European Journal of Pharmaceutical Sciences 83 (2016) 114–119

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Thalidomide analogues: Tumor necrosis factor-alpha inhibitors and their evaluation as anti-inflammatory agents

Juan José Casal [a,1], Mariela Bollini [a,1], María Elisa Lombardo [b,*], Ana María Bruno [a,*]

[a] Departamento de Química Orgánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113. Ciudad Autónoma de Buenos Aires, Argentina
[b] Centro de Investigaciones sobre Porfirias y Porfirinas (CIPYP, UBA-CONICET), Hospital de Clínicas José de San Martín, Avenida Córdoba 2351, 1120. Ciudad Autónoma de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 16 September 2015
Received in revised form 28 November 2015
Accepted 11 December 2015
Available online 12 December 2015

Keywords:
Phthalimides
Phthalazinone derivate
TNF-α
Anti-inflammatory effect

ABSTRACT

A series of related thalidomide derivatives (**2**–**9**) were synthesized by microwave irradiation and evaluated for anti-inflammatory activity. Such activity was assessed in vivo and ex vivo. Compounds **2**, **8** and **9** showed the highest levels of inhibition of TNF-α production. On rat paw edema and hyperalgesia assays, compound **9**, (1,4-phthalazinedione) demonstrated the highest in vivo anti-inflammatory activity. Thus, compound **9** can be considered as a promising compound to be subjected to further modification to obtain new agents for the treatment of inflammatory diseases.

1. Introduction

Thalidomide (2-(2,6-dioxo-3-piperidyl)isoindoline-1,3-dione), compound **1,** is a synthetic glutamic acid derivative developed as a sedative hypnotic agent to treat emesis during pregnancy (Fig. 1). Despite its early successful clinical results, this compound had to be withdrawn from the market due to its teratogenic effects (Ito et al., 2011). Nevertheless, over the past years, the interest in this drug has resurged due to its potential usefulness in the treatment of erythema nodosum leprosum (Sampaio et al., 1993; Haslett et al., 2005), multiple myeloma (Singhal et al., 1999), AIDS as well as various cancers (Jacobson et al., 1999; Singhal and Mehta, 2001). Thalidomide has been found to have several biological activities, including, the inhibition of tumor necrosis factor-α (TNF-α) production (Sampaio et al., 1991, Moreira et al., 1993), as well as anti-inflammatory, anti-angiogenic (D'Amato et al., 1994), and cyclooxygenase inhibitory activities (Sano et al., 2005).

TNF-α was originally described as a circulating factor that can cause necrosis of tumors. Later, it has been identified as a key regulator of the inflammatory response. The central role of TNF-α in inflammation has been demonstrated through the ability of TNF-α blocking agents to treat a wide range of inflammatory conditions.

TNF-α production inhibitory activity was initially considered to be one of the key thalidomide action mechanisms. This cytokine plays a critical role in several physiological immunological processes, causing severe damage when produced in excess.

A number of phthalimide derivatives have been previously synthesized employing different strategies in order to obtain molecules that can act as modulators of the over production of TNF-α (Niwayama et al., 1998; Muller et al., 1998; Lima et al., 2002; Cupertino Da Silva et al., 2010; Stewart et al., 2007; Zhu et al., 2003; Machado et al., 2005; Chaulet et al., 2011; Tweedie et al., 2011). In an attempt to maintain the beneficial properties while avoiding its side effects, new achiral synthetic thalidomide analogues have been designed.

In this work we optimized the synthesis process and evaluated the anti-inflammatory activity of a series of phthalimides that are, structurally related to thalidomide (Fig. 1). To test the anti-inflammatory activity in vivo (rat paw edema, hyperalgesia and myeloperoxidase activity) and ex vivo (TNF-α production) assays were employed. A phthalazinone derivative was synthesized and its anti-inflammatory activity was also evaluated.

2. Materials and Methods

Melting points (uncorrected) were determined in a capillary with an Electrothermal 9100 SERIES-Digital apparatus. The microwave-assisted reaction was carried out in Monowave 300 Anton Paar. Reactions were monitored by thin-layer chromatography (TLC) in silica gel plates (F245 Merck) and the products visualized under ultraviolet light (254 and 365 nm).

IR spectra were recorded with a FT Perkin Elmer Spectrum One employing KBr discs. ^{1}H- and ^{13}C-NMR spectra were determined in DMSO-d_6 and $CDCl_3$ solutions using a Bruker 300 MHz and Bruker Biospin 600 MHz AVIII600 spectrometers at room temperature with

* Corresponding authors.
E-mail addresses: elombardo@qb.fcen.uba.ar (M.E. Lombardo), anabruno@ffyb.uba.ar (A.M. Bruno).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ejps.2015.12.017
0928-0987/

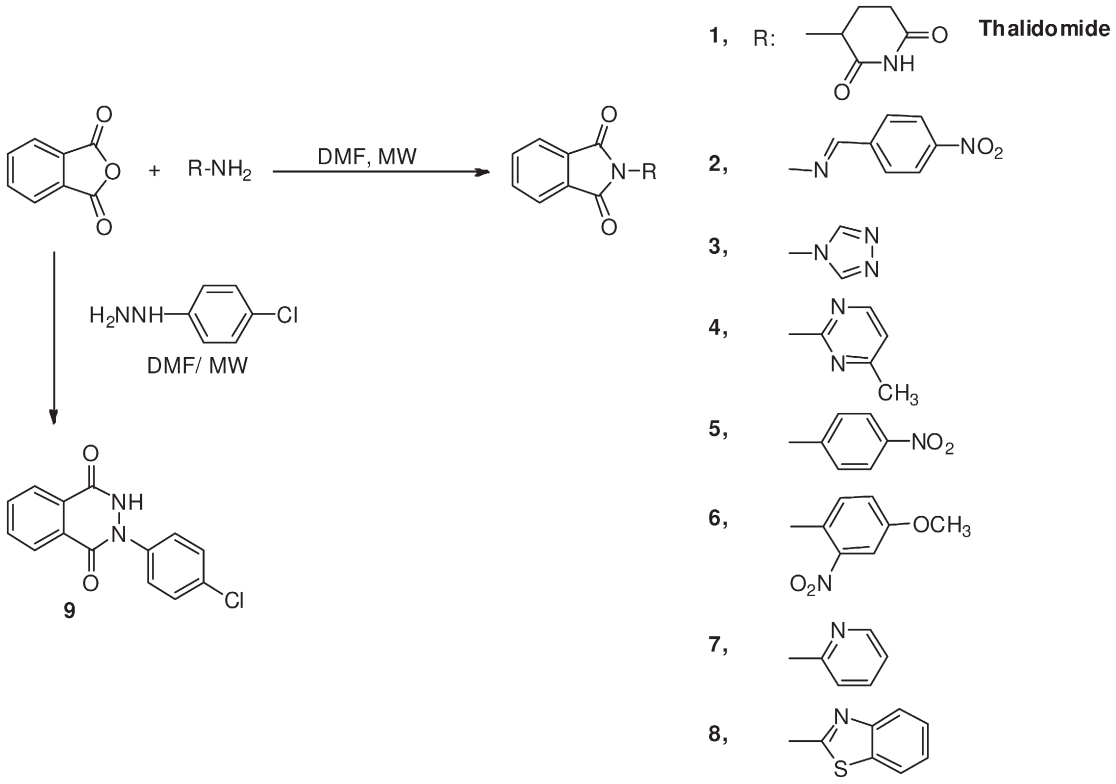


Fig. 1. Structure of thalidomide (compound **1**). Synthesis of phthalimide derivatives (compounds **2–9**) from phthalic anhydride and the corresponding amines.

tetramethylsilane as internal standard. Chemical shifts (δ) are reported in ppm and coupling constants (*J*) in Hertz.

2.1. Chemistry

2.1.1. General Procedure for the Synthesis of Phthalimide Derivatives

A mixture of phthalic anhydride (2.5 mmol, 0.37 g), 2.5 mmol of the corresponding amine and 0.1 mL of DMF were subjected to microwave irradiation (Fig. 1). Reaction time and temperature varies for each derivative being synthesized. The reaction was monitored by TLC employing Cl_2CH_2: MeOH (8:2) as mobile phase. After complete conversion, the reaction mixture was triturated with ethanol and the solid product was recrystallized from a solvent mixture (EtOH: H_2O, 2:1).

2.1.1.1. (E)-2-((4-Nitrobenzylidene)amino)isoindoline-1,3-dione ***(2)***. Reaction temperature: 170 °C, reaction time: 2 min. A pale yellow solid was obtained after recrystallization. Yield: 0.45 g (62%). Mp: 260–264 °C. Mp Lit. (Hearn and Lucero, 1982; Salman and Ray, 1981): 301–305 °C and 290 °C. IR (KBr, ν cm^{-1}): 1724 (CO), 1597 (CN), 1513 and 1345 (NO_2), 837 (C—H). ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.48 (s, 1 H, *H*CN), 8.28 (d, 2 H, *J* = 8.2 Hz, Ar*H*), 7.76–7.84 (m, 3 H, Ar*H*), 7.50 (dd, 3 H, *J* = 7.8 1.6 Hz, Ar*H*).

^{13}C NMR (151 MHz, DMSO-d_6): δ 163.9, 148.5, 136.7, 135.6, 129.5, 129.3, 125.3, 124.7, and 124.1.

2.1.1.2. 2-(4 H-1,2,4-Triazol-4-yl)isoindoline-1,3-dione ***(3)***. Reaction temperature: 170 °C, reaction time: 4:00 min. A white solid was obtained after recrystallization. Yield: 0.52 g (87%). Mp: 266–269 °C. Mp Lit. (Sena et al., 2003): 269.7–270.4 °C. ^{1} H NMR (300 MHz, DMSO-d_6): δ 8.44 (s, 2 H, *H-3′*, *H-5′*), 7.84 (d, 2 H, *J* = 7.5 Hz, Ar*H*), 7.74 (d, 2 H, *J* = 7.5 Hz, Ar*H*). ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.9, 141.2, 135.5, 133.8, 132.9, 128.9, 119.4, and 118.7.

2.1.1.3. 2-(4-Methylpirimidin-2-yl)isoindolin-1,3-dione ***(4)***. Reaction temperature: 160 °C, reaction time: 4:00 min. A white solid was obtained after recrystallization. Yield: 0.13 g (22%). Mp: 178–180 °C. Mp Lit. (Cingolani et al., 1976): 188–190 °C. ^{1} H NMR (300 MHz, DMSO-d_6): δ 8.78 (d, 1 H, *J* = 6.0 Hz, *H-6′*), 7.99–7.97 (m, 2 H, Ar*H*), 7.84–7.80 (m, 2 H, Ar*H*, *H-5′*), 7.25 (dt, 1 H, *J* = 6.0, 0.51 Hz, Ar*H*), 2.89 (s, 3 H, *CH*$_3$). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.8, 167.6, 158.5, 135.2, 133.9, 132.9, 128.9, 126.1, and 21.1.

2.1.1.4. 2-(4-Nitrophenyl)isoindolin-1,3-dione ***(5)***. Reaction temperature: 160 °C, reaction time: 2:00 min. A yellow solid was obtained after recrystallization. Yield 0.54 g (82%). Mp: 273–275 °C. Mp. Lit. (Aliabadi et al., 2014): 265 °C. IR (KBr, ν cm^{-1}): 1738 (CO), 1523 and 1346 (NO_2), 837 (C—H). ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.42 (d, 2 H, *J* = 8.6 Hz, Ar*H*), 8.02 (dt, 2 H, *J* = 7.6, 3.9 Hz, Ar*H*), 7.95 (dd, 2 H, *J* = 5.5, 3.1 Hz, Ar*H*), 7.81 (d, 2 H, *J* = 8.6 Hz, Ar*H*).^{13}C NMR (151 MHz, DMSO-d_6): δ 166.8, 146.6, 138.2, 135.4, 131.9, 128.2, 124.6, and 124.2.

2.1.1.5. 2-(4-Methoxy-2-nitrophenyl)isoindolin-1,3-dione ***(6)***. Reaction temperature: 160 °C, reaction time: 4:00 min. A yellow solid was obtained after recrystallization. Yield: 0.65 g (88%). Mp: 132–134 °C. Mp Lit. (Davood et al., 2013): 148–153 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.03 (dd, 2 H, *J* = 5.5, 3.0 Hz, Ar*H*), 7.97 (td, 2 H, *J* = 5.2, 2.1 Hz, Ar*H*), 7.76 (d, 1 H, *J* = 2.9 Hz, Ar*H*), 7.70 (d, 1 H, *J* = 8.8 Hz, Ar*H*), 7.53 (dd, 1 H, *J* = 8.8, 2.9 Hz, Ar*H*), 3.94 (s, 3 H, O*CH*$_3$). ^{13}C NMR (151 MHz, DMSO-d_6): δ 166.9, 160.2, 146.8, 135.7, 132.8, 131.8, 124.3, 120.8, 117.5, 110.9, and 56.8.

2.1.1.6. 2-(Pyridin-2-yl)isoindolin-1,3-dione ***(7)***. Reaction temperature: 170 °C, Reaction time: 4:00 min. A white solid was obtained after recrystallization. Yield 0.49 g (88%). Mp: 227–230 °C. Mp Lit. (Guirado et al., 1997): 225–226 °C. IR (KBr, ν cm^{-1}): 1713 (CO), 1586 and 1571 (CC and CN), 717 (C—H).^{1}H NMR (600 MHz, DMSO-d_6): δ 8.66 (dd, 1 H, *J* = 7.1, 3.1 Hz, Ar*H*), 8.06 (t, 1 H, *J* = 7.0 Hz, Ar*H*), 8.01 (dd, 2 H, *J* = 7.1, 3.1 Hz, Ar*H*), 7.95 (dd, 2 H, *J* = 7.3, 3.0 Hz, Ar*H*), 7.58–7.53 (m, 2 H, Ar*H*). ^{13}C NMR (151 MHz, DMSO-d_6): δ 166.9, 149.8, 146.3, 139.1, 135.4, 131.8, 124.5, 124.1, and 123.5.

*2.1.1.7. 2-(Benzo[d]tiazol-2-yl)isoindolin-1,3-dione **(8)***. Reaction temperature: 170 °C, reaction time: 4:00 min. A white solid was obtained after recrystallization. Yield: 0.64 g (92%). Mp: 147–148 °C. Mp Lit. (El Sadek et al., 1989): 143–147 °C. IR (KBr, ν cm^{-1}): 1725 (CO), 1594 and 1512 (CC and CN), 838 (C—H). ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.16 (dd, 1 H, J = 7.4, 3.1 Hz, Ar*H*), 8.07–8.03 (m, 3 H, Ar*H*), 7.98 (d, 2 H, J = 7.4 Hz, Ar*H*), 7.57 (t, 1 H, J = 7.4 Hz, Ar*H*), 7.48 (t, 1 H, J = 7.4 Hz, Ar*H*). ^{13}C NMR (151 MHz, DMSO-d_6): δ 165.0, 152.5, 149.4, 135.7, 133.1, 131.5, 126.9, 125.7, 124.6, 122.8, and 122.4.

*2.1.1.8. 2-(4-Chlorophenyl)2,3-dihydrophthalazin-1,4-dione **(9)***. Reaction temperature: 180 °C, reaction time: 4:00 min. A white solid was obtained after recrystallization. Yield: 0.49 g (74%). Mp: 182–184 °C. (Prime et al., 2011). IR (KBr, ν cm^{-1}): 3165 (NH), 1663 (CO), 1496 (NH) and 790 (C—Cl). ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.54 (s, 1 H, N*H*), 7.98 (dt, 2 H, J = 7.4, 3.7 Hz, Ar*H*), 7.93–7.92 (m, 2 H), 7.62 (d, J = 8.6 Hz, 2 H), 7.51 (d, J = 8.6 Hz, 2 H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.0, 160.6, 135.2, 133.0, 132.3, 131.3, 129.6, 129.4, and 124.0.

2.2. Biology

2.2.1. Animals

Male BALB/c mice (25–30 g) and male Sprague–Dawley rats (240–270 g) were obtained from the LASSBio breeding unit (Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Brazil). Animals were maintained in a temperature-controlled room and received water and food ad libitum. Animals received human care and were treated according to the "Principles of Laboratory Animal Care and Use in Research" (Colégio Brasileiro de Experimentacão Animal - COBEA/Instituto Brasileiro Carlos Chagas Filho — IBCCF, Brazil) that is based upon the International Guidelines for the Care and Use of Laboratory Animals and Ethical Guidelines for the Investigation of Experimental Pain in Conscious Animals (Zimmerman, 1983).

2.2.2. Ex Vivo Anti-Inflammatory Activity Assay: TNF-α Production

Male BALB/c mice were used throughout this study. Animals were pretreated with saline solution (vehicle) ip. 45 min before LPS inhalation. The inhalation procedure was performed as previously described (Corti et al., 1994). After aspiration, airspaces were washed with saline to obtain 4 ml of bronchoalveolar lavage fluid (BALF). Aliquots of BALF were used for TNF-α assay after incubation with either drugs (10–100 μM) or with thalidomide (100–300 μM). The concentration of TNF-α was determined by ELISA according to the manufacturer's instructions. The assay had a detection limit of 250 pg/ml (Genzyme, Cambridge, MA, USA).

2.2.3. In Vivo Anti-Inflammatory Activity Assays

2.2.3.1. Rat Paw Edema. Male Sprague–Dawley rats were divided in different groups which received an ip. injection of test compound (10 mg/kg as a suspension in 1 ml of 0.5% methylcellulose). Control animals received an equal volume of vehicle. An hour later each group of animals received a subplantar injection of 0.2 ml of either 1% carrageenan solution in saline (w/v) or sterile saline (0.9% NaCl) on the right and left hind paws respectively. Paw volumes were measured with a plethysmometer (UGO Basile, Italy) immediately before the subplantar injection (0 h) and 3 h after. The edema (μl) was calculated by subtracting the difference between the carrageenan- and saline-treated paws evaluated at 0 h to the same difference measured at 3 h.

2.2.3.2. Hyperalgesia. Carrageenan-induced hyperalgesia was quantified in separate groups of animals as a measure of the nociceptive response to a thermal stimulus using the hot-plate test with the temperature adjusted to 51 ± 2 °C (Bannon, 2001). For this study male Sprague–Dawley rats were used and test drugs were administered orally at a dose of 10 mg/kg, 1 h before carrageenan administration. The control group received only vehicle (10 ml/kg). Animals were then injected subplantarly with 0.2 ml of either 1% carrageenan solution in saline (w/v) or sterile saline (0.9% NaCl) on the right and left hind paws respectively. Each hind paw was placed on the heated surface at intervals of 1 h for 4 h after carrageenan injection and the time between placement and the first sign of paw licking or withdrawal was recorded as latency. The latency time (withdrawal time) of each hind paw was determined and the anti-inflammatory activity defined as a decrease in the Δ latency (s) calculated as difference between carrageenan and saline paw latency times. A cut-off time of 40 s was established to prevent injury to the paws.

2.2.3.3. Myeloperoxidase Activity. Animals were treated as described above for the rat paw edema test. The methodology used to measure MPO activity was adapted from the one previously described by Posadas et al. (2004). Briefly, rats were killed 6 h after carrageenan administration and inflamed paws were weighed, cut and suspended in hexadecyl trimethylammonium bromide (HTBA) buffer (containing 5 g HTBA in 1 l potassium phosphate buffer 50 mM pH 6.0) in ratio of 50:1 (mg tissue:ml buffer). Resuspended tissues were homogenized at 4 °C with a Turrax ® at 13,000 rpm for 3 min. After centrifugation at 13,000 rpm for 2 min, supernatants were assayed for MPO activity. Samples (100 μl) were mixed with 50 mM potassium phosphate buffer pH 6.0 (100 μl) containing 2 mM *o*-dianisidine dihydrochloride and 0.002% hydrogen peroxide in a microtiter plate. The absorbance at 450 nm was measured after incubation at 25 °C with constant stirring for 15 min. One unit of MPO is defined as the amount of enzyme converting 1 μmol of hydrogen peroxide to water in 1 min at 25 °C. MPO activity was expressed as units/mg fresh inflamed tissue.

2.2.4. Data Analysis

Results are presented as means ± n S.E.M. of measurements made on at least four to six animals in each group. Differences between responses were evaluated by the Student *t*-test and two ways analysis of variance (ANOVA). Statistical differences were considered significant from $P < 0.05$.

To calculate physicochemical descriptors PaDEL-descriptor software were performed. (Yap, 2011).

3. Results and Discussion

3.1. Chemistry

The general method for the synthesis of *N*-substituted (alkyl, aryl, heteroaryl) phthalimides employing the corresponding amines and phthalic anhydride requires long reflux reaction times. The synthesis methods using microwave irradiation shorten reaction times and provide higher yields. Therefore, the target compounds were obtained within minutes by a microwave-assisted method. With the exception of compound **4**, good yields and excellent purities were obtained (Fig. 1). Reaction conditions were optimized by using different solvents (dioxane, acetic acid and DMF) observing that DMF as an aprotic reaction solvent at 160–170 °C gave the best yields (compounds **2**–**8**).

By applying microwave radiation, compound **9** was synthesized in one step obtaining a very good yield.

The synthesis of these compounds involving the reaction of phthalic anhydride with electron-rich phenylhydrazines affords the corresponding phthalazine-1,4-diones in one step, though with moderate yields (30–50%), whereas electron poor phenylhydrazines require an additional ring-expansion step under harsh conditions after the formation of the initial isoindole-1,3-diones to give the target phthalazine-1,4-diones in good yields (70–80%) (Prime et al., 2011).

The structures of compounds **2–9** were established on the basis of spectral (^{1}H NMR, ^{13}C NMR, FTIR) analyses and they were identical to those found in literature.

All synthesized compounds meet the rule of Oprea (Oprea et al., 2001) which considers the physicochemical descriptors required to generate leadlike compounds (Table 1).

3.2. Anti-Inflammatory Activity

TNF-α is a cytokine considered to be a primary mediator of the inflammatory response. To evaluate if the compounds **2–9** had anti-inflammatory activity, its effect on TNF-α production was investigated. The results (Fig. 2A) revealed that all compounds tested at 10 and 100 μM had higher anti-inflammatory activity than thalidomide at 100 and 300 μM (in our experiments thalidomide was employed for comparison purposes rather than as a positive control). Compound **2** was not tested at 100 μM due to its low solubility in DMSO. Compound **5** showed, independently of the concentration, equal inhibitory activity. A different behavior was observed for compound **3**, which showed a lower inhibitory activity at the highest concentration.

Fig. 2B shows the IC_{50} (50% inhibitory concentration) values obtained for the different thalidomide derivatives. Compounds **2**, **8** and **9** with IC_{50} values of 8.4, 1.2 and 2.1 μM respectively, were selected for further testing since they were the ones displaying the highest inhibitory activities on TNF-α production.

Finally, we investigated the possible correlation between the IC_{50} values and the physicochemical properties (Table 1). A good inverse correlation ($r = -0.8738$, $P < 0.05$) was observed between inhibitory activity on TNF-α production and CLogP, indicating that higher ClogP values (high lipophilicity and ability to cross membranes) correlated with low IC_{50} values (higher anti-inflammatory activity). However, no relationship was found with the other physicochemical descriptors.

Results for the determination of anti-inflammatory activity of compounds **2**, **8** and **9** on the rat paw edema are illustrated in Fig. 3. At the concentration tested (10 mg/kg), compound **9** was the only one which exerted inhibition of the paw edema (36%). The inhibition produced by the compounds **2**, **8** and thalidomide showed no statistically significant differences as compared to control animals ($P < 0.05$).

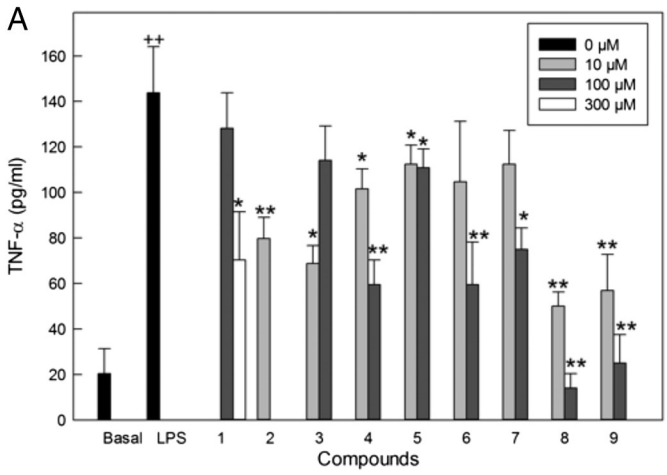
Fig. 4 depicts the effect of the treatment with compounds **2**, **8** and **9** (10 mg/kg) on carrageenan-induced hyperalgesia (compound **1** was used for comparison). During the period spanning from 0 to 4, the injection of carrageenan induced a substantial increase in Δ latency, which was significantly lower (about 45%) in rats treated with compound **9**. No significant differences with the control group were found for animals treated with compounds **1**, **2**, or **8**.

Finally, the values of MPO activity, a neutrophil sequestration marker enzyme, were determined (Fig. 5). All the three compounds tested

Table 1
Calculated physicochemical properties.

Compound	CLogP[a]	nRB[b]	nHBA[c]	nHBD[d]	MW[e]	TopoPSA[f]	CMR[g]
1	0.11	1	6	1	258.232	83.55	66.36
2	2.45	3	7	0	295.059	92.88	83.93
3	0.18	1	6	0	214.049	68.09	56.36
4	2.26	2	5	0	239.069	63.16	68.01
5	2.75	2	6	0	268.048	80.52	76.00
6	2.76	3	7	0	298.059	89.75	82.55
7	2.38	1	4	0	224.059	50.27	65.26
8	4.00	1	4	0	280.031	78.51	81.69
9	3.14	2	4	1	272.035	49.41	75.62

[a] CLogP: Crippen's logP.
[b] nRB: number rotatable bonds.
[c] nHBA: number hydrogen bond acceptors.
[d] nHBD: number of hydrogen bond donors.
[e] MW: molecular weight (Dalton).
[f] TopoPSA: Topological polar surface area.
[g] CMR: Crippen's molar refractivity.



B

Compounds	IC_{50} (μM)
1	239.1 ± 23.4
2	8.4 ± 1.2
3	ND
4	28.9 ± 10.9
5	> 100
6	31.2 ± 15.2
7	64.1 ± 14.2
8	1.2 ± 0.9
9	2.1 ± 0.9

Fig. 2. A: Effect of the thalidomide (compound **1**) and a series of phthalimides (compounds **2–9**) on TNF-α production in BALF from mice pre-treated with LPS. (++) $P < 0.01$: significant difference between LPS-induced TNF-α levels and basal level. (*) $P < 0.05$ and (**) $P < 0.01$ significant difference between TNF-α production in BALF from mice pre-treated with LPS in the presence and absence of test compounds. B: IC_{50} values for tested compounds. IC_{50}: concentration that inhibits 50% of TNF-α production.

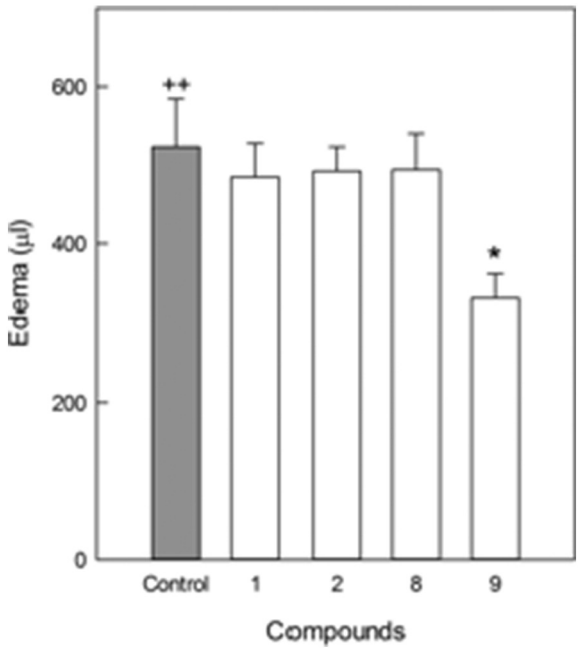


Fig. 3. Effect of the thalidomide (compound **1**) and the most active compounds (**2**, **8**, and **9**) administered intraperitoneally on the carrageenan-induced rat paw edema. (++) $P < 0.01$: significant difference between carrageenan-induced group and group treated with the vehicle. (*) $P < 0.05$ significant difference between carrageenan-induced group in the presence and absence of test compounds.

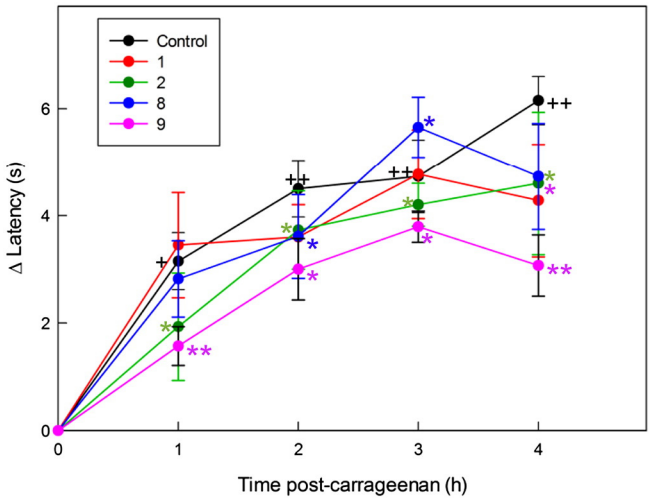


Fig. 4. Time course effect of thalidomide (compound **1**) and selected phthalimides (**2**, **8**, and **9**) administered orally on carrageenan-induced rat hyperalgesia. (+) $P < 0.05$ and (++) $P < 0.01$: significant difference between carrageenan-induced group and the group treated with the vehicle. (*) $P < 0.05$ and (**) $P < 0.01$ significant difference between carrageenan-induced group in the presence and absence of test compounds.

reduced MPO activity significantly as compared to the control group (between 48 and 60%, $P = 0.05$). As expected, inhibition of this enzyme was correlated with decreased production of TNF-α. For animals treated with thalidomide, the MPO activity values were similar ($P = 0.05$) to those corresponding to the control group.

Our results suggested that replacement of the glutarimide ring of thalidomide by *N*-phenyl functionalized and *N*-heteroaromatic derivatives improved the inhibitory activity on TNF-α production. Compounds **2** and **8** exhibited remarkable anti-inflammatory activity and were 30- and 200-fold more potent than thalidomide ($IC_{50} = 8.4$ and 1.2 μM vs 239.1 μM) respectively. Differently, compounds **3** and **5** did not show inhibitory activities on TNF-α production.

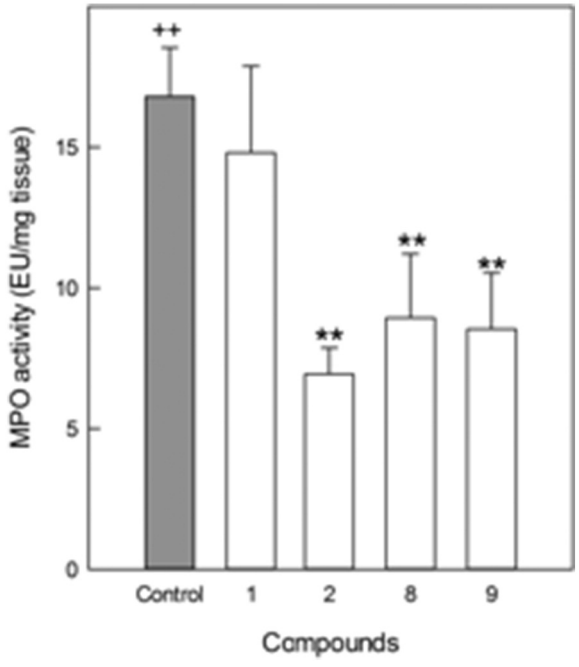


Fig. 5. Effect of pretreatment with compounds **1**, **2**, **8** and **9** on MPO in carrageenan-induced inflammation of rat paws. (++) $P < 0.01$: significant difference between carrageenan-induced group and the group treated with the vehicle. (**) $P < 0.01$ significant difference between carrageenan-induced group in the presence and absence of test compounds.

When the phthalimide ring was replaced by a phthalazinone structure (compound **9**) afforded the most potent in vivo anti-inflammatory agent of all synthesized compounds. It can be considered to be scaffold for the future design and development of anti-inflammatory agents.

4. Conclusion

In this work, we report the use of microwave irradiation to carry out the synthesis of compounds **2–9**. These compounds were screened for their anti-inflammatory activity (inhibition of TNF-α production, rat paw edema, hyperalgesia and myeloperoxidase activity). Compound **9**, a phthalazin-1,4-dione, may be considered as a new prototype molecule with excellent anti-inflammatory features that manifest both in vivo and ex vivo.

The importance of this compound cannot be ignored, as it is an intermediary in the lead optimization process.

Acknowledgments

Authors wish to thank Dr. A. L. Palhares de Miranda and Dr. L. Moreira Lima for hosting us in your laboratory. JJC is also thankful to the PROSUL-CNPq (CNP-q 490600/07) (Programa Sul Americano de Apoio às Atividades de Cooperação em Ciência e Tecnología- Conselho Nacional de Desenvolvimento Científico e Tecnológico-Brazil), the grant received to carry out anti-inflammatory tests at Laboratório de Avaliação e Síntese de Substâncias Bioativas (LASSBio), Departamento de Fármacos, Faculdade de Farmácia, UFRJ, Brazil of E. J. Barreiro. This work was supported by a grant from University of Buenos Aires UBACyT 20020100100770BA.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejps.2015.12.017.

References

Aliabadi, A., Gholamine, B., Karimi, T., 2014. Synthesis and antiseizure evaluation of isoindoline-1,3-dione derivatives in mice. Med. Chem. Res. 23 (6), 2736–2743.
Bannon, A.W., 2001. Models of pain: hot-plate and formalin test in rodents. Current Protocols in Pharmacology. John Wiley and Sons, Inc. (00:5.7:5.7.1–5.7.11).
Chaulet, C., Croix, C., Alagille, D., Normand, S., Delwail, A., Favot, L., Lecron, J.C., Viaud-Massuard, M.C., 2011. Design, synthesis and biological evaluation of new thalidomide analogues as TNF-α and IL-6 production inhibitors. Bioorg. Med. Chem. Lett. 21, 1019–1022.
Cingolani, G.M., Giardina, D., Carotti, A., Casini, G., Ferappi, M., 1976. Phthalimide derivatives of pyrimidine and thiazole heterocycles. Farmaco, Edizione Scientifica 31(2), pp. 133–139.
Corti, A., Poiesi, C., Merli, S., Cassani, G., 1994. Tumor necrosis factor (TNF) α quantification by ELISA and bioassay: effects of TNF α-soluble TNF receptor (p55) complex dissociation during assay incubations. J. Immunol. Methods 177, 191–198.
Cupertino Da Silva, Y.K., Villarinho Augusto, C., de Castro Barbosa, M.L., Muniz de Albuquerque Melo, G., de Queiroz, C., de Lima Matos Freire Dias, T., Bispo Júnior, W., Barreiro, E.J., Lima, L.M., Alexandre-Moreira, M.S., 2010. Synthesis and pharmacological evaluation of pyrazine N-acylhydrazone derivatives designed as novel analgesic and anti-inflammatory drug candidates. Bioorg. Med. Chem. 18, 5007–5015.
D'Amato, R.J., Loughnan, M.S., Flynn, E., Folkman, J., 1994. Thalidomide is an inhibitor of angiogenesis. Proc. Natl. Acad. Sci. U. S. A. 91, 4082–4085.
Davood, A., Amini, M., Azimidoost, L., Rahmatpour, S., Nikbakht, A., Iman, M., Shafaroodi, H., Ansari, A., 2013. Docking, synthesis, and pharmacological evaluation of isoindoline derivatives as anticonvulsant agents. Med. Chem. Res. 22 (7), 3177–3184.
El Sadek, M.M., El Essawi, M.M., Abdel Baky, S.A., 1989. Synthesis and spectra of some phthalimido derivatives. J. Chem. Eng. Data 34 (2), 257–259.
Guirado, A., Zapata, A., Ramirez de Arellano, M.C., 1997. The reaction of phthalidylidene dichloride with primary amines. Synthesis and X-ray molecular structure of *N*-substituted phthalisoimides. Tetrahedron 53 (14), 5305–5324.
Haslett, P.J., Roche, P., Butlin, C.R., Macdonald, M., Shrestha, N., Manandhar, R., Lemaster, J., Hawksworth, R., Shah, M., Lubinsky, S., Albert, M., Worley, J., Kaplan, G., 2005. Effective treatment of erythema nodosum leprosum with thalidomide is associated with immune stimulation. J. Infect. Dis. 192, 2045–2053.
Hearn, M.J., Lucero, E.R., 1982. Reactions of *N*-aminophthalimide with electrophiles. II. Preparation and properties of araldehyde hydrazones. J. Heterocycl. Chem. 19, 1537–1539.
Ito, T., Ando, H., Handa, H., 2011. Teratogenic effects of thalidomide: molecular mechanisms. Cell. Mol. Life Sci. 68, 1569–1579.

Jacobson, J.M., Spritzler, J., Fox, L., Fahey, J.L., Jackson, J.B., Chernoff, M., Wohl, D., Wu, W., Hooton, T.M., Sha, B.E., Shikuma, C.M., MacPhail, L., Simpson, D.M., Trapnell, C.B., Basgoz, N., 1999. Thalidomide for the treatment of esophageal aphthous ulcers in patients with human immunodeficiency virus infection. National Institute of Allergy and Infectious Disease AIDS Clinical Trials Group. J. Infect. Dis. 180, 61–67.

Lima, L.M., Castro, P., Machado, A.L., Fraga, C.A.M., Lugnier, C., De Moraes, V.L.G., Barreiro, E.J., 2002. Synthesis and anti-inflammatory activity of phthalimide derivatives, designed as new thalidomide analogues. Bioorg. Med. Chem. 10, 3067–3073.

Machado, A.L., Lima, L.M., Araújo Jr., J.X., Fraga, C.A., Koatz, V.L., Barreiro, E.J., 2005. Design, synthesis and antiinflammatory activity of novel phthalimide derivatives, structurally related to thalidomide. Bioorg. Med. Chem. Lett. 15, 1169–1172.

Moreira, B.A.L., Sampaio, E.P., Zmuidzinas, S.A., Frindt, P., Smith, K.A., Kaplan, G., 1993. Thalidomide exerts its inhibitory action on tumor necrosis factor α by enhancing mRNA degradation. J. Exp. Med. 177, 6–11.

Muller, G.W., Shire, M.G., Wong, L.M., Corral, L.G., Patterson, R.T., Chen, Y., Stirling, D.I., 1998. Thalidomide analogs and PDE4 inhibition. Bioorg. Med. Chem. Lett. 8, 2669–2674.

Niwayama, S., Loh, C., Turk, B.E., Liu, J.O., Miyachi, H., Hashimoto, Y., 1998. Enhanced potency of perfluorinated thalidomide derivatives for inhibition of LPS-induced tumor necrosis factor-α production is associated with a change of mechanism of action. Bioorg. Med. Chem. Lett. 8, 1071–1076.

Oprea, T.I., Davis, A.M., Teague, S.J., Leeson, P.D., 2001. Is there a difference between leads and drugs? A historical perspective. J. Chem. Inf. Comput. Sci. 41, 1308–1315.

Posadas, I., Bucci, M., Roviezzo, F., Rossi, A., Parente, L., Sautebin, L., Cirino, G., 2004. Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. Br. J. Pharmacol. 142, 331–338.

Prime, M.E., Courtney, S.M., Brookfield, F.A., Marston, R.W., Walker, V., Warne, J., Boyd, A.E., Kairies, N.A., von der Saal, W., Limberg, A., Georges, G., Engh, R.A., Goller, B., Rueger, P., Rueth, M., 2011. Phthalazinone pyrazoles as potent, selective, and orally bioavailable inhibitors of aurora-a kinase. J. Med. Chem. 54 (1), 312–319.

Salman, M., Ray, S., 1981. Studies in antifertility agents: part XXXVI-syntheses of *N*-(substituted benzylidene)-aminophthalimides, -dihydroisoindoles and -1,2,3,4-tetrahydro-isoquinolines. Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem. 20B (6), 477–479.

Sampaio, E.P., Sarno, E.N., Galilly, R., Cohn, Z., Kaplan, G., 1991. Thalidomide selectively inhibits tumor necrosis factor alpha production by stimulated human monocytes. J. Exp. Med. 173, 699–703.

Sampaio, E.P., Kaplan, G., Miranda, Nery, J., Miguel, C.P., Viana, S.M., Sarno, E.N., 1993. The influence of thalidomide on the clinical and immunologic manifestation of erythema nodosum leprosum. J. Infect. Dis. 168, 408–414.

Sano, H., Noguchi, T., Tanatani, A., Hashimoto, Y., Miyachi, H., 2005. Design and synthesis of subtype-selective cyclooxygenase (COX) inhibitors derived from thalidomide. Bioorg. Med. Chem. 13, 3079–3091.

Sena, V.L.M., Srivastava, R.M., Silva, R.O., Lima, V.L.M., 2003. Synthesis and hypolipidemic activity of *N*-substituted phthalimides. Part V. Farmaco 58, 1283–1288.

Singhal, S., Mehta, J., 2001. Thalidomide in cancer. Biomed. Pharmacother. 56, 4–12.

Singhal, S., Mehta, J., Desikan, R., Ayers, D., Roberson, P., Eddlemon, P., Munshi, N., Anaissie, E., Wilson, C., Dhodapkar, M., Zeldis, J., Barlogie, B., 1999. Antitumor activity of thalidomide in refractory multiple myeloma. N. Engl. J. Med. 341, 1565–1571.

Stewart, S.G., Spagnolo, D., Polomska, M.E., Sin, M., Karimi, M., Abraham, L.J., 2007. Synthesis and TNF expression inhibitory properties of new thalidomide analogues derived via heck cross coupling. Bioorg. Med. Chem. Lett. 17, 5819–5824.

Tweedie, D., Frankola, K.A., Luo, W., Li, Y., Greig, N.H., 2011. Thalidomide analogues suppress lipopolysaccharide-induced synthesis of TNF-α and nitrite, an intermediate of nitric oxide, in a cellular model of inflammation. Open Biochem. J. 5, 37–44.

Yap, C.W., 2011. PaDEL-descriptor: an open source software to calculate molecular descriptors and fingerprints. J. Comput. Chem. 32, 1466–1474.

Zhu, X., Giordano, T., Yu, Q.S., Holloway, H.W., Perry, T.A., Lahiri, D.K., Brossi, A., Greig, N.H., 2003. Thiothalidomides: novel isosteric analogues of thalidomide with enhanced TNF-alpha inhibitory activity. J. Med. Chem. 46, 5222–5229.

Zimmerman, M., 1983. Ethical guidelines for investigation of experimental pain in conscious animals. Pain 16, 109–110.