Abstract

Herein, we describe the synthesis and pharmacological evaluation of novel N-phenylpyrazolyl-N-glycinyl-hydrazide derivatives that were designed as novel prototypes of p38 mitogen-activated protein kinase (MAPK) inhibitors. All of the novel synthesized compounds described in this study were evaluated for their in vitro capacity to inhibit tumor necrosis factor α (TNF-α production in cultured macrophages) and in vitro MAPK p38α inhibition. The two most active anti-TNF-α derivatives, \((E)-2-(3\text{-}tert\text{-}butyl\text{-}1\text{-}phenyl\text{-}1H\text{-}pyrazol\text{-}5\text{-}yl)\text{-}N\text{-}((4\text{-}(2\text{-}morpholinooethoxy)naphthalen\text{-}1\text{-}yl)methylene)acetohydrazide (4a) and \((E)-2-(3\text{-}tert\text{-}butyl\text{-}1\text{-}phenyl\text{-}1H\text{-}pyrazol\text{-}5\text{-}yl)\text{-}N\text{-}((4\text{-}chlorobenzylidene)acetohydrazide (4f), were evaluated to determine their in vivo anti-hyperalgesic profiles in carrageenan-induced thermal hypernociception model in rats. Both compounds showed anti-inflammatory and antinociceptive properties comparable to SB-203580 used as a standard drug, by oral route at a dose of 100 μmol/kg. This bioprofile is correlated with the ability of NAH derivatives (4a) and (4f) suppressing TNF-α levels in vivo by 57.3 and 55.8%, respectively.

Introduction

The production of proinflammatory cytokines, e.g., TNF-α, IL-1β and IL-6, is a key factor in chronic inflammatory diseases, such as rheumatoid arthritis, Crohn’s disease, psoriasis and asthma [1,2]. Moreover, evidence exists that supports the involvement of cytokines in other diseases, including cardiac heart failure, ischemic retinopathy [3] and the development of insulin resistance in diabetes [4]. Due to the role of cytokines in various inflammatory diseases, many pharmaceutical companies have made efforts to develop new orally active substances that can modulate the production of proinflammatory cytokines.

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic cytokine that possesses proinflammatory and osmoregulator actions [5]. It is the major cytokine mediator of acute inflammation, it activates platelets, and it is also involved in the genesis of fever and anemia. TNF-α also mediates many inflammatory events in rheumatoid arthritis, including immune cell activation, proliferation, apoptosis and regulation of leukocyte movement [6], which has led to the development of strategies to block TNF-α-mediated effects. The currently available anti-TNF-α strategies involve either administration of anti-TNF-α antibodies or soluble TNF receptors to remove circulating TNF-α [7]. These inhibitors act by binding to TNF-α and preventing it from binding to its receptors on nearby cells, thus preventing the initiation of apoptosis or an inflammatory response [8].

Despite the approval of anti-TNF-α drugs, e.g., infliximab, etanercept and adalimumab, which demonstrated the effectiveness of therapeutic strategies based on the depletion of TNF-α, the appearance of side effects resulting from the debilitating actions of these drugs on the immune system highlights the necessity of identifying new alternative mechanisms to modulate the actions of pro-inflammatory cytokines [9,10].

One of the most promising targets involved in modulating the production of pro-inflammatory cytokines is the mitogen-activated protein kinase (MAPK) pathway, particularly p38 MAPK, a serine-threonine protein kinase that has been identified as a molecular target of the pyridinyl-imidazole derivatives SB-203580 (1) and SB-202190 (2) [11,12]. These terphenyl-heterocyclic derivatives, which have been widely used to study p38 MAPK function, competitively bind at the ATP-binding pocket of p38 MAPK and inhibit TNF-α and IL-1β production.

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Over the years, a large number of structurally diverse p38α and p38β MAPK inhibitors have been developed with both enhanced potency and specificity. Most of the p38 MAPK inhibitors are ATP competitors [13], but a new class of allosteric inhibitors has also been reported [14]. For example, BIRB-796 [15] (3) produces a conformational reorganization of the kinase that prevents ATP binding and activation.

In this context, the present work describes the synthesis of novel N-phenylpyrazolyl-N-glycinyl-hydrazine derivatives 4a-g, which were designed as structural analogues of the p38 MAP kinase inhibitor BIRB-796 (3), and the investigation of their anti-cytokine and anti-inflammatory properties. For the proposed derivatives (4a–g), we investigated the replacement of the urea subunit of BIRB-796 (3) by a N-acylhydrazine unit [16] (A, Figure 2), which was attached to the N-phenyl-pyrazole nucleus through an NHCH₂ spacer (B, Figure 2).

Furthermore, we performed a series of molecular simplifications in the functionalized naphthyl framework attached to the imine unit of the NAH group of compound 4a to better understand the structure-activity relationships (Figure 2).

**Results and Discussion**

The first step to obtain the N-phenylpyrazolyl-N-glycinyl-hydrazine derivatives 4a–g consisted of preparing the derivative 3-tert-butyl-1-phenyl-1H-pyrazol-5-amine (5) [15] from the condensation reaction between 4,4-dimethyl-3-oxopentanenitrile and phenylhydrazine (6) in refluxing toluene. The amino-pyrazole derivative 5 was subjected to alkylation with ethyl 2-bromoacetate in toluene and triethylamine under reflux, which gave rise to the corresponding amino ester 7 with a 60% yield. Next, the hydrazinolysis of the ester 7 with hydrazine hydrate in ethanol under reflux produced the corresponding hydrazide intermediate 8 with an 80% yield. The novel N-phenyl-pyrazolyl-N-glycinyl hydrazine derivatives 4a–g (Table 1) were then prepared in satisfactory yields through the acid catalyzed condensation of hydrazide 8 with aromatic aldehydes at room temperature (Figure 3).

The structures of the N-phenyl-pyrazolyl-N-glycinyl-hydrazones 4a–g were completely characterized by common spectroscopic methods and the analytical results for C, H and N were within ± 0.4% of the calculated values.

According to the literature, N-acetylhydrazones (NAHs) may exist as E/Z geometrical isomers about the C=N double bond and syn/anti amide conformers [17]. For most NAH derivatives described herein, the 1H-NMR spectra were recorded at room temperature, and they indicated the presence of two isomers, whereas only one species was detected by reversed-phase HPLC (Figure S22). In a study involving compound 4 g, the 1H-NMR spectrum in DMSO-d₆ at 90 °C showed that the two isomers were in rapid equilibrium (Figure 4A and Figure S13) [18]. Interestingly, complete coalescence of the signals was reached at 90°C, and the reversibility of the changes was verified, indicating the presence of conformational isomers (Figure 5).

Moreover, the 1D NOESY showed spatial relationships of amide and imine hydrogens of compound 4 g that were compatible with the relative configuration (E) at the imine double bond (Figure S14, Figure S15 and Figure S23).

Another approach that was used to evaluate the presence of mixtures of conformers in our series of NAH derivatives 4a–g was based on the work of Wyrzykiewicz and Palla [17,18]. A ¹H-NMR spectrum of the compound 2-(3-tert-butyl-1-phenyl-hydrazol-5-ylmino)-N-(propan-2-ylidene)acetylbis(9), which was obtained by a reaction of the previously obtained hydrazide 8 with acetone (Figure 3), was performed because compound 9 cannot exist as E/Z geometrical isomers about the imine double bond. Nevertheless, the ¹H-NMR spectrum of compound 9 displayed duplicate signals for amide, methylene and pyrazole hydrogens, which completely coalesced at 90°C (Figure 4B and Figure S18).

To evaluate whether the amino spacer exerts some influence on the stabilization of the conformational isomers in solution, we inserted a methyl group into the amino spacer, as described in Figure 6. The protection of the primary amine group [19] of compound 5 by treatment with acetic anhydride in acetic acid and sodium acetate resulted in the acetamide compound 10 with an 80% yield. Subsequent N-methylation was performed by deprotonation of compound 10 with NaH in THF followed by the addition of CH₃I, which resulted in a 90% yield of N-methylacetamide 11. The next step consisted of the removal of the protecting acetyl group to obtain the 3-tert-butyl-N-methyl-1-phenyl-1H-pyrazol-5-amine (12), which showed a 90% yield. The alkylation of the monomethylamine derivative 12 with ethyl 2-bromoacetate in ethanol and sodium carbonate under reflux provided the corresponding ethyl ester 13 with a 60% yield. Hydrazinolysis of the ester 13 followed by condensation of the corresponding hydrazide 14 with benzaldehyde under acid catalysis resulted in the desired N-acetylhydrazine derivative 15 with a 60% yield.

The ¹H-NMR spectrum of the N-methyl derivative 15 showed the same pattern of duplicity that was observed for the other synthesized N-acetylhydrazones 4a-g. We were able to observe conformational isomers of the amide unit of compound 15, suggesting that the amino spacer does not participate as a hydrogen bond donor in the stabilization of the conformational isomers in solution (Figure S20 and Figure S21).

We also performed the chemoselective N-alkylation of the N-acetylhydrazine derivative 4g (Figure 3) to investigate the influence of an alkyl group on the observation of conformational isomers in solution.

The pattern of duplication observed in the ¹H NMR spectrum of the N-acetylhydrazine derivative 4g disappeared after methylation of the NAH framework, i.e., we did not observe conformational isomers for the corresponding N-methyl N-acetylhydrazine derivative 4h. These results suggest that the insertion of the methyl group at the amide nitrogen leads to a sterically or electronic effect that does not allow the distinction of conformational isomers in solution by ¹H-NMR (Figure S19), as previously reported by Kummerle and co-workers [20].

We initially investigated the capacity of our N-acetylhydrazine derivatives 4a–g to inhibit TNF-α production in vitro [21]. The p38 MAPK inhibitor SB-203580 (1) was chosen as a standard. As depicted in Table 2, six NAH compounds 4a, 4b, 4c, 4d, 4f and 4g inhibited the in vitro LPS-induced production of TNF-α in cultured mouse peritoneal macrophages at a concentration of 10 μM. Among them, 4f (93.2%, IC₅₀ = 1.6 μM), 4a (96.9%, IC₅₀ = 3.6 μM) and 4b (75.4%, IC₅₀ = 4.3 μM) showed the most potent inhibitory effects. Compared with the unsubstituted phenyl

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**Figure 1. Pyridinyl-imidazole inhibitors of p38 MAPK.**
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ring compound 4g (cLogP = 5.3), the inhibitory potency increased when lipophilic groups \textit{para}-chloro for 4f (cLogP = 6.1), naphthyl for 4c (cLogP = 6.6), 4-hydroxynaphthyl for 4b (cLogP = 6.3) and 4-(2-(naphthalen-1-yloxy)ethyl)morpholine for 4a (cLogP = 6.0) were added. These results indicate that the differences in hydrophobicity of the imine-attached framework play an important role for the \textit{in vitro} anti-TNF-\alpha activity of \textit{N}-phenylpyrazolyl-\textit{N}-glycinyl-hydrazone derivatives.

Because the novel \textit{N}-acylhydrazones 4a–g were designed based on the p38\alpha MAPK inhibitor BIRB-796 (3), they were all evaluated for their \textit{in vitro} capacity to inhibit p38\alpha MAPK activity [23] at a concentration of 10 \muM. Interestingly, only compounds 4b and 4e were active, and they inhibited approximately 30\% of p38\alpha activity (Table S1).

To evaluate the \textit{in vivo} anti-inflammatory and antinociceptive profile of the NAH derivatives 4a, 4b, 4c and 4f, we employed the carrageenan-induced thermal hypernociception model [24]. Compounds were orally administered at a dose of 100 \mu mol/kg. SB-203580 (1) (100 \mu mol/kg, p.o.) was used as a standard. Figure 7 shows that compounds 4a and 4f were effective anti-hypernociceptive agents. Although these two compounds have shown similar capacities to inhibit TFN-\alpha production \textit{in vitro} (Table 2), compound 4a was more effective \textit{in vivo}. In addition, compound 4a was able to completely inhibit the hypernociceptive response, whereas compound 4f was only able to partially inhibit this response.

We then investigated whether the inhibition of carrageenan-induced thermal hypernociception by 4a and 4f occurs through the inhibition of TNF-\alpha. Four hours after carrageenan injection, the TNF-\alpha level in the paw was elevated by more than two times that of the saline control. Interestingly, pretreatment with 4a and 4f (100 \mu mol/kg) suppressed the elevation of tissue TNF-\alpha level by 57.3 and 55.8\%, respectively (Figure 8).

About the best anti-hypernociceptive profile of the compound 4a in comparison to derivative 4f, we decided to investigate the molecular reasons associated with a probable distinction in the

\begin{table}
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\begin{tabular}{llll}
\hline
\textbf{Compound} & \textbf{Molecular Formula}\textsuperscript{a} & \textbf{MW} & \textbf{Yield (\%)}\textsuperscript{b} & \textbf{M.P. (\degree C)} \\
\hline
4a & C\textsubscript{32}H\textsubscript{38}N\textsubscript{6}O\textsubscript{3}.(H\textsubscript{2}O) & 572.70 & 70 & 180 \\
4b & C\textsubscript{26}H\textsubscript{27}N\textsubscript{5}O\textsubscript{2} & 441.52 & 70 & 270 \\
4c & C\textsubscript{26}H\textsubscript{27}N\textsubscript{5}O & 425.53 & 90 & 140 \\
4d & C\textsubscript{28}H\textsubscript{36}N\textsubscript{6}O\textsubscript{3} & 504.62 & 60 & 158 \\
4e & C\textsubscript{22}H\textsubscript{25}N\textsubscript{5}O\textsubscript{2} & 391.47 & 70 & 280 \\
4f & C\textsubscript{22}H\textsubscript{24}ClN\textsubscript{5}O & 409.91 & 70 & 193 \\
4g & C\textsubscript{22}H\textsubscript{25}N\textsubscript{5}O & 375.47 & 70 & 148 \\
\hline
\end{tabular}
\caption{Yields and physical properties of \textit{N}-phenylpyrazolyl-\textit{N}-glycinyl-hydrazone derivatives 4a–g.}
\textsuperscript{a}The analytical results for C, H and N were within 0.4\% of the calculated values.
\textsuperscript{b}Yields obtained for the condensation step of hydrazide (8) with the corresponding aromatic aldehydes.
\end{table}
respective pharmacokinetic behaviors. The physicochemical property cLogP doesn’t seem to explain the better in vivo profile of derivative 4a since both compounds, 4a and 4f, have the same theoretical lipophilicity, i.e. cLogP = 6.0 and 6.1, respectively. Considering that an adequate balance between the lipophilicity and aqueous solubility is essential for a good oral

Figure 3. Synthesis of N-phenylpyrazolyl-N-glycinyl-hydrazone derivatives 4a–h.
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Figure 4. 1H-NMR spectra of NAH derivatives 4g (A) and 9 (B) in DMSO-d$_6$ at 40°C and 90°C.
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absorption of a drug candidate, we decided to determine experimentally the solubility of compounds 4a and 4f in buffer solutions of pH 6.4 and 7.4 (Figure 9). The derivative 4a, which contains the ethoxymorpholine-naphthyl group, exhibited an improvement in solubility at both pH values when compared with para-chlorophenyl derivative 4f, i.e. ca. 5 times at pH 7.4 and ca. 12 times at pH 6.4. As expected, at pH 6.4 only compound 4a showed to present an improvement in aqueous solubility (ca. three times), due to the partial ionization of its basic morpholine subunit. These solubility results allow us to rationalize that the improved in vivo activity of compound 4a is due to its better water solubility, which could favor its gastrointestinal absorption.

Moreover, we also evaluated the in vitro metabolic stability of derivatives 4a and 4f when placed in contact with preparations of liver and plasma of rats. The two NAH derivatives were resistant to oxidative microsomal metabolism, but the derivative 4a was about four times more resistant than derived 4f to plasma degradation, as described in Table 3. Taken together, these results indicate that the plasma stability associated to the better aqueous solubility is responsible for the better in vivo pharmacological profile shown by the NAH derivative 4a when given orally.

This study describes the synthesis and pharmacological evaluation of novel N-phenylpyrazol-yl-glycinyl-hydrazono derivatives that were designed as novel prototypes of p38 MAPK inhibitors. All novel synthesized compounds were evaluated for their in vitro capacity to inhibit TNF-α production in cultured macrophages and their in vivo p38α MAPK inhibition. The two most active anti-TNF-α derivatives were (E)-2-(3-tert-butyl-1-phenyl-1H-pyrazol-5-ylamino)-N-((4-[2-morpholinoethoxy]naphthalen-1-yl)methylenecetohydrazide (4a) and (E)-2-(3-tert-butyl-1-phenyl-1H-pyrazol-5-ylamino)-N-(4-chlorobenzylidene)acetohydrazide (4f). These two compounds were evaluated for their in vivo anti-hypernociceptive profiles. Both compounds showed anti-inflammatory and anti-hypernociceptive properties that were comparable to SB-203580 (1), which was used as a standard.

**Materials and Methods**

Reactions were routinely monitored by thin-layer chromatography (TLC) in silica gel (F245 Merck plates) and the products visualized with ultraviolet lamp (254 and 365 nm). NMR spectra were recorded on a 200/500 MHz Bruker DPX-200, 250/ 62.5 MHz Bruker DPX-250, 400/100 MHz Varian 400-Mr, 300/75 MHz Varian Unity-300 spectrometer at room temperature. Peak positions are given in parts per million (δ) from tetramethylsilane as internal standard, and coupling constant values (J) are given in Hz. Infrared (IR) spectra were obtained using a Nicolet Magna IR 760 spectrometer. Samples were examined as potassium bromide (KBr) disks. Elemental analyses were carried out on a Thermo Scientific Flash EA 1112 Series CHN-Analyzer. Melting points were determined using a Quimis instrument and are uncorrected and the compounds 4a-f had their melting points determined using a differential scanning calorimeter (Shimadzu DSC-60). Column chromatography purifications were performed using silica gel Merck 230-400 mesh. All described products showed 1H and 13C NMR spectra according to the assigned structures.

All organic solutions were dried over anhydrous sodium sulfate and all organic solvents were removed under reduced pressure in rotary evaporator.

HPLC for purity determinations were conducted using Shimadzu LC-20AD with a SHIM-PACK CLC-ODS analytical column (4.6 mm x 250 mm) or Kromasil 100-5C18 (4.6 mm x 250 mm) and a Shimadzu SPD-M20A detector at 254 nm wavelength. The solvent systems for HPLC purity analyses was acetonitrile:phosphate buffer solution pH7 = 70:30. The isocratic HPLC mode was used, and the flow rate was 1.0 mL/min.

**Procedure for Preparation of 3-tert-butyl-1-phenyl-1H-pyrazol-5-amine (5)**

A round-bottomed flask charged with phenylhydrazine (0.83 mmol), 4,4-dimethyl-3-oxo-pentanenitrile (2.0g; 8.0 mmol) and toluene (3 mL) was stirred and heated at reflux for 24 hours. The resulting mixture was concentrated on a rotary evaporator and the residue was purified by column chromatography on silica gel (hexane/ethyl acetate, gradient), to yield the title compound (1.38g, 80%) as a white solid (mp: 50-52°C). <sup>1</sup>H NMR (200 MHz, DMSO-d<sub>6</sub>) δ = 7.59 (d, 2H, J = 8 Hz, H2 and H6-phenyl); 7.44 (t, 2H, J = 8 Hz, H3 and H5-phenyl); 7.26 (t, 1H, J = 8 Hz, H4-phenyl); 5.39 (s, 1H, CH-pyrazole); 5.19 (s, 2H, NH2); 1.22 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>). <sup>13</sup>C NMR (50 MHz, CDCl<sub>3</sub>) δ = 160.7 (C3-pyrazole), 146.9 (C5-pyrazole), 139.6 (C1-phenyl), 129.9 (C3 and C5-phenyl), 125.5 (C4-phenyl), 122.4 (C2 and C6-phenyl), 87.0 (CH-pyrazole), 31.8 (CH<sub>3</sub>), 30.2 (3xCH<sub>3</sub>). IR (KBr): 3412, 3284, 3146, 2961, 1597, 1556, 1507, 1382, 1243, 798, 696 cm<sup>-1</sup>.

**Procedure for the Preparation of Ethyl 2-(3-tert-butyl-1-phenyl-1H-pyrazol-5-ylamino)acetate (7)**

To a solution of amine 5 (100 mg; 0.465 mmol) in toluene (3.0 mL) and triethylamine (0.1 mL), was added ethyl 2-bromooacetate (1.5 eq, 0.697 mmol, 0.077 mL). The resulting mixture was stirred and heated at reflux for 4 hours. The residue was partitioned between water and ethyl acetate. The combined organic phases were dried over Na<sub>2</sub>SO<sub>4</sub> filtered, and concentrated. The brown residue was purified by silica gel chromatography hexane/ethyl acetate (gradient) to give the title compound (84 mg, 60%) as a brown oil. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>) δ = 5.75-7.44 (m, 4H, HA<sub>a</sub>); 7.31 (m, 1H, HA<sub>b</sub>); 5.71 (t, 1H, J = 6Hz, NH<sub>2</sub>); 5.37 (s, 1H, CH-pyrazole); 4.12 (q, 2H, J = 8 Hz, CH<sub>2</sub>); 3.81 (d, 2H, CH<sub>2</sub>, J = 7.57-7.44 Hz); 3.81 (d, 2H, CH<sub>2</sub>, J = 7.57-7.44 Hz); 2.55-2.7 (m, 4H, CH<sub>2</sub>); 1.21-1.19 (m, 12H, CH<sub>3</sub> and CH<sub>2</sub>).

**Procedure for the Preparation of 2-(3-tert-butyl-1-phenyl-1H-pyrazol-5-ylamino)acetohydrazide (8)**

A round-bottomed flask charged with 600 mg (2 mmol) of ester (7), hydrazine hydrate 100% (20 eq) and ethanol (5 mL) was stirred and heated at reflux for 2 hours. To the resulting mixture was added water and the aqueous phase was extracted with ethyl acetate to give the title compound (430 mg, 80%) as a yellow oil.
1H NMR (200 MHz, DMSO-d₆) \( \delta = 9.17 \) (s, 1H, NHCO); 7.58 (d, \( J = 8 \) Hz, H2 and H6-phenyl); 7.47 (d, \( J = 8 \) Hz, H3 and H5-phenyl); 7.31 (t, \( J = 8 \) Hz, H4-phenyl); 5.56 (t, \( J = 6 \) Hz, NH); 5.35 (s, 1H, CH-pyrazole); 4.25 (s, 2H, NH₂); 3.57 (d, \( J = 6 \) Hz, CH₂); 1.37 (s, 9H, (CH₃)₃). 13C NMR (50 MHz, DMSO-d₆) \( \delta = 168.9 \) (CO), 160.9 (C3-pyrazole), 148.1 (C5-pyrazole), 139.2 (C1-phenyl), 129.1 (C3 and C5-phenyl), 126.1 (C4-phenyl), 123.2 (C2 and C6-phenyl), 84.4 (CH-pyrazole), 47.5 (CH₂), 31.2 (C(CH₃)₃), 30.2 (CH₃). IR (NaCl) 3319, 2960, 1669, 1596, 1567, 1520, 1373, 1247, 991, 764 cm⁻¹.

**General Procedure for the Preparation of N-phenylpyrazole N-glycinyl-N-acylhydrazones (4a–g)**

In a round flask containing hydrazide 8 (1.6 mmol) in ethanol (10 mL), was added aldehyde (1.68 mmol; 1.05 eq) and catalytic concentrated hydrochloric acid. The mixture was stirred for about 2 hours at room temperature. At the end of the reaction the volume of ethanol was reduced, saturated solution of sodium bicarbonate and ice were added to the reaction. The precipitate formed was filtered, or the mixture was extracted with dichloromethane.

(E)-2-(3-tert-butyl-1-phenyl-1H-pyrazol-5-ylamino)-N’-(4-(2-morpholinoethoxy)naphthalen-1-yl)methylene)acetohydrazide (4a)

(Yield = 75%) (mp. 180°C).

1H NMR (300 MHz, DMSO-d₆) \( \delta = 11.37 \) and 11.28 (s, 1H, NHCO); 8.91 and 8.72 (d, \( J = 9 \) Hz, CH-naphthyl); 8.72 and 8.53 (s, 1H, N = CH); 8.26 (m, 1H, CH-naphthyl); 7.77 (m, 1H, CH-naphthyl); 7.40-7.60 (m, 6H, ArH); 7.33 (m, ArH); 7.04 (d, \( J = 9 \) Hz, ArH); 6.54 and 5.35 (t, \( J = 5.7 \) Hz, NH); 5.45 (s, CH-pyrazole); 4.33 (t, \( J = 4.6 \) Hz, OCH₂-ethoxyl); 4.24 and 3.78 (d, 2H, \( J = 5.7 \) Hz, CH₂); 3.56 (t, \( J = 4.7 \) Hz, 2xCH₂-morpholine); 2.88 (t, 2H, \( J = 4.7 \) Hz, CH₂-ethoxyl); 2.56 (t, \( J = 4.7 \) Hz, 2xCH₂-morpholine); 1.23 (s, 9H, (CH₃)₃) [Figure S1]. 13C NMR (50MHz, DMSO, TMS) \( \delta = 170.6 \) and 166.1 (C = O); 161.0 (C3-pyrazol); 157.7 (C4-naphthyl); 141.8 (C5-pyrazol); 147.7 and 144.3 (N = CH); 139.2 (C1-phenyl); 131.0 (C9-naphthyl); 129.2 (ArCH); 127.8 (ArCH); 126.2 (ArCH); 125.7...
Table 2. Effects of N-phenylpyrazolyl-N-glycinyl-hydrazone derivatives 4a-g on TNF-α production and cell viability in murine peritoneal macrophages.

| Compound | TNF-α Inhibition[a] | IC50 (μM)[b] | % of Cell Viability [c] | cLogP[d] |
|----------|---------------------|--------------|-------------------------|---------|
|          | % inhibition at 10 μM | IC50 (μM) | (at 3 μM) | (at 10 μM) |
| 4a       | 96.9*               | 3.6 (0.1–30) | 100          | 61.5*    | 6.0   |
| 4b       | 75.4*               | 4.3 (0.1–30) | 100          | 100      | 6.3   |
| 4c       | 61.2*               | 5.5 (0.1–30) | 100          | 90.4     | 6.6   |
| 4d       | 52.5*               | –            | 100          | 100      | 4.8   |
| 4e       | 8.1                 | –            | 100          | 97.8     | 5.1   |
| 4f       | 93.2*               | 1.6 (0.03–10) | 100        | 46.6*    | 6.1   |
| 4g       | 50.4*               | –            | 100          | 99.8     | 5.3   |
| SB-203580 (1) | 90.0*           | 0.22 (0.03–10) | 100      | 44.3*    | –     |

Results are expressed as percentage of inhibition[a] and percentage of cell viability[c] compared with vehicle (DMSO), n = 3 independent experiments in duplicate, *p<0.05 using student’s t test.

[b]IC50 were determined using at least five concentrations, the range concentration are showed in parentheses.

c[Values calculated using ACDLABS program.

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Figure 7. Effects of 4a, 4b, 4c, 4f and SB-203580 (100 μmol/kg, p.o.) on carrageenan-induced thermal hyperalgesia. n = 5–10 animals per group, the test groups were compared to the vehicle control group using two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test, *p<0.05; ***p<0.001. doi:10.1371/journal.pone.0046925.g007

Figure 8. Effects of the NAH derivatives 4a and 4f (100 μmol/kg, p.o.) on the TNF-α level in carrageenan-injected paws. n = 8–10 animals per group, the test groups were compared to the vehicle control group using student’s t test, *p<0.05. doi:10.1371/journal.pone.0046925.g008
1H NMR (200 MHz, DMSO-d6) δ = 11.60 and 11.53 (s, 1H, NHCO); 8.90 and 8.69 (s, 1H, N = CH); 8.84 and 8.64 (d, 1H, J = 8.0 Hz, H8-naphthyl); 8.03-7.88 (m, 3H, HAr); 7.65-7.49 (m, 7H, HAr); 5.78 and 5.45 (t, 1H, J = 6.0 Hz, NH); 5.50 (s, 1H, CH-pyrazole); 4.30 e 3.84 (d, 2H, J = 6.0 Hz, CH2); 1.23 (s, 9H, (CH3)3) [Figure S5]. 13C NMR (50 MHz, DMSO-d6) δ = 171.5 and 167.1 (CO); 161.3 (C3-pyrazole); 148.7 (C5-pyrazol); 147.6 and 144.1 (HC = N); 139.8 (C1-phenyl); 134.1 (C4-naphthyl); 131.0 (C10-naphthyl); 130.6 (C4-naphthyl); 129.8 (C3 e C5-phenyl); 129.4 (C5-naphthyl); 128.5 (C4-phenyl); 127.9 (C2-naphthyl); 126.8 (C7-naphthyl); 126.1 (C2 and C6-phenyl); 124.8 (C6-naphthyl); 124.2 (C9-naphthyl); 123.7 (C3-naphthyl); 85.1 (CH-pyrazole); 46.6 and 47.0 (CH2); 32.49 (C(CH3)3); 30.75 (CH2), 30.55 (CH2) [Figure S6]. IR (KBr): 3339, 3196, 2948, 1687, 1588, 1479, 1344, 1273, 1164 cm⁻¹. Anal. Calcd for C28H36N6O3: C 66.64; H 7.19; N 17.04; Found: C 66.47; H 7.08; N 16.63.

\( \text{E}=2\text{-(3-tert-butyl-1-phenyl-1H-pyrazol-5-ylamino)-N'}\text{-}(4-chlorobenzylidene)acetohydrazide (4f) \)

White solid; recr. from ethanol; Yield = 70% (mp. 193°C). 1H NMR (200 MHz, DMSO-d6) δ = 11.62 and 11.60 (s, 1H, NHCO); 8.27 and 8.01 (s, 1H, N = CH); 7.75-7.50 (m, 9H, H-Ar); 5.33 and 5.46 (s, 1H, CH-pyrazole); 4.21 and 3.78 (s, 2H, CH2); 1.23 (s, 9H, (CH3)3) [Figure S11]. 13C NMR (50 MHz, CDCl3) δ = 171.6 (CO); 161.50 (C3-pyrazole); 148.9 (C5-pyrazole); 143.1 (HC = N); 139.1 (C1-phenylpyrazole); 134.9 (C4-pyrazol); 133.5 (C1-phenyl); 129.8 (2xCH-Ar); 129.4 (2xCH-Ar); 129.2 (2xCH-Ar); 127.0 (CH-Ar); 125.9 (2xCH-Ar); 85.3 (CH-pyrazole); 46.7 (CH2); 32.5 (C(CH3)3); 30.7 (CH2), 23.1 (CH3) [KBr]: 3374, 2956, 1684, 1547, 1324, 1285, 1163, 835, 695 cm⁻¹. Anal. Calcd for C22H19ClN3O: C 67.50; H 6.44; N 17.89. Found: C 67.27; H 6.33; N 17.88.

### Table 3. In vitro stability of compounds 4a and 4f in rat liver microsome and rat plasma.

| Compound | Rat liver microsomes | Rat plasma |
|----------|----------------------|------------|
|          | % Recovered amount after 60 min[a] |
| 4a       | 90.1                  | 40.7       |
| 4f       | 86.7                  | 13.3       |

[a]The percentage of compound remaining was calculated by ratio of peak area at 60 min to peak area found at 0 min multiplied by 100. The values are the mean of at least two experiments in duplicate.
(E)-N'-benzylidene-2-(3-tert-butyI-1-phenyl-1H-pyrazol-5-ylaminio)acetoxyhydrazide (4g)

(White solid; Yield = 70%; mp. 148°C)

1H NMR (300 MHz, DMSO-d6) δ = 11.49 and 11.38 (s, 1H, NHCO); 8.27 and 8.02 (s, 1H, N = CH); 7.60-7.30 (m, 10H, H-Ar); 5.49 and 5.43 (s, 1H, CH-pyrazole); 2.00 and 3.77 (d, 2H, J = 5.7 Hz, CH2); 1.23 (s, 9H, (CH3)3) [Figure S12]. 13C NMR (50 MHz, CDCl3) δ = 172.9 and 166.5 (CO); 160.9 (C3-pyrazole); 143.8 (C5-pyrazole); 147.0 and 143.8 (CH = N); 139.2 (C1-phenylpyrazole); 133.9 (C1-phenyl); 129.8 (CH-Ar); 129.2 (2xCH-Ar); 128.7 (2xCH-Ar); 126.8 (2xCH-Ar); 126.0 (CH-Ar); 123.0 (2xCH-Ar); 84.4 (CH-pyrazole); 47.9 and 46.2 (CH3); 51.9 (C(CH3)3); 30.1 (C(CH3)3) [Figure S16]. IR (KBr): 3446, 2952, 1684, 1595, 1278, 753 cm−1. 

Procedure for Preparation of (E)-N'-benzylidene-2-(3-tert-butyI-1-phenyl-1H-pyrazol-5-ylamino)-N-methylacetohydrazide (4h)

Iodomethane (1.5mmol) was added dropwise to the magnetically stirred solution of 8g (375 mg, 1 mmol) in anhydrous THF (5 mL). The mixture was recrystallized from acetone (262 mg, 80%, mp: 181–183°C). 1H NMR (400 MHz, DMSO-d6) δ = 8.01 (s, 1H, N = CH); 7.78 (d, 2H, J = 8 Hz, ArH); 7.59 (d, 2H, J = 7 Hz, H-Ar); 7.51-7.44 (m, 5H, H-Ar); 7.32 (t, 1H, J = 8 Hz, H-Ar); 5.47 (s, CH-pyrazole); 5.39 (t, 1H, J = 6 Hz, NH); 4.34 (d, 2H, J = 6 Hz, CH2); 3.34 (s, 3H, CH3); 1.22 (s, 9H, (CH3)3). 13C NMR (50 MHz, DMSO-d6) δ = 163.8 (CO); 160.7 (C3-pyrazole); 138.9 (C1-phenyl); 136.0 (C5-pyrazole); 129.0 (C3 and C5-phenyl); 126.9 (C4-phenyl); 123.3 (C2 and C6-phenyl); 99.0 (C4-pyrazole); 32.0 (C(CH3)3); 30.1 (C(CH3)3); 22.8 (CO2H). IR (KBr): 9255, 3218, 3058, 2946, 1671, 1554, 1373, 1399, 1277, 1235, 687 cm−1.

Procedure for Preparation of N-[(3-tert-butyI-1-phenyl-1H-pyrazol-5-y1)-N'-methylacetamide (11)

Sodium hydroxide (100 mg, 4.2 mmol) was added to the magnetically stirred acetamide 10 (900 mg, 3.5 mmol) in anhydrous THF (5 mL), and iodomethane (0.26 mL, 4.2 mmol) was added dropwise to the mixture, which was maintained be below 5°C for 0.5 h, and stirred at room temperature. The reaction was monitored by TLC. After the reaction was complete, the reaction mixture was partitioned between saturated aqueous NH4Cl and ethyl acetate. The organic layer was separated and the aqueous layer was extracted with ethyl acetate. The organic layers were washed with water, dried over Na2SO4, and concentrated to give the N-[(3-tert-butyI-1-phenyl-1H-pyrazol-5-yl)-N'-methylacetamide (11) as a solid in (854 mg, 90%): mp: 108–110°C. 1H NMR (200 MHz, DMSO-d6) δ = 8.36-7.99 (m, 5H-phenyl); 0.48 (s, 3H, CH3-pyrazole); 3.01 (NHCO-CH3); 1.67 (NCH3COCH3); 1.30 (s, 9H, (CH3)3). 13C NMR (50 MHz, DMSO-d6) δ = 169.7 (C=O); 161.5 (C3-pyrazole); 141.6 (C5-pyrazole); 138.3 (C1-phenyl); 129.4 (C3 and C5-phenyl); 127.5 (C4-phenyl); 122.6 (C2 and C6-phenyl); 101.0 (C4-pyrazole); 35.8 (NCH3COCH3); 35.0 (OCH3); 30.0 (CH3); 21.4 (NCH3); 13C NMR, IR (KBr): 2969, 2867, 1677, 1594, 1566, 1502, 1366, 1354, 758, 688, 635 cm−1.
ethyl acetate (gradient) to give the title compound (642 mg, 60%) as a brown oil. 1H NMR (200 MHz, DMSO-d$_6$) $\delta$ = 7.69 (d, 2H, $J$ = 0 Hz, H$_2$ and H6-phenyl); 7.44 (t, 2H, $J$ = 0 Hz, H3 and H5-phenyl); 2.96 (s, 1H, CH-pyrazole); 1.65 (s, CH$_2$); 1.57 (q, 2H, $J$ = 8 Hz, H2 and H6-phenyl); 7.44 (t, 2H, $J$ = 0 Hz, H$_3$ and H5-phenyl), 92.6 (C4-pyrazole); 65.9 (CH$_2$ ester); 60.0 (CH$_2$ spacer); 41.1 (C3-pyrazole); 150.8 (C5-pyrazole); 140.0 (C1-phenyl); 128.9 (C3 and C5-phenyl); 126.2 (C4-phenyl); 122.6 (C2 and C6-phenyl); 91.1 (C4-pyrazole); 65.9 (CH$_2$ ester); 60.0 (CH$_2$ spacer); 41.1 (NCH$_3$); 31.9 (C(CH$_3$)$_3$); 30.0 (CH$_3$). IR (NaCl): 3315, 2960, 2862, 1682, 1592, 1500, 1402, 1314, 1267, 759, 693 cm$^{-1}$.

Procedure for Preparation of 2-((3-tert-butyl-1-phenyl-1H-pyrazol-5-yl)(methyl)amino)acetohydrazide (14)

A round-bottomed flask charged with 472.5 mg (1.5 mmol) of ester (13), hydrazine hydrate 100% (20 eq) and ethanol (5 mL) was stirred and refluxed for 2 hours. To the resulting mixture was added water and the aqueous phase was extracted with dichloromethane to give the title compound (361 mg, 50%) as a yellow oil after solvent concentration. 1H NMR (200 MHz, DMSO-d$_6$) $\delta$ = 9.00 (s, 1H, NHCO); 7.71 (m, 2H, H$_2$ and H6-phenyl); 7.38 (m, 2H, H3 and H5-phenyl); 7.24 (m, 1H, H4-phenyl); 5.82 (s, 1H, CH-pyrazole); 4.18 (s, 2H, CH$_2$); 2.56 (NCH$_3$); 1.19 (s, 9H, (CH$_3$)$_3$). 13C NMR (50 MHz, DMSO-d$_6$) $\delta$ = 167.7 (CO); 160.5 (C3-pyrazole); 151.4 (C5-pyrazole); 140.0 (C1-phenyl); 129.9 (C3 and C5-phenyl); 126.1 (C4-phenyl); 122.7 (C2 and C6-phenylpyrazole); 122.9 and 122.6 (C2 and C6-phenyl); 127.0 and 126.7 (C3 and C5-phenyl); 126.2 and 126.0 (C2 and C6-phenylpyrazole); 122.9 and 122.6 (C2 and C6-phenyl); 91.1 (C4-pyrazole); 65.9 (CH$_2$ ester); 60.0 (CH$_2$ spacer); 41.1 (NCH$_3$); 31.9 (C(CH$_3$)$_3$); 30.0 (CH$_3$). IR (NaCl): 3315, 2960, 2870, 1749, 1595, 1556, 1502, 1454, 1373, 1198, 1137, 1030, 765, 693 cm$^{-1}$.

Procedure for Preparation of 6-((3-tert-butyl-1-phenyl-1H-pyrazol-5-yl)(methyl)amino)acetohydrazide (15)

In a round flask containing hydrazide 14 (0.93 mmol) in ethanol (5 mL), was added benzaldehyde (0.1 mL; 0.97 mmol; 1.05 eq) and catalytic concentrated hydrochloric acid. The mixture was stirred and heated at reflux for 2 hours. To the resulting mixture was added water and the aqueous phase was extracted with dichloromethane to give the title compound (361 mg, 50%) as a yellow oil after solvent concentration. 1H NMR (200 MHz, DMSO-d$_6$) $\delta$ = 9.00 (s, 1H, NHCO); 7.71 (m, 2H, H$_2$ and H6-phenyl); 7.38 (m, 2H, H3 and H5-phenyl); 7.24 (m, 1H, H4-phenyl); 5.82 (s, 1H, CH-pyrazole); 4.18 (s, 2H, CH$_2$); 2.56 (NCH$_3$); 1.19 (s, 9H, (CH$_3$)$_3$). 13C NMR (50 MHz, DMSO-d$_6$) $\delta$ = 167.7 (CO); 160.5 (C3-pyrazole); 151.5 (C5-pyrazole); 140.0 (C1-phenyl); 129.9 (C3 and C5-phenyl); 126.1 (C4-phenyl); 122.7 (C2 and C6-phenylpyrazole); 122.9 and 122.6 (C2 and C6-phenyl); 127.0 and 126.7 (C3 and C5-phenyl); 126.2 and 126.0 (C2 and C6-phenylpyrazole); 122.9 and 122.6 (C2 and C6-phenyl); 91.1 (C4-pyrazole); 65.9 (CH$_2$ ester); 60.0 (CH$_2$ spacer); 41.1 (NCH$_3$); 32.0 (C(CH$_3$)$_3$); 30.2 (CH$_3$). IR (NaCl): 3315, 2960, 2870, 1749, 1595, 1556, 1502, 1454, 1373, 1198, 1137, 1030, 765, 693 cm$^{-1}$.
experiments were performed in duplicate. The samples were centrifuged and filtered for HPLC-UV analysis.

**Rat Plasma Stability Studies**

The rat plasma was obtained from blood by centrifugation and diluted in phosphate buffer (pH 7.4). The test compounds were added at final concentration of 50 μM with 0.25 mL of final volume and incubated at 37°C for 60 min under agitation. At the end of the incubation time the reaction was stopped by the addition of 375 μL of MeOH and 375 μL of CH3CN. The experiments were performed in duplicate. The samples were centrifuged and filtered for HPLC-UV analysis.

**HPLC-UV Analysis**

The organic fraction was analyzed with the Shimadzu Prominence HPLC system (Shimadzu, Tokio, Japan) consisting of a vacuum degasser (DGU-20A5), a binary pump (LC-20AD), a autosampler (SIL-20A), UV/VIS Photodiode Array Detector (SPD-M20A) and fitted with a guard column (CLC-G-ODS) and a Shimadzu (CLC-ODS, M) column (250 mm x 4.6 mm i.d.) running at room temperature. Isocratic elution was performed with acetonitrile-water (40:60 v/v), at a flow rate set at 1 mL/min. The mobile phase pH was adjusted to 8.0 with NH4OH solution. The detection was carried out at 285 nm and 330 nm wavelength for compound 4f and 4a, respectively.

**Statistical Analysis**

Data obtained from experiments were expressed as mean ± S.E.M., compared with vehicle control groups and statistically analyzed by the ANOVA one-way (Bonferroni post hoc test) for carrageenan-induced thermal hyperalgesia and Student’s t test or for the others experiments. In all cases p<0.05 was considered significant (*p<0.05; **p<0.01; ***p<0.001). When appropriate, the IC50 values (i.e. the concentration able to inhibit 50% of the maximum effect observed) were determined by non-linear regression using GraphPad Prism software v. 5.0.

**Supporting Information**

**Figure S1** ¹H NMR spectrum of 4a (DMSO-d6, 300 MHz). (TIF)

**Figure S2** ¹³C NMR spectrum of 4a (DMSO-d6, 75 MHz). (TIF)

**Figure S3** ¹H NMR spectrum of 4b (DMSO-d6, 200 MHz). (TIF)

**Figure S4** ¹³C NMR spectrum of 4b (DMSO-d6, 50 MHz). (TIF)

**Figure S5** ¹H NMR spectrum of 4c (DMSO-d6, 200 MHz). (TIF)

**Figure S6** ¹³C NMR spectrum of 4c (DMSO-d6, 50 MHz). (TIF)

**Figure S7** ¹H NMR spectrum of 4d (DMSO-d6, 400 MHz). (TIF)

**Figure S8** ¹³C NMR spectrum of 4d (DMSO-d6, 100 MHz). (TIF)

**Figure S9** ¹H NMR spectrum of 4e (DMSO-d6, 200 MHz). (TIF)

**Figure S10** ¹³C NMR spectrum of 4e (DMSO-d6, 50 MHz). (TIF)

**Figure S11** ¹H NMR spectrum of 4f (DMSO-d6, 200 MHz). (TIF)

**Figure S12** ¹H NMR spectrum of 4 g (DMSO-d6, 300 MHz, t~40°C). (TIF)

**Figure S13** ¹H NMR spectrum of 4 g (DMSO-d6, 300 MHz, t~90°C). (TIF)

**Figure S14** 1D NOESY spectrum of 4 g (DMSO-d6, 300 MHz). Irradiation at 11.48 ppm. (TIF)

**Figure S15** 1D NOESY spectrum of 4 g (DMSO-d6, 300 MHz). Irradiation at 8.26 ppm. (TIF)

**Figure S16** ¹³C NMR spectrum of 4 g (DMSO-d6, 75 MHz). (TIF)

**Figure S17** ¹H NMR spectrum of 9 (DMSO-d6, 200 MHz, t~40°C). (TIF)

**Figure S18** ¹H NMR spectrum of 9 (DMSO-d6, 300 MHz, t~90°C). (TIF)

**Figure S19** ¹H NMR spectrum of 4 h (DMSO-d6, 400 MHz). (TIF)

**Figure S20** ¹H NMR spectrum of 15 (DMSO-d6, 200 MHz). (TIF)

**Figure S21** ¹³C NMR spectrum of 15 (DMSO-d6, 50 MHz). (TIF)

**Figure S22** Chromatogram of compound 4 g obtained from reversed-phase HPLC studies. (TIF)

**Figure S23** 1D NOESY spectrum of [(E)-N'-benzylidene-2-(3-tetra-tert-butyl-1-phenyl-1H-pyrazol-5-ylamino)acetohydrazide (4 g) in DMSO-d6 (300 MHz). Irradiation at 11.48 ppm (A) and 8.26 ppm (B). (TIF)

**Table S1** p38 MAPK inhibitory activity of compounds (4a–g) at 10 μM. (DOC)

**Author Contributions**

Conceived and designed the experiments: RBL, ALPM, SAL, EJB, CAMF. Performed the experiments: RBL, LLdS, CKFdL, EM, SAL. Analyzed the data: RBL, LLdS, CKFdL, ALPM, EJB, CAMF. Contributed reagents/materials/analysis tools: ALPM, SAL, EJB, CAMF. Wrote the paper: RBL, LLdS, CKFdL, EM, SAL, EJB, CAMF.

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