kutlar, A.; Meiler, S.; Swerdlow, P.; Knight, R. Thalidomide and its analogs for hemoglobinopathies: two birds with one stone? *Expert Rev. Hematol.* **2012**, *5* (1), 9−11.

(22) Ribeiro, R. A; Vale, M. L.; Ferreira, S. H.; Cunha, F. Q. Analgesic effect of thalidomide on inflammatory pain. *Eur. J. Pharmacol.* **2000**, *391*, 97−103.

(23) Xu, Y.; Li, J.; Ferguson, G. D.; Mercurio, F.; Khambatta, G.; Morrison, L.; Lopez-Girona, A.; Corral, L. G.; Webb, D. R.; Bennett, B. L.; Xie, W. Immunomodulatory drugs reorganize cytoskeleton by modulating Rho GTPases. *Blood* **2009**, *114* (2), 338−345.

(24) Dos Santos, J. L.; Lanaro, C.; Lima, L. M.; Gambero, S.; Franco-Penteado, C. F.; Alexandre-Moreira, M. S.; Wade, M.; Yerigenahally, S.; Kutlar, A.; Meiler, S. E.; Costa, F. F.; Chung, M. C. Design, synthesis, and pharmacological evaluation of novel hybrid compounds to treat sickle cell disease symptoms. *J. Med. Chem.* **2011**, *54* (16), 5811−5819.

(25) Cerecetto, H.; Porcal, W. Pharmacological properties of furoxans and benzofuroxans: recent developments. *Mini-Rev. Med. Chem.* **2005**, *5* (1), 57−71.

(26) Scatena, R.; Bottoni, P.; Pontoglio, A.; Giardina, B. Pharmacological modulation of nitric oxide release: new pharmacological perspectives, potential benefits and risks. *Curr. Med. Chem.* **2010**, *17*, 61−73.

(27) Cerecetto, H.; Dias, E.; Di Maio, R.; González, M.; Pacce, S.; Saenz, P.; Seoane, G.; Suescun, L.; Mombrú, A.; Fernández, G.; Lema, M.; Villalba, J. Synthesis and herbicidal activity of *N*-oxide derivatives. *J. Agric. Food Chem.* **2000**, *48*, 2995−3002.

(28) Sorba, G.; Medana, C.; Fruttero, R.; Cena, C.; Di Stilo, A.; Galli, U.; Gasco, A. Water Soluble Furoxan Derivatives as NO Prodrugs. *J. Med. Chem.* **1997**, *40*, 463−469; 2288.

(29) Gasco, A.; Mortarini, V.; Rua, G.; Serafino, A. Unsymmetrically substituted furoxans. III. Methylnitrofuroxan: Its structure and behaviour toward nucleophilic substitution. *J. Heterocycl. Chem.* **1973**, *10*, 587−590.
(30) Gasco, A.; Boulton, A. J. Furazans and furazan oxides. Part IV. The structures and tautomerism of some unsymmetrically substituted furoxans. *J. Chem. Soc., Perkin Trans. 2* **1973**, 1613−1617.
(31) Ovchinnikov, I. V.; Strelenko, Y. A.; Popov, N. A.; Finogenov, A. O.; Makhova, N. N. A study of the reaction mechanism of 3-nitro-4-*R*-furoxans formation by nitrosation of dipotassium salts of 1-hydroxyimino-2,2-dinitro-1-*R*-ethanes. *Russ. Chem. Bull.* **2011**, *60* (5), 855−860.
(32) Tsikas, D. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2007**, *851*, 51−70.
(33) Collier, H. O. J.; Dinneen, L. C.; Johnson, C. A.; Schneider, C. The abdominal constriction response and its suppression by analgesic drugs in the mouse. *Br. J. Pharmacol.* **1968**, *32*, 285−291.
(34) Hayashi, M.; Morita, T.; Kodama, Y.; Sofuni, T.; Ishidate, M. J. The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. *Mutat. Res.* **1990**, *245*, 245−249.
(35) Cartron, J. P.; Elion, J. Erythroid adhesion molecules in sickle cell disease: effect of hydroxyurea. *Transfus. Clin. Biol.* **2008**, *15*, 39−50.
(36) Lou, T. F.; Singh, M.; Mackie, A.; Li, W.; Pace, B. S. Hydroxyurea generates nitric oxide in human erythroid cells: mechanisms for gamma-globin gene activation. *Exp. Biol. Med.* **2009**, *234*, 1374−1382.
(37) Santos, J. L.; Varanda, E. A.; Lima, L. M.; Chung, M. C. Mutagenicity of new lead compounds to treat sickle cell disease symptoms in Salmonella/microsome assay. *Int. J. Mol. Sci.* **2010**, *11*, 779−788.
(38) Santos, J. L.; Bosquesi, P. L.; Varanda, E. A.; Lima, L. M.; Chung, M. C. Assessment of the in vivo genotoxicity of new lead compounds to treat sickle cell disease. *Molecules* **2011**, *16*, 2982−2989.
(39) Meiler, S. E.; Wade, M.; Kutlar, F.; Yerigenahally, S. D.; Xue, Y.; Moutouh-de Parseval, L. A.; Corral, L. G.; Swerdlow, P. S.; Kutlar, A. Pomalidomide augments fetal hemoglobin production without the myelosuppressive effects of hydroxyurea in transgenic sickle cell mice. *Blood* **2011**, *118* (4), 1109−1112.
(40) Morris, C. R.; Gladwin, M. T.; Kato, G. J. Nitric oxide and arginine dysregulation: a novel pathway to pulmonary hypertension in hemolytic disorders. *Curr. Mol. Med.* **2008**, *8* (7), 620−632.
(41) Mack, A. K.; McGowan, I. V. R.; Tremonti, C. K.; Ackah, D.; Barnett, C.; Machado, R. F.; Gladwin, M. T.; Kato, G. J. Sodium nitrite promotes regional blood flow in patients with sickle cell disease: a phase I/II study. *Br. J. Hamaetol.* **2008**, *142* (6), 971−978.
(42) Kaul, D. K.; Zhang, X.; Dasgupta, T.; Fabry, M. E. Arginine therapy of transgenic-knockout sickle mice improves microvascular function by reducing non-nitric oxide vasodilators, hemolysis, and oxidative stress. *Am. J. Physiol.: Heart Circ. Physiol.* **2008**, *295* (1), H39−H47.
(43) Head, C. A.; Swerdlow, P.; McDade, W. A.; Joshi, R. M.; Ikuta, T.; Cooper, M. L.; Eckman, J. R. Beneficial effects of nitric oxide breathing in adult patients with sickle cell crisis. *Am. J. Hematol.* **2010**, *85* (10), 800−802.
(44) Gladwin, M. T.; Kato, G. J.; Weiner, D.; Onyekwere, O. C.; Dampier, C.; Hsu, L.; Hagar, R. W.; Howard, T.; Nuss, R.; Okam, M. M.; Tremonti, C. K.; Berman, B.; Villella, A.; Krishnamurti, L.; Lanzkron, S.; Castro, O.; Gordeuk, V. R.; Coles, W. A.; Peters-Lawrence, M.; Nichols, J.; Hall, M. K.; Hildesheim, M.; Blackwelder, W. C.; Baldassarre, J.; Casella, J. F. Nitric oxide for inhalation in the acute treatment of sickle cell pain crisis: a randomized controlled trial. *JAMA, J. Am. Med. Assoc.* **2011**, *305* (9), 893−902.
(45) Canalli, A. A.; Franco-Penteado, C. F.; Saad, S. T.; Conran, N.; Costa, F. F. Increased adhesive properties of neutrophils in sickle cell disease may be reversed by pharmacological nitric oxide donation. *Haematologica* **2008**, *93* (4), 605−609.
(46) Cokic, V. P.; Smith, R. D.; Beleslin-Cokic, B. B.; Njoroge, J. M.; Miller, J. L.; Gladwin, M. T.; Schechter, A. N. Hydroxyurea induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl cyclase. *J. Clin. Invest.* **2003**, *111* (2), 231−239.
(47) Vandy Black, L.; Smith, W. R. Evidence-based mini-review: Are systemic corticosteroids an effective treatment for acute pain in sickle cell disease? *Hematology Am. Soc. Hematol. Educ. Program* **2010**, 416−417.
(48) Ribeiro, R. A; Vale, M. L.; Ferreira, S. H.; Cunha, F. Q. Analgesic effect of thalidomide on inflammatory pain. *Eur. J. Pharmacol.* **2000**, *391*, 97−103.
(49) Conran, N.; Oresco-Santos, C.; Acosta, H. C.; Fattori, A.; Saad, S. T.; Costa, F. F. Increased soluble guanylate cyclase activity in the red blood cells of sickle cell patients. *Br. J. Hamaetol.* **2004**, *124*, 547−554.
(50) Burney, S.; Caulfield, J.; Niles, J.; Wishnok, J.; Tannenbaum, S. The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutat. Res.* **1999**, *424*, 37−49.
(51) Felley-Bosco, E. Role of nitric oxide in genotoxicity: Implication for carcinogenesis. *Cancer Metastasis Rev.* **1998**, *17*, 25−37.
(52) Balbo, S.; Lazzarato, L.; Di Stilo, A.; Fruttero, R.; Lombaert, N.; Kirsch-Volders, M. Studies of the potential genotoxic effects of furoxans: the case of CAS 1609 and of the water-soluble analogue of CHF 2363. *Toxicol. Lett.* **2008**, *178*, 44−51.
(53) Cabrera, M.; López, G. V.; Gómez, L. E.; Breijo, M.; Pintos, C.; Botti, H.; Raymondo, S.; Vettorazzi, A.; Ceráin, A. L.; Monge, A.; Rubbo, H.; González, M.; Cerecetto, H. Genetic toxicology and preliminary in vivo studies of nitric oxide donor tocopherol analogs as potential new class of antiatherogenic agents. *Drug Chem. Toxicol.* **2011**, *34*, 285−293.
(54) Machado, A. L.; Lima, L. M.; Araújo, J. X.; Fraga, C. A.; Koatz, V. L.; Barreiro, E. J. Design, synthesis and antiinflammatory activity of novel phthalimide derivatives, structurally related to thalidomide. *Bioorg. Med. Chem. Lett.* **2005**, *15* (4), 1169−1172.
(55) Cerecetto, H.; Di Maio, R.; Ibarruri, G.; Seoane, G.; Denicola, A.; Peluffo, G.; Quijano, C.; Paulino, M. Synthesis and Antitrypanosomal Activity of Novel 5-Nitro-2-furaldehyde and 5-Nitrothiophene-2-carboxaldehyde Semicarbazone Derivatives. *Farmaco* **1998**, *53* (2), 89−94.
(56) Cerecetto, H; Di Maio, R; González, M; Risso, M; Saenz, P; Seoane, G; Denicola, A; Peluffo, G; Quijano, C; Olea-Azar, C. 1,2,5-Oxadiazole *N*-oxide derivatives and related compounds as potential antitrypanosomal drugs: structure−activity relationships. *J. Med. Chem.* **1999**, *42*, 1941−1950.
(57) López, G. V.; Batthyány, C.; Blanco, F.; Botti, H.; Trostchansky, A.; Migliaro, E.; Radi, R.; González, M.; Cerecetto, H.; Rubbo, H. Design, synthesis, and biological characterization of potential antiatherogenic nitric oxide releasing tocopherol analogs. *Bioorg. Med. Chem.* **2005**, *13*, 5787−5796.
(58) Vandesompele, J.; De Preter, K.; Pattyn, F.; Poppe, B.; Van Roy, N.; De Paepe, A.; Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **2002**, *3* (7), research0034.1−research0034.11.