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Design, Synthesis, and Pharmacological Evaluation of Novel Hybrid Compounds To Treat Sickle Cell Disease Symptoms

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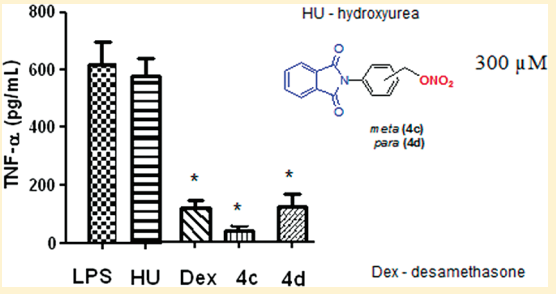
ABSTRACT: A novel series of thalidomide derivatives (**4a**−**f**) designed by molecular hybridization were synthesized and evaluated in vitro and in vivo for their potential use in the oral treatment of sickle cell disease symptoms. Compounds **4a**−**f** demonstrated analgesic, anti-inflammatory, and NO-donor properties. Compounds **4c** and **4d** were considered promising candidate drugs and were further evaluated in transgenic sickle cell mice to determine their capacity to reduce the levels of the proinflammatory cytokine tumor necrosis factor α (TNFα). Unlike hydroxyurea, the compounds reduced the concentrations of TNFα to levels similar to those induced with the control dexamethasone (300 μmol/kg). These compounds are novel lead drug candidates with multiple beneficial actions in the treatment of sickle cell disease symptoms and offer an alternative to hydroxyurea treatment.



INTRODUCTION

Sickle cell disease (SCD) is one of the most common genetic disorders worldwide, caused by a point mutation that changes glutamic acid (Glu6) to valine (Val6) in the β chain of hemoglobin. At low oxygen tensions, after constant cycles of oxygenation−deoxygenation, the sickle hemoglobin molecule (HbS) polymerizes inside the red blood cells, altering the erythrocyte cytoskeletal structure and leading to hemolysis or the formation of sickle-shaped red blood cells. The abnormal adhesion of these blood cells to the vascular endothelium contributes to a vaso-occlusive process.[1−3] Patients with SCD present a multifaceted pathophysiology characterized by painful vaso-occlusive crises, acute chest syndrome, priapism, pulmonary hypertension, stroke, and many other disorders that reduce their life expectancy.[4−6]

The cruelest characteristic of SCD is that there is no specific drug for its treatment. Pharmacological therapies are based on drugs that alleviate the main symptoms of the disease. Hydroxyurea (HU), a well-known ribonucleotide reductase inhibitor, is the only drug approved by the U.S. Food and Drug Administration (FDA) to treat SCD. HU is commonly used to treat a variety of myeloproliferative disorders, and its effectiveness in SCD has been attributed to its capacity to stimulate the production of fetal hemoglobin (HbF).[7,8] After its metabolism, HU can act as an exogenous nitric oxide (NO) source, which is responsible for numerous HU benefits, including the induction of γ-globin gene expression, vasodilatation, and the inhibition of platelet aggregation.[9−11] Individuals with SCD have shown reduced physiological NO levels in the vascular endothelium, resulting from the hemolytic process.[12] Much interest has centered on understanding the positive benefits of NO in the treatment of SCD after some studies demonstrated that NO inhalation reduced the severity and duration of vaso-occlusive crises in children.[10,13]

SCD patients demonstrate a chronic inflammatory response, with significantly increased levels of circulating proinflammatory cytokines, such as tumor necrosis factor α (TNFα).[14−16] High

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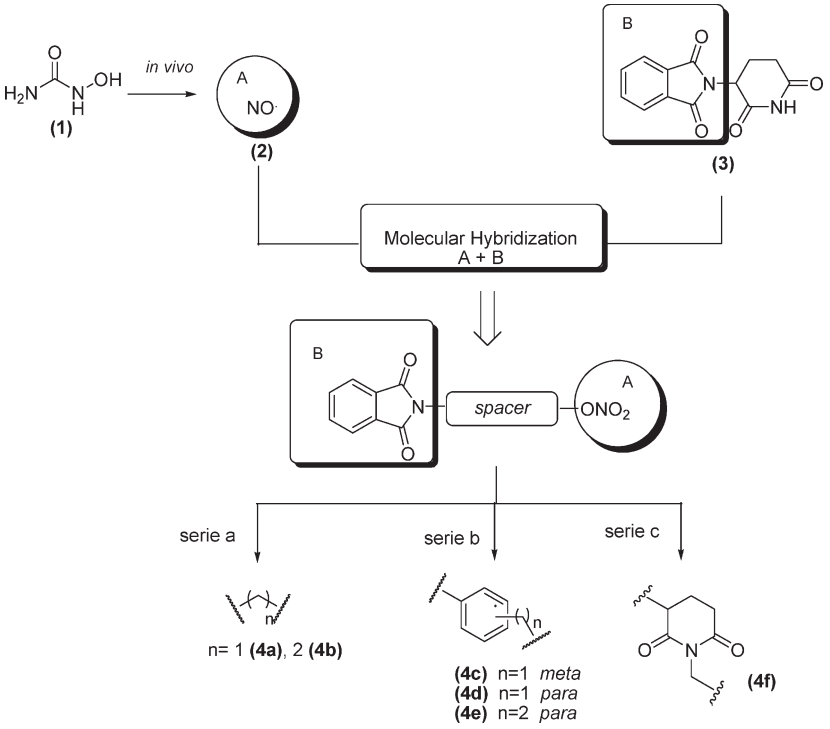
Scheme 1. Structural Planning Using Molecular Hybridization



levels of TNFα increase the chemotactic properties of neutrophils and their adhesion to the vascular endothelium, contributing to the vaso-occlusion process. It has been reported that HU treatment increases TNFα levels, counteracting the beneficial effects of the drug.[17] TNFα also stimulates the production of free radicals and the synthesis of other inflammatory mediators, such as interleukin 1 (IL1) and prostaglandin E_2, induces changes in coagulants and anticoagulants properties, and sensitizes nociceptive terminals, aggravating pain crises.[18,19] Increased blood levels of TNFα in patients with sickle cell anemia can aggravate painful vaso-occlusive crises and lead to infection and inflammatory episodes.[20,21]

Thalidomide, first described as a hypnotic and sedative drug in the late 1950s, presents a well-known TNFα-inhibitor property. Nowadays the drug is used as an immunomodulatory agent to treat some diseases, such as multiple myeloma and leprosy. Thalidomide was also demonstrated to increase the production of reactive oxygen species, inducing γ-globin mRNA expression in a dose-dependent manner. Aerbajinai and co-workers (2007) postulated that this effect could be mechanistically associated with the p38 mitogen-activated protein kinase (MAPK) signaling pathway and histone H4 acetylation.[22] Other thalidomide derivatives with TNFα-inhibitory properties, such as pomalidomide and lenalidomide, induce HbF expression and modulate erythroid differentiation during the specific commitment of $CD34^+$ progenitor cells.[23] It has been hypothesized that pomalidomide regulates HbF by modifying the chromatin structure, which results from histone H3 acetylation. In vivo studies with transgenic sickle mice treated with pomalidomide showed augmented HbF expression similar to that produced by treatment with HU.[24]

In a continuing effort to develop new candidate drugs to treat SCD symptoms, we report here the design, synthesis, and pharmacological evaluation of thalidomide derivatives (**4a**−**f**) by the structural modification of the prototypes hydroxyurea (**1**) and thalidomide (**3**) (Scheme 1).

The design concept was based on the molecular hybridization of hydroxyurea (**1**) and thalidomide (**3**). Specifically, NO resulting from the bioconversion of HU was inserted into nitrate ester subunits to produce novel hybrid derivatives (**4a**−**f**) containing both pharmacophoric elements: NO-donor properties and TNFα-inhibitory properties. We thus combined analgesic, anti-inflammatory, and vasodilatory activities in a single structure.

CHEMISTRY

The synthetic routes for the preparation of the phthalimide derivatives (**4a**−**f**) are summarized in Schemes 2 and 3. The alkyl and aryl derivatives (Scheme 2) were obtained in two or three synthetic steps, with overall yields of 51%−80%. Compound **4f** was produced by the derivatization of the thalidomide molecule (Scheme 3).

The initial stage of the reactions used to produce the phthalimide derivatives (**6c**−**e**) involved the coupling of functionalized amino derivatives with phthalic anhydride in pyridine, with variable yields (68−89%). Compound **8a** was produced with a good yield (80%) by treating phthalimide with 37% formaldehyde under reflux conditions.[25] The phthalimide derivative **8b** was acquired commercially. Compound **10** was synthesized by the procedure described by Hess and co-workers (2001).[26]

The compounds containing alcohol functions (**6c**−**e**, **8a**,**b**, and **10**) were then reacted in dichloromethane with a mixture of glacial acetic acid and fuming nitric acid to produce the final organic nitrate ester compounds **4a**−**f**. The structures of all the compounds were established by mass spectrometry, elemental analysis, IR spectroscopy, and ^1H and ^{13}C NMR. All compounds

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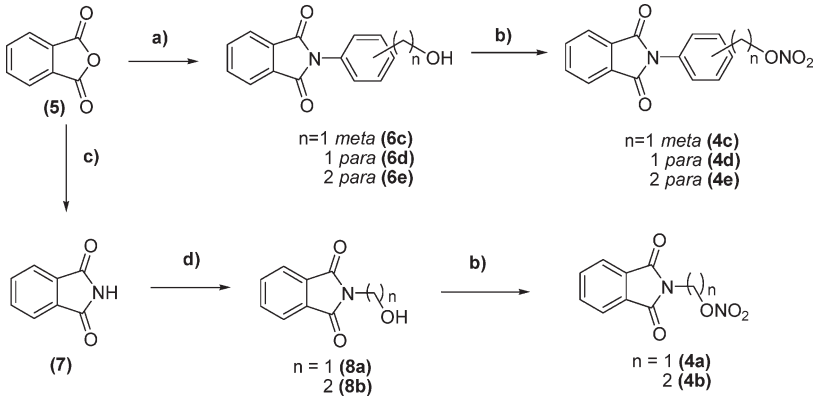
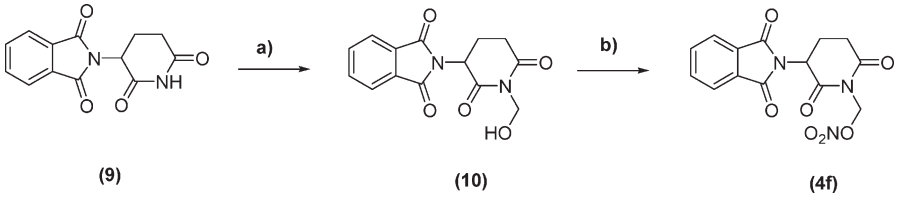
Scheme 2[a]



[a] Reagents and conditions: (a) pyridine, reflux, 2 h, 68−89%; (b) HNO_3/Ac_2O, CH_2Cl_2, room temp, overnight, 74−90%; (c) urea, 140°C, 20 min, 80%; (d) HCOH, H_2O, reflux, 1 h, 93%.

Scheme 3[a]



[a] Reagents and conditions: (a) HCOH, H_2O, reflux, 70%; (b) HNO_3/Ac_2O, CH_2Cl_2, room temp, overnight, 70%.

were analyzed by HPLC, and their purity was confirmed to be over 98.5%.

RESULTS

Detection of Nitrite (Griess Reaction). The in vitro ability of compounds **4a**−**f** to act as NO donors was measured using the Griess reaction by quantifying the nitrite produced from the oxidative reaction of nitric oxide, oxygen, and water.[27] The extent of thiol-induced NO generation was determined similarly after incubation for 1 h in the presence of a large excess of cysteine (1:50). The results, expressed as percentage nitrite (NO_2^-, mol/mol), are summarized in Table 1.

All organic nitrate ester compounds (**4a**−**f**) were capable of inducing nitrite formation at concentrations between 5.3% and 6.2%. Isosorbide dinitrate (DNS), used as the control, induced 11.7% nitrite formation. However, DNS has two organic nitrate ester groups (ONO_2) that can release NO, whereas compounds **4a**−**f** have only one group that releases NO.

Capsaicin-Induced Ear Edema. The anti-inflammatory activity of compounds **4a**−**f** was evaluated using the in vivo capsaicin-induced ear edema model,[28] and they were administered orally to mice at doses of 300 μmol/kg. Compounds **4a**−**f** all inhibited ear edema by about 43−65%, producing a significant anti-inflammatory effect similar to that produced by indomethacin, which was used as the positive control (Figure 1).

Thioglycollate-Induced Peritonitis in Mice. The anti-inflammatory effects of compounds **4a**−**f** were confirmed by testing their effects on thioglycollate-induced peritonitis in mice.[29] The thioglycollate stimulus induces a time-dependent accumulation of polymorphonuclear cells (PMNs) into the peritoneal cavity, with maximal cell influx after 4 h. The exudates obtained from the peritoneal cavities of rats 4 h after a thioglycollate injection were confirmed to contain >98% neutrophils (data not shown). As demonstrated in Figure 2, the accumulation of PMNs in the peritoneal cavity in response to thioglycollate was delayed by derivatives **4a**−**f**, indicating the ability of these compounds to inhibit leukocyte migration.

Antinociceptive Activity. The antinociceptive profiles of compounds **4a**−**f** were evaluated using acetic acid induced abdominal constrictions in mice.[30] The compounds were administered orally at 100 μmol/kg. Compound **4d** was the most active antinociceptive compound, remarkably reducing the acetic acid induced abdominal constrictions by 66%. Compounds **4a**, **4c**, **4e**, and **4f** showed antinociceptive activities similar to that of the control, dypirone, at the same dose. Compound **4b** reduced the induced abdominal constrictions by only 25% (Figure 3).

In Vitro TNFα Measurements. Levels of the cytokine TNFα were measured in the supernatants of monocyte cultures of adult knockout−transgenic sickle cell mice. The purity of the monocytes was >90%, as determined with May−Giemsa staining. Compounds **4c** and **4d** were added to the monocyte cultures at different concentrations (100 or 300 μM). Dexamethasone (Dex) was used as the positive anti-inflammatory control, and HU was used as the SCD drug control. After treatment with 100 or 300 μM HU, the levels of TNFα were 463.3 ± 130.9 and 579.8 ± 60.2 pg/mL, respectively. Dexamethasone (1 μM) inhibited 83% of TNFα. Only compound **4c** reduced the in vitro level of TNFα. Compound **4c** (100 and 300 μM) inhibited 45% and 91% of TNFα production, respectively, and compound **4d** (100 and 300 μM)

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Table 1. NO Release Data

compd	% NO_2^- (mol/mol),[a] 50 × 10^{-4} M L-Cys
DNS[b]	11.7 ± 0.3
4a	5.4 ± 0.1
4b	6.2 ± 0.2
4c	6.0 ± 0.05
4d	5.5 ± 0.08
4e	6.1 ± 0.06
4f	5.8 ± 0.15

[a] All values are the mean ± SEM. 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. [b] DNS: isosorbide dinitrate. DNS possesses two ONO_2 groups that may release NO, whereas compounds **4a**−**f** possess only one group that may release NO.

inhibited 67% and 84% of TNFα production, respectively (Figure 4).

DISCUSSION

Several strategies have been developed to identify compounds useful in the treatment of SCD.[31] These strategies involve (a) agents that reduce iron overload (chelating agents),[32] (b) agents that induce γ-globin expression and HbF synthesis (i.e., cytidine, azacytidine, short-chain fatty acids),[33] (c) agents that prevent hemoglobin dehydration (i.e., Gardos channel inhibitors),[34] (d) agents that bind covalently to hemoglobin (i.e., aldehyde derivatives),[3,35] (e) agents that modify rheological blood properties (i.e., poloxamer-188),[36] and (f) agents that increase NO availability.[37] In general, the compounds used in these strategies are available for therapeutic uses other than the treatment of SCD. In fact, the only drug approved by the regulatory agency U.S. FDA for the treatment of SCD is HU. Despite the beneficial effects of HU, long-term treatment with it is associated with several deleterious effects, which limit its life-long utility for SCD patients.[38−40]

Therefore, to find new therapeutic alternatives with which to treat SCD symptoms, we have designed and synthesized a series of thalidomide derivatives containing an organic nitrate ester function as an NO-donor subunit (Scheme 1). The NO-donor properties of the organic nitrate ester are well established in the literature. Therefore, because the most beneficial effect of HU is its ability to generate NO, we evaluated this effect for all the synthesized compounds (**4a**−**f**). All compounds (**4a**−**f**) demonstrated a similar ability to generate nitrite in the cell culture medium in the presence of cysteine, measured with the Griess reaction. Interestingly, it has been reported that NO donors such as *S*-nitrosocysteine and deta-NONOate can increase γ-globin mRNA in K562 cells.[41] The same kind of property was observed in these compounds, which induced the expression of γ-globin mRNA in K562 cells at lower concentrations than were used for HU (unpublished results).

The inflammatory pathway in SCD is an important therapeutic target. Recurrent inflammation causes the increased expression of adhesion molecules by activated leukocytes and vascular endothelial cells. Several studies have associated high levels of leukocyte adhesion molecules with the clinical severity of the disease.[42] The vaso-occlusion process is also frequently triggered by inflammatory processes.[43−45] Because inflammation plays a deleterious role in SCD, resulting in progressive damage to most organs including the lungs, brain, kidneys, bones, and

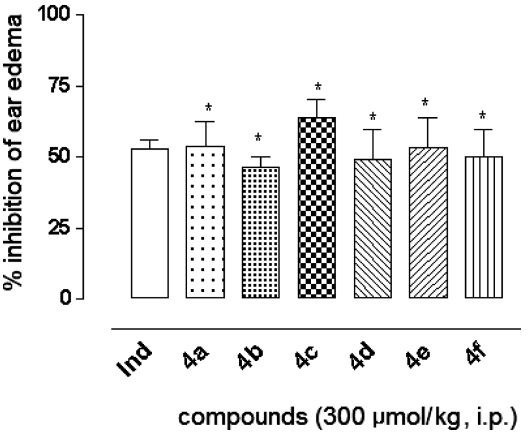

Figure 1. Effects of compounds **4a**−**f** and indometacin (Ind) on edema ear capsaicin-induced in mice. All compounds were administered po at a dose of 300 μmol/kg. Results are expressed as the mean ± SEM for $n = 8$ animals (triplicate). The % of inhibition was obtained by comparison with vehicle control group (data not shown): ∗, $P < 0.01$ (ANOVA followed by Dunnett's test).

cardiovascular system, compounds with anti-inflammatory activity should contribute to the control of some disease symptoms.

All the compounds (**4a**−**f**) demonstrated anti-inflammatory activity, with compound **4c** demonstrating activity higher than that of the positive control indomethacin. All the compounds (**4a**−**f**) inhibited leukocyte migration in a similar manner. It is well established that stimulated neutrophils produce superoxide and other highly reactive oxygen products capable of inducing cellular injury, so the inhibition of leukocyte migration is important in the prevention of the damage associated with the inflammatory processes. Both experiments showed that the synthesized compounds have important anti-inflammatory activities.

The inflammatory response also involves an increase in proinflammatory cytokines. It has been reported that patients with SCD present with high levels of TNFα, IL1, and IL6.[14] Malavé and co-workers (1993) reported an inverse correlation between the percentage of HbF and levels of TNFα in humans, demonstrating the importance of controlling cytokine levels.[16] Furthermore, Laurance and co-workers (2010) demonstrated that HU therapy stimulates the production of proinflammatory gene expression and levels of cytokines, such as TNFα, IL1A, IL1B, IL6, and IL8.[17] These cytokines increase the expression of vascular cell adhesion molecules on leukocytes, encouraging the initiation of the vaso-occlusion process.

To characterize the analgesic activity of compounds **4a**−**f**, we examined their effects after their oral administration in a mouse model of acetic acid induced abdominal constriction.[30] All the thalidomide derivatives (**4a**−**f**) showed analgesic activity, but the most active compound was **4d**, which reduced the acetic acid induced constrictions by 66%. Ribeiro and co-workers (2002) demonstrated that TNFα also plays an important role in the nociceptive writhing response in mice and that the analgesic effect of thalidomide results from the inhibition of this cytokine.[19] It has been observed that therapies that inhibit TNFα production have an important role in pain management.

The genotoxicity of the compounds was evaluated in vitro using a *Salmonella*/microsome assay and in vivo using the micronucleus test.[46] An interesting mutagenic relationship was observed

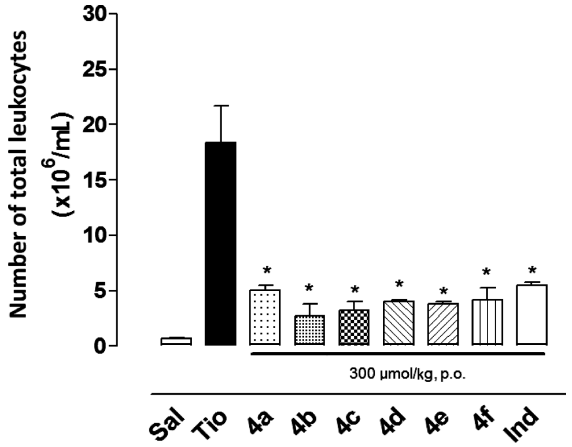

Figure 2. Effect of compounds **4a**−**f** and indomethacin (Ind) on cell migration in peritonite 3% thioglycollate-induced in mice. All compounds were administered po at a dose of 300 μmol/kg. Mice were killed at the time-point of 4 h after % thioglycollate-induced peritonitis. Total cell migration was counted using a Neubauer chamber. Data represent the mean ± SEM from at least six animals: ∗, $P < 0.01$ (ANOVA followed by Dunnett's test).

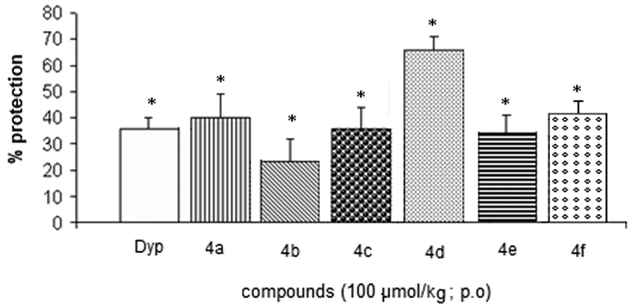

Figure 3. Antinociceptive effect of dypirone (Dyp) and compounds **4a**−**f** (all in doses of 100 μmol/kg, po) in the 0.6% acetic acid (AcOH) induced abdominal constrictions observed for 20 min after the administration of acetic acid. Data are expressed as the inhibition percentage of total writhings calculated from eight animals: ∗, $P < 0.01$ (ANOVA followed by Dunnett's test).

and allowed us to characterize compounds **4c** and **4d** as less mutagenic in the TA100 and TA102 strains of *Salmonella typhimurium* than the other compounds.[46] An in vivo micronucleus test using mouse peripheral blood demonstrated that all the compounds were less genotoxic than HU. All the compounds induced an average frequency of less than six micronucleated reticulocytes (MNRET) after their oral administration at 100 mg/kg, whereas HU induced an average frequency of 33.7 MNRET at the same concentration.[47]

Considering all these results, we selected compounds **4c** and **4d** for further evaluation of their TNFα inhibitory activity in adult knockout−transgenic sickle cell mice. Compounds **4c** and **4d** reduced the level of TNFα relative to that in the lipopolysaccharide (LPS) treated control mice. Both 100 and 300 μM showed dose-dependent effects. At 300 μM, compound **4c** decreased TNFα levels more than compound **4d** at the same concentration. Dexamethasone, the positive control, reduced TNFα but not to the same degree as compound **4c.** These results are consistent with the literature, which has demonstrated that the analgesic and anti-inflammatory activities of phthalimide

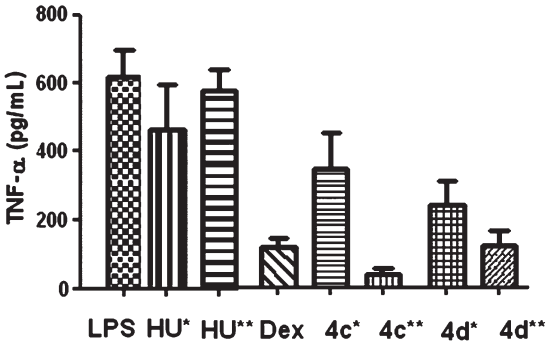

Figure 4. Level of proinflammatory mediator TNF-α, determined by ELISA in the supernatant of mononuclear culture treated with LPS and co-incubated with test drugs (**4c** and **4d**; 100 and 300 μM, 24 h), was used a positive anti-inflammatory control. Dexamethasone (Dex) at 1 μM was used a positive anti-inflammatory control. Hydroxyurea was used to determine its anti-inflammatory capacity (100 and 300 μM). Data are represented as the mean ± SEM; $P < 0.05$, compared to LPS (ANOVA followed by Dunnett's test); $N \geq 4$ experiments; ∗, 100 μM; ∗∗, 300 μM.

derivatives are related to their inhibition of TNFα.[48] HU showed no anti-inflammatory effect but an increase in the production of the inflammatory mediator TNFα at a concentration similar to that of the positive control (LPS). Lanaro and co-workers reported similar data, indicating that HU has proinflammatory properties.[14]

These results together demonstrate that combining the properties of thalidomide derivatives with NO-donor capacity represents a successful new approach to the treatment of some sickle cell disease symptoms and could be a therapeutic alternative to HU treatment.

CONCLUSIONS

A novel series of compounds (**4a**−**f**), designed by molecular hybridization and containing organic nitrate ester functions, was synthesized and characterized by mass spectrometry, elemental analysis, IR spectroscopy, and ^{1}H and ^{13}C NMR. Compounds **4a**−**f** inhibited leukocyte migration in a similar manner in a thioglycollate-induced model of peritonitis. All compounds demonstrated analgesic and anti-inflammatory activities and NO-donor properties. Compound **4d** was the most active antinociceptive compound, reducing acetic acid induced abdominal constrictions by 66%. Compound **4c** was more active than indomethacin, inhibiting 65% of ear edema. Compounds **4c** and **4d**, given at 300 μmol/kg, reduced TNFα levels in the supernatants of monocyte cultures from transgenic sickle cell mice, whereas HU showed no activity. Compounds **4c** and **4d** have emerged from these in vivo and in vitro studies as novel lead drug candidates 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 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−visible 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 Shimadzu Shim-pack CLC-ODS (M) column (5 μm particle, 250 mm × 4.6 mm i.d.). 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 (254−265 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 **7**, **8a**, and **10** were synthesized according to a previously described methodology.[25,26] Compound **8b** was purchased commercially.

General Procedure for the Synthesis of Phthalimide Derivatives (6c−e). A mixture of the selected amine (2.02 mmol), phthalic anhydride (**5**) (0.3 g, 2.02 mmol), and 10 mL of pyridine was stirred under nitrogen at 140 °C for 3 h. The mixture was then cooled and 50 mL of dichloromethane added; successive washes with a 10% copper sulfate solution were performed until a blue color was produced in aqueous phase. The organic phase was washed twice with 30 mL of water and dried with sodium sulfate or magnesium sulfate. After filtration, the organic phase was concentrated under reduced pressure to produce compounds with variable yields (70%−90%).

If necessary, the samples were further purified with silica gel column chromatography, using hexane/ethyl acetate (6:4) as the mobile phase.

*2-(3-(Hydroxymethyl)phenyl)isoindoline-1,3-dione (**6c**).* Yield: 82%. Mp: 149−151 °C. IR ν_{max} (cm^{-1}, KBr pellets): 3541 (O−H), 2933 (C−H), 1772 and 1716 (C═O imide). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.94 (2H; dd, H_6 and H_9; J = 8.4 Hz); 7.78 (2H; dd, H_7 and H_8; J_{orto} = 8.4 Hz); 7.49 (1H; d, $H_{2'}$); 7.41 (2H; dd, $H_{6'}$; J = 7.6 Hz); 7.37 ($H_{4'}$ and $H_{5'}$); 4.76 (2H; s, $H_{1''}$) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ: 166.9; 141.9; 123.4; 131.5; 125.4; 116.8; 126.3; 134.1; 132.0; 128.9; 64.4 ppm. Anal. Calcd for $C_{15}H_{11}NO_3$: C, 71.1; H, 4.38; N, 5.53. Found: C, 71.4; H, 4.11; N, 5.7.

*2-(4-(Hydroxymethyl)phenyl)isoindoline-1,3-dione (**6d**).* Yield: 63%. Mp: 155−158 °C. IR ν_{max} (cm^{-1}, KBr pellets): 3516 (O−H), 2916 (C−H), 1768 and 1710 (C═O imide). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.99 (2H; dd, H_6 and H_9; J = 8.5 Hz); 7.80 (2H; dd, H_7 and H_8; J = 8.5 Hz); 7.52 (2H; d, $H_{2'}$ and $H_{6'}$; J = 8.5 Hz); 7.44 (2H; d, $H_{3'}$ and $H_{5'}$; J = 8.5 Hz); 4.77 (2H; s, $H_{1''}$) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ: 167.2; 140.9; 134.4; 128.2; 131.7; 127.5; 123.7; 126.7; 64.8 ppm. Anal. Calcd for $C_{15}H_{11}NO_3$: C, 71.14; H, 4.38; N, 5.53. Found: C, 71.2; H, 4.21; N, 5.7.

*2-(4-(2-Hydroxyethyl)phenyl)isoindoline-1,3-dione (**6e**).* Yield: 74%. Mp: 172−175 °C. IR ν_{max} (cm^{-1}, KBr pellets): 3516 (O−H), 2916 (C−H), 1768 and 1701 (C═O imide). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.95 (2H; dd, H_6 and H_9; J = 8,5 Hz); 7.81 (2H; dd, H_7 and H_8); 7.38 (4H; m, $H_{2'}$, $H_{3'}$, $H_{5'}$ and $H_{6'}$); 3.9 (2H; t, $H_{2''}$); 2.93 (2H; t, $H_{1''}$) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ: 167.7; 139.1; 134.8; 132.1; 130.2; 127.1; 124.1; 117.6; 63.9; 39.3 ppm. Anal. Calcd for $C_{16}H_{13}NO_3$: C, 71.9; H, 4.9; N, 5.24. Found: C, 71.8; H, 4.8; N, 5.01.

General Procedure for the Synthesis of Phthalimide Derivatives (4a−e). A mixture of 2.61 mmol of properly functionalized phthalimide derivative was added to 17 mL of dichloromethane. After dissolution, 0.0062 g (0.103 mmol) of urea was added. The reaction mixture was cooled to 0 °C in an ice bath and was added dropwise to a previously prepared mixture of 3 mL of dichloromethane containing 0.42 mL of HNO_3 (9.56 mmol) and 1.16 mL (10.44 mmol) of acetic anhydride. The mixture was stirred continuously for 24 h at room temperature under nitrogen atmosphere. The compounds were isolated by the addition of 40 mL of dichloromethane and 30 mL of ice−water. The organic phase was isolated and washed three more times with 20 mL of saturated sodium bicarbonate solution. The organic phase was evaporated at reduced pressure to obtain compounds **4a−e** with variable yields (65−85%).

If necessary, the compounds were further purified with silica gel column chromatography using hexane/ethyl acetate (6:4) as the mobile phase.

*(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl Nitrate (**4a**).* Yield: 74%. Mp: 75−77 °C. IR ν_{max} (cm^{-1}, KBr pellets): 2929 (C−H), 1768 and 1701 (C═O imide), 1622 and 1278 (O-NO_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.93 (2H; m, H_6 and H_9); δ 7.80 (2H; m, H_7 and H_8; J = 8,1 Hz); 6.08 (2H; s, H_{10}) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ: 166.0; 135.0; 131.5; 124.3; 66,7 ppm. Anal. Calcd for $C_9H_6N_2O_5$: C, 48.6; H, 2.7; N, 12.6. Found: C, 48.4; H, 2.5; N, 12.5. MS/ESI m/z: $[M]^+$ 222.0, $[M - ONO_2]^+$ 161.05 (100%).

*2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)ethyl Nitrate (**4b**).* Yield: 80%. Mp: 87−89 °C. IR ν_{max} (cm^{-1}, KBr pellets): 2925 (C−H), 1762 and 1716 (C═O imide), 1622 and 1278 (O−NO_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.85 (2H; m, H_6 and H_9); 7.73 (2H; m, H_7 and H_8; J = 7.9 Hz); 4.66 (2H; t, H_{11}); 4.05 (2H; t, H_{10}) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ: 167.82; 134.3; 131.8; 123.6; 69.52; 35.22 ppm. Anal. Calcd for $C_{10}H_8N_2O_5$: C, 50.85; H, 3.41; N, 11.86. Found: C, 50.92; H, 3.5; N, 11.9. MS/ESI m/z: $[M]^+$ 236.0, $[M - ONO_2]^+$ 175 (100%).

*3-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)benzyl Nitrate (**4c**).* Yield: 90%. Mp: 126−128 °C. IR ν_{max} (cm^{-1}, KBr pellets): 2926 (C−H), 1774 and 1722 (C═O imide), 1620 and 1279 (O−NO_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.98 (2H; dd, H_6 and H_9; J = 8.1 Hz); 7.82 (2H; dd, H_7 and H_8; J = 8.1 Hz); 7.57 (1H; d, $H_{2'}$); 7.53 (1H; dd, $H_{5'}$ J = 7.8 Hz); 7.55 (1H; d, $H_{6'}$); 7.45 (1H; d, $H_{4'}$); 5.5 (2H; s, $H_{1''}$) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ: 167; 124.1; 133.7; 131.5; 127.6; 127.0; 128.9; 134.8; 132.5; 129.8; 74.2 ppm. Anal. Calcd for $C_{15}H_{10}N_2O_5$: C, 60.4; H, 3.38; N, 9.39. Found: C, 60.1; H, 3.5; N, 9.45. MS/ESI m/z: $[M]^+$ 298, $[M - ONO_2]^+$ 237 (54%).

*4-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)benzyl Nitrate (**4d**).* Yield: 88%. Mp: 159−161 °C. IR ν_{max} (cm^{-1}, KBr pellets): 2924 (C−H), 1786 and 1718 (C═O imide), 1620 and 1279 (O−NO_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.98 (2H; dd; H_6 and H_9; J = 8.4 Hz); 7.81 (2H; dd, H_7 and H_8; J = 8.4 Hz); 7.5 (2H; d, $H_{3'}$ and $H_{5'}$; J = 8.4 Hz); 7.53 (2H; d, $H_{2'}$ and $H_{6'}$; J = 8.4 Hz); 5.48 (2H; s, $H_{1''}$) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ: 167.2; 124.1; 133.0; 132.3; 131.9; 137.8; 126.9; 129.9; 74.1 ppm. Anal. Calcd for $C_{15}H_{10}N_2O_5$: C, 60.4; H, 3.38; N, 9.39. Found: C, 60.6; H, 3.22; N, 9.53. MS/ESI m/z: $[M]^+$ 298, $[M - ONO_2]^+$ 237 (65%).

*2-[4-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)phenyl]ethyl nitrate (**4e**).* Yield: 76%. Mp: 141−144 °C. IR ν_{max} (cm^{-1}, KBr pellets): 2924 (C−H), 1786 and 1718 (C═O imide), 1610 and 1283 (O−NO_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.95 (2H; m, H_6 and H_9; J = 8.4 Hz); 7.79 (2H; m, H_7 and H_8); 7.41 (2H; d, $H_{2'}$ and $H_{6'}$); 7.37 (2H; d, $H_{3'}$ and $H_{5'}$; J = 8.5 Hz); 4.66 (2H; t, $H_{2''}$); 3.08 (2H; t, $H_{1''}$) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ: 167.7; 132.1; 134.8; 131.1; 130.1; 127.3; 124.2; 117.6; 73.4; 33.4 ppm. Anal. Calcd for $C_{16}H_{12}N_2O_5$: C, 61.54; H, 3.87; N, 8.97. Found: C, 61.6; H, 3.72; N, 8.8. MS/ESI m/z: $[M]^+$ 312, $[M - ONO_2]^+$ 251 (47%).

[3-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl nitrate (**4f**). Yield: 70%. Mp: 162−165 °C. IR ν_{max} (cm^{-1}, KBr pellets): 2930 (C−H), 1785 and 1715 (C═O imide), 1613 and 1281 (O−NO_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.0 (4H; m, H_6, H_7, H_8, H_9); 6.2 (2H; s, $H_{1''}$); 5.3 (1H; t, $H_{1'}$); 2.8 (2H; q, $H_{2'}$); 2.2 (2H; t, $H_{3'}$) ppm. ^{13}C NMR (400 MHz, $CDCl_3$) δ: 171.5, 170.3, 168.4; 134.8; 131.1; 124.2; 69.5; 49; 30.2, 19.1 ppm. Anal. Calcd for $C_{14}H_{11}N_3O_7$: C, 50.46; H, 3.33; N, 12.61. Found: C, 50.67; H, 3.11; N, 12.8. MS/ESI m/z: $[M]^+$ 333, $[M - ONO_2]^+$ 272 (29%).

Pharmacology. Drugs and Reagents. The agents used here were acetic acid and indomethacin (Merck), arabic gum, and dipyrone (Sigma Chemical). A solution of 2.5% formalin was prepared with formaldehyde (Merck) in saline (NaCl 0.9%). The compounds and standards were used as suspensions in arabic gum in all experiments.

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 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.[27]

Animals. Adult male and female 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 (lights on at 0600) in a controlled-temperature room (22 ± 2 °C). The mice had free access to food and water. Groups of six animals were used in each test group, and the control animals received vehicle only. The experiments were performed after the protocol was approved by the local Institutional Ethics Committee (No. 006443/2005/78). 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.

Capsaicin-Induced Ear Edema. Ear edema was induced in female Swiss mice (8 weeks old) as previously described.[28] The mice were divided into groups of six and had access to food and water ad libitum. Each mouse received 12.5 μg/mL capsaicin in acetone on the right ear. The phlogistic agent was applied with an automatic pipet in 10 μL volumes to both the inner and outer surfaces of the ear. The test compounds (100 μmol/kg) and indomethacin, which was used as the standard reference drug (100 μmol/kg), were administrated po in arabic gum 1 h before treatment. The control group received vehicle only (10 mL/kg, po). The left ear was treated with acetone, delivered in the same manner. Thirty minutes after the capsaicin application, the mice were killed and both ears were removed. Circular sections were taken, using a cork borer with a diameter of 7 mm, and weighed. The increase in weight caused by the irritant was measured by subtracting the weight of the untreated left ear section from that of the treated right ear sections. Ear edema was measured as the differences in weight between the challenged and unchallenged ears.

Percentage inhibition was calculated using the formula $[(C - T)/(3C)] \times 100$, where *C* and T indicate the untreated (vehicle) control edema and drug-treated edema, respectively. The significance of the differences between the experimental groups and the control group was determined using ANOVA in the program Prisma. The differences were considered significant when $P < 0.05$. The results are expressed as the mean ± SEM, as indicated in the captions to the figures.

Thioglycollate-Induced Peritonitis in Mice. This series of experiments was performed according to a previously described method.[29] The animals were treated with the thalidomide derivatives or indomethacin (300 μmol/kg, po in arabic gum) and 1 h afterward were administered ip 1 mL of 3% thioglycollate (Difco-BD Biosciences). After 4 h, the animals were killed by cervical dislocation and their peritoneal cavities were washed with 1.5 mL of cold PBS and gentle manual massage. The cells were retrieved from the peritoneal exudates and their volumes measured. The significance of the differences between the experimental groups and the control group was calculated with ANOVA in the program Prisma. Differences were considered significant when $P < 0.05$. The results are expressed as the mean ± SEM, as indicated in the captions to the figures.

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.[30] Swiss mice of both sexes (18−23 g) were used. 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 minutes 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 the mean ± SEM of 10 animals per group. The data were analyzed statistically with Student's *t* test at a significance level of $P < 0.05$.

Monocyte Stimulation and in Vitro TNFα Measurement. Adult knockout−transgenic sickle cell mice were anesthetized with ketamine/xylazine, and their blood was collected by intracardiac puncture. The mononuclear cells were purified from the peripheral blood using Ficoll gradient separation. The mononuclear cells were placed in plastic dishes with Dulbecco's modified Eagle's medium and 10% calf serum and incubated for 2 h in humidified air (5% CO_2 at 37 °C). The purity of the monocytes was >90%, as determined by May−Giemsa staining. Compounds **4c** and **4d** in the same medium containing calf serum were added to the monocyte cultures at different concentrations (100 or 300 μM) 30 min before the addition of LPS (1 μg/mL). Dexamethasone (1 μM) was used as the positive anti-inflammatory control. The possible anti-inflammatory effect of HU was evaluated at two different concentrations (100 and 300 μM). After incubation for 20 h, the supernatants were collected and stored at −80 °C. The levels of the cytokine TNFα were measured in the supernatants of the monocyte cultures. The concentration of TNFα was determined by enzyme-linked immunosorbent assay (ELISA), with a commercially available ELISA kit (R&D Systems, Minneapolis, MN, U.S.).

Statistical Analysis. Data were analyzed statistically using the INSTAT software, version 3.0. The results were compared before and after treatment with the new compounds using ANOVA followed by Dunnett's test. A *P* value of less than 0.05 was considered to be statistically significant.

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ABBREVIATIONS USED

SCD, sickle cell disease; Hb, hemoglobin; HU, hydroxyurea; FDA, Food and Drug Administration; NO, nitric oxide; TNFα, tumor necrosis factor α; DNS, isosorbide dinitrate; IL, interleukin; LPS, lipopolysaccharides

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