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Synthesis and biological evaluation of novel pyrazolyl-2,4-thiazolidinediones as anti-inflammatory and neuroprotective agents

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ABSTRACT

Novel pyrazolyl-2,4-thiazolidinediones were prepared via the reaction of appropriate pyrazolecarboxaldehydes with 2,4-thiazolidinediones and substituted benzyl-2,4-thiazolidinediones. The resultant compounds were first evaluated for their anti-inflammatory and neuroprotective properties in vitro. The active compounds were further studied in vivo by using the formalin-induced paw edema and the turpentine oil-induced granuloma pouch bioassays. We identified four novel compounds that showed protective effects in vitro at non-toxic concentrations, and were also effective in the animal models of acute and sub-acute inflammation.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for the treatment of a wide range of inflammatory conditions. However, their long-term use is associated with significant morbidity which limits their clinical applications.[1] Therefore there is a need for the development of novel drugs with better safety profiles that could be used long term to relieve chronic inflammatory conditions. Recent evidence suggests that anti-inflammatory drugs could also be beneficial in a number of neurodegenerative disorders, including Alzheimer's and Parkinson's diseases, which have a prominent neuroinflammatory component.[2,3] In this context, the present study was designed to evaluate the anti-inflammatory and neuroprotective properties of novel pyrazole and thiazolidinedione derivatives.

Pyrazole derivatives are known to possess a wide spectrum of biological activities including anti-inflammatory,[4] antitumor,[5] antinociceptive[6] and antimicrobial[7] actions. Aryl pyrazoles have also been shown to be neuroprotective due to their ability to block sodium channels.[2] The high profile NSAID celecoxib (Celebrex; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide) contains the pyrazole nucleus. This cyclooxygenase (COX)-2 inhibitor is considered to be a potent anti-inflammatory and analgesic agent, which has relatively few gastrointestinal side effects. However, there is evidence of an increase in cardiovascular injury with its prolonged use.[8]

Celecoxib is considered as a typical model of the 1,5-diaryl heterocyclic template that selectively inhibits the COX-2 isoenzyme.[9] Several studies have reported the development of novel pyrazole analogues.[1] Two examples of newly described pyrazole derivatives include SC-58125 (3-(trifluoromethyl)-5-(4-fluorophenyl)-1-(4-methylsulphonyl)phenyl)-1*H*-pyrazole)[10] and SC-558 (5-(4-bromophenyl)-3-(trifluoromethyl)-1-(4-methylsulphonyl)phenyl)-1*H*-pyrazole).[11] Both these compounds exhibit selective COX-2 inhibitory activity.

Thiazolidinediones (TZDs) have been the subject of extensive research because of their involvement in the regulation of different physiological processes. TZDs, such as rosiglitazone and pioglitazone, lower plasma glucose levels by acting as ligands for the gamma peroxisome proliferator-activated receptors (PPARs). In addition, this class of compounds has several other potentially beneficial effects including improvement in lipid profile, blood pressure lowering and anti-inflammatory effects.[12] TZDs target vascular cells[13] and monocytes/macrophages[14,15] to inhibit the production of pro-inflammatory cytokines as well as the expression of inducible nitric oxide synthase and cell adhesion molecules. These drugs may also be beneficial in multiple sclerosis and neurodegenerative diseases, including Alzheimer's and Parkinson's, at least partially due to their anti-inflammatory activity.[16]

Herein we describe the synthesis of some novel structures incorporating both the pyrazole moiety and thiazolidinedione ring

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systems. The objective of this study was to investigate the benefits of such hybridization on the anticipated biological activities, and to determine whether this would lead to added synergistic biological activity of the target molecules.

We employed in vitro assays relevant to inflammatory processes in the central nervous system as well as animal models of peripheral inflammation to demonstrate that four out of thirteen compounds possessed biological activity both in vitro and in vivo. In addition, the ulcerogenic and acute toxicity profiles of the biologically active compounds were examined.

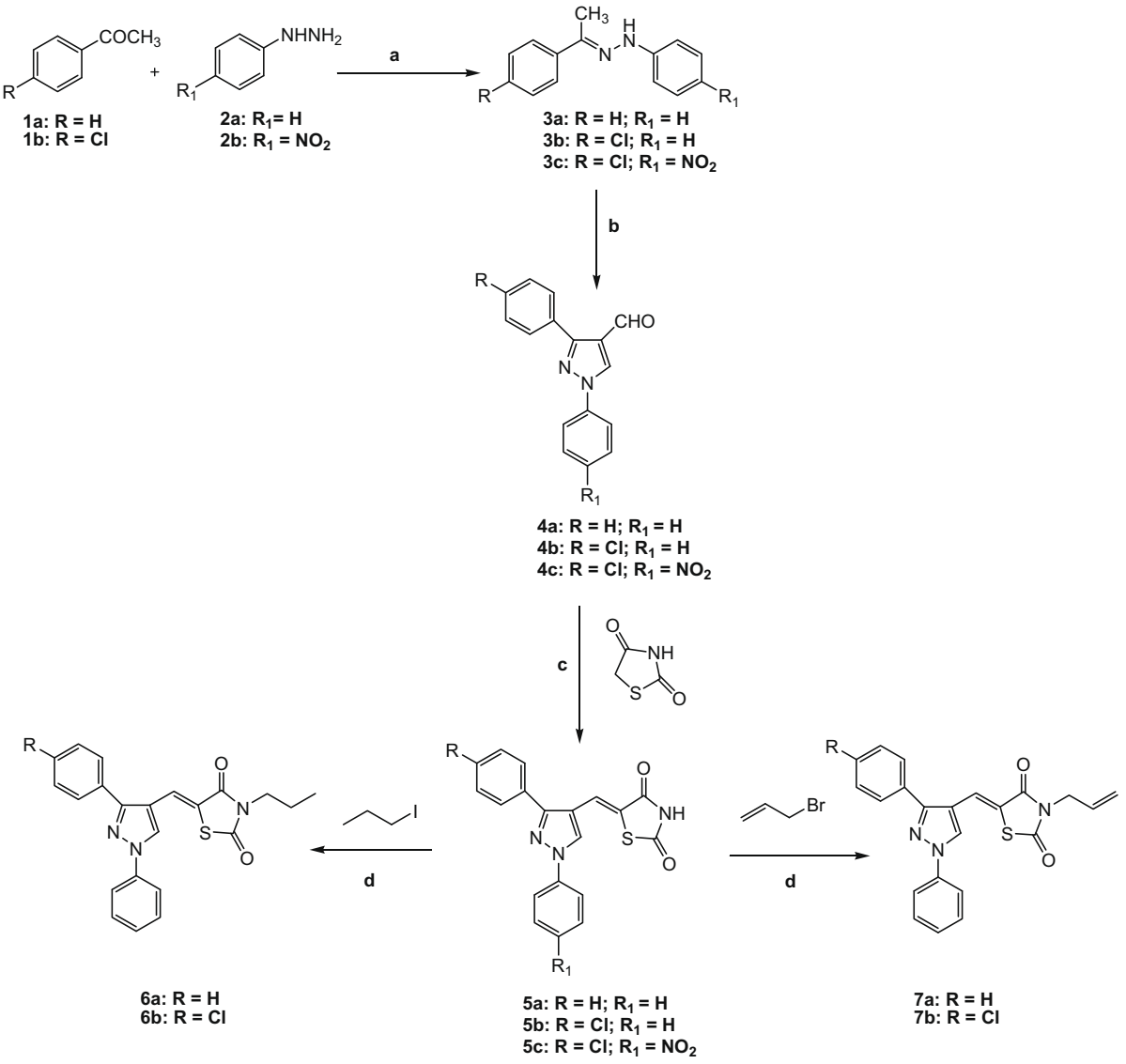
2. Results and discussion

2.1. Chemistry

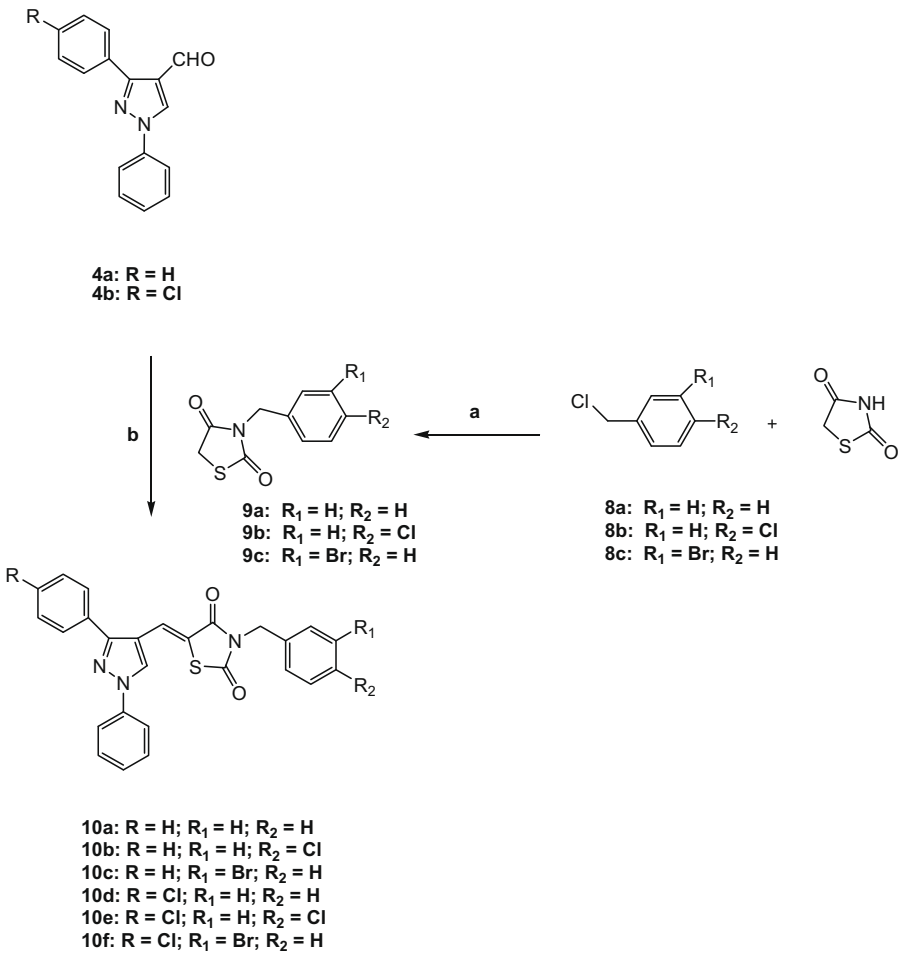
The synthesis of the desired compounds was accomplished as described in Schemes 1 and 2. The key starting materials 1,3-diphenyl-1*H*-pyrazole-4-carbaldehyde **(4a)**,[17] 3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazole-4-carbaldehyde **(4b)**[17] and 3-(4-chlorophenyl)-1-(4-nitrophenyl)-1*H*-pyrazole-4-carbaldehyde **(4c)**,[18] were prepared by the reaction of acetophenone and 4-chloroacetophenone with phenyl hydrazine and 4-nitrophenyl hydrazine to produce the corresponding hydrazones **(3a–c)**, followed by Vilsmeier–Haack reaction.[19]

Condensation of substituted pyrazolecarboxaldehydes **(4a–c)** with 2,4-thiazolidinedione in anhydrous toluene in the presence of glacial acetic acid and piperidine as catalysts produced 5-((1,3-substituted-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-diones **(5a–c)**. The products were then reacted with electrophilic reagents like iodopropane and allyl bromide in anhydrous dimethylformamide (DMF) in the presence of potassium carbonate to give 5-((3-substituted-1-phenyl-1*H*-pyrazol-4-yl)methylene)-3-propylthiazolidine-2,4-diones **(6a**,**b)** and 3-allyl-5-((3-substituted-1-phenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-diones **(7a**,**b)** respectively, (Scheme 1).

Substituted benzyl-2,4-thiazolidinediones **(9a–c)** were obtained by reacting 2,4-thiazolidinedione with appropriate benzyl halide derivatives **(8a–c)** in NaOH/ethanol. 3-Substituted benzyl-5-((3-substituted-1-phenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-diones **(10a–f)** were prepared via the reaction between the pyrazolecarboxaldehydes **(4a**,**b)** and appropriately substituted benzyl-2,4-thiazolidinediones **(9a–c)** in anhydrous toluene in the presence of glacial acetic acid and piperidine as catalysts (Scheme 2).



Scheme 1. Reagents and conditions: (a) EtOH, AcOH; (b) $POCl_3$, DMF; (c) toluene, AcOH, piperidine; (d) DMF, K_2CO_3, rt.



Scheme 2. Reagents: (a) NaOH, 50% EtOH, reflux; (b) toluene, AcOH, piperidine.

2.2. In vitro assays

The novel compounds were initially tested in several in vitro assays to assess their cytotoxic, anti-inflammatory, anti-neurotoxic and neuroprotective properties. Table 1 summarizes the effects of all thirteen of the new compounds, as well as the standard anti-inflammatory drug celecoxib on three of the cellular parameters.

2.2.1. Monocytic cell viability and chemokine secretion

First, human monocytic THP-1 cells were pre-incubated with various concentrations of experimental drugs (Table 1) or the vehicle solution dimethylsulfoxide (DMSO) for 15 min before their stimulation with a combination of lipopolysaccharide (LPS) and interferon (IFN)-γ. Previous studies showed that these stimuli induced maximal pro-inflammatory response in human monocytic cells.[20] Different concentration ranges of the compounds were tested due to their various solubilities in DMSO. Viability of THP-1 cells was assessed 24 h later by the lactate dehydrogenase (LDH) and MTT (3(-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays.[21] A minimum of three independent experiments were performed from which concentration curves were plotted and the corresponding EC_{50} values were estimated from both assays. We have reported the lower of the two EC_{50} values if the two assay results differed. An example of a concentration curve measuring LDH content in **7b**-treated THP-1 cell cultures is presented in Figure 1A. In addition to the viability of THP-1 cells, secretion of a pro-inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1) in the presence of various compounds was measured after the 24 h incubation. None of the compounds reduced this parameter of cellular activation (data not shown), which is a well-established assay used to assess the activation status of human monocytes/macrophages.[22]

Table 1
Comparative in vitro biological activity of pyrazolyl-2,4-thiazolidinedione derivatives

Compound	Concentration range tested (μM)	THP-1 cells, cytotoxicity towards EC_{50}[a] (μM)	THP-1 cells toxicity towards SH-SY5Y cells, IC_{50} (μM)	HL-60 cell respiratory burst, IC_{50} (μM)
5a	1–500	50	No effect	8
5b	0.1–100	>100	No effect	0.8
5c	0.1–100	>100	2	1
6a	1–100	>100	No effect	No effect
6b	0.1–100	>100	No effect	No effect
7a	1–100	>100	50	No effect
7b	0.1–250	120	10	No effect
10a	1–100	>100	No effect	No effect
10b	5–100	50	80	No effect
10c	0.5–100	>100	No effect	No effect
10d	0.1–100	1	8	No effect
10e	0.1–100	4	No effect	No effect
10f	0.1–100	5	8	No effect
Celecoxib	5–100	50	5	No effect

[a] Cytotoxicity and inhibition of THP-1 toxicity towards SH-SY5Y cells were assessed as described for **7b** in Figure 1.

2.2.2. Anti-neurotoxic activity

In order to assess the anti-neurotoxic activity of the novel compounds, supernatants from THP-1 cells stimulated in the presence

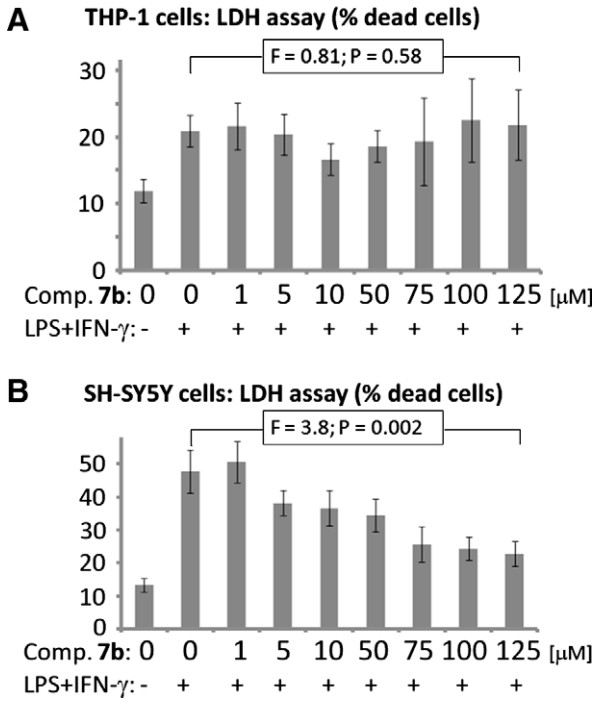


Figure 1. Compound **7b** has no direct toxic effects on human THP-1 cells (A), but it inhibits THP-1 cell toxic secretions toward SH-SY5Y neuroblastoma cells in a concentration-dependent manner (B). (A) THP-1 cells were seeded into 24-well plates at a concentration of 4.5×10^5 cells per well in 0.9 mL of DMEM-F12 medium containing 5% FBS. They were pretreated with various concentrations (μM) of **7b** for 15 min before stimulation with LPS (0.5 μg/mL) and IFN-γ (150 Units/mL). After 24 h incubation, the THP-1 cell viability was assessed by the LDH assay. (B) The cell-free supernatants from the above THP-1 cell cultures were transferred to the wells containing SH-SY5Y cells. Viability of SH-SY5Y cells was assessed after 72 h by the LDH assay. Data (means ± SEM) from 6 to 12 independent experiments are presented. The concentration-dependent effects of **7b** were assessed by randomized block design ANOVA.

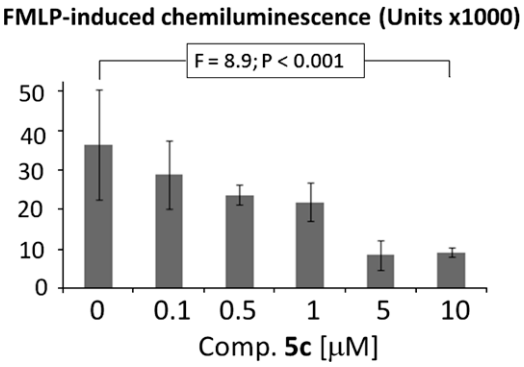


Figure 2. Compound **5c** in a concentration-dependent manner inhibits phagocyte respiratory burst activity. Human promyelocytic HL-60 cells were first differentiated for 6–seven days in the presence of DMSO. They were then washed and transferred into DMEM-F12 medium without phenol red and seeded into 96-well plates at a concentration of 8×10^4 per well. Luminol-dependent chemiluminescence response of HL-60 cells in the presence or absence of drugs was recorded for 30 min after injection of 1 μM FMLP. Data (means ± SEM) from 4 independent experiments are presented in Relative Light Units. The concentration-dependent effects of **5c** were assessed by randomized block design ANOVA.

of various concentrations of compounds were transferred to SH-SY5Y cells and the viability of neuronal cells was measured 72 h later by the LDH and MTT assays.[21] The IC_{50} of the anti-neurotoxic effect are presented in Table 1. Figure 1B shows as an example the data obtained by using **7b**, which indicate that this compound acts in a concentration-dependent manner to effectively reduce THP-1 cell-induced killing of neuronal cells. Similarly to our previous studies,[20,21] supernatants from unstimulated THP-1 cells did not cause reduction of SH-SY5Y cell viability and LDH levels were comparable to those obtained after incubation of SH-SY5Y cells in growth medium only (data not shown).

2.2.3. Inhibition of the respiratory burst

The effects of the compounds on phagocyte respiratory burst were studied by using differentiated human promyelocytic HL-60 cells that have previously been reported to express functional NADPH oxidase complex.[23] We recorded luminol-enhanced chemiluminescence signals to measure the respiratory burst activity of Formyl-Met-Leu-Phe (FMLP)-stimulated HL-60 cells in the presence or absence of various drugs. In a series of preliminary experiments, we confirmed that the observed chemiluminescence signal was suppressed by a selective NADPH oxidase inhibitor apocynin as well as superoxide dismutase. Figure 2 illustrates the inhibitory effect of **5c** in the chemiluminescence assay. All of the drugs were also studied in a luminol-enhanced chemical assay generating oxygen free radicals (Fenton's reaction, data not shown).[24] None of the drugs inhibited the chemiluminescence signal in this chemical assay, which indicated that suppression of the light signal in the HL-60 cell assay was due to an inhibition of NADPH oxidase, and not because of either light absorption by the compounds or due to their reactive oxygen species (ROS) scavenging properties. Table 1 summarizes chemiluminescence assay data for all of the compounds and illustrates that three of them (**5a–c**) significantly inhibited the respiratory burst of HL-60 cells. The effective concentrations for **5b** and **5c** in this assay were at least 100-fold lower than their corresponding toxic activity, while in the case of **5a** this difference was sixfold.

Table 1 illustrates that three of the compounds **(10d–f)** were highly toxic to THP-1 cells with EC_{50} = 1, 4 and 5 μM, respectively. Compounds **5a**, **10b** and celecoxib were less toxic since their EC_{50} values were approximately 50 μM. For all other drugs, the EC_{50} of toxic effects were above 100 μM, which in most cases was the highest concentration tested for these compounds. Table 1 also shows that several compounds inhibited THP-1 cell-induced killing of SH-SY5Y cells. In the case of **10b**, **10d** and **10f**, the IC_{50} for their anti-neurotoxic effects were comparable to the EC_{50} of the toxic effects; therefore these drugs most likely inhibited THP-1 cell neurotoxicity by reducing their viability. However, **5c**, **7b** and celecoxib, although toxic to THP-1 cells at high concentrations, inhibited monocytic cell neurotoxicity at significantly lower concentrations. An approximate 10-fold difference was found between the toxic and anti-neurotoxic effects for **7b** (120 μM vs 10 μM) and celecoxib (50 μM vs 5 μM). This difference increased to at least 50-fold for **5c** with no toxicity to THP-1 cells at the concentrations tested.

Due to their anti-neurotoxic activity, compounds **5c** and **7b** were selected for further in vitro experiments. First, we measured the anti-neurotoxic effects of these compounds after extending the incubation time with THP-1 cells to 48 h instead of 24 h. Figure 3 illustrates that under these experimental conditions **5c** was again able to reduce neuronal cell killing. Both the LDH (Fig. 3A) and MTT assays (Fig. 3B) produced IC_{50} values comparable to the 24 h experiments (Table 1).

2.2.4. Neuroprotective activity

The beneficial effects of the compounds in the above assays could be due to inhibition of THP-1 cell toxic secretions, or due to a direct protective effect on the neuronal cells. In order to distinguish between these two possibilities, a series of experiments were performed where compounds **5c** and **7b** were added directly to SH-SY5Y cells at the time of transfer of supernatants from stimulated THP-1 cells. In this case, no reduction of neuronal killing was

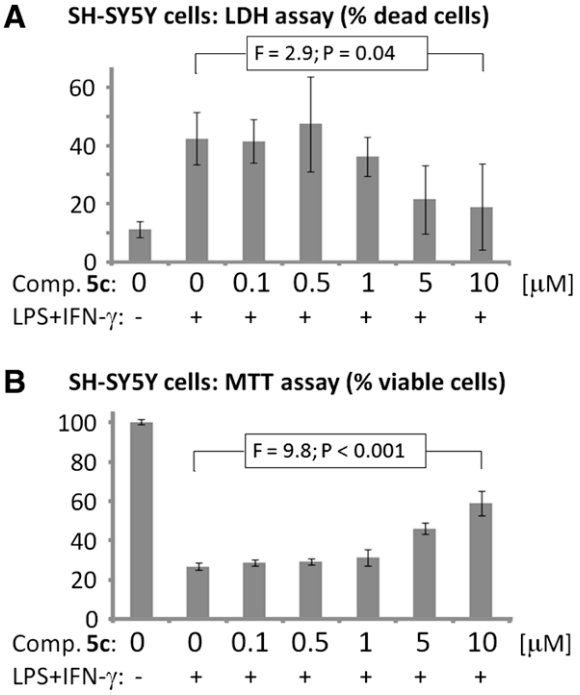


Figure 3. Compound **5c** in a concentration-dependent manner inhibits THP-1 cell toxicity toward SH-SY5Y neuroblastoma cells. THP-1 cells were seeded into 24-well plates and stimulated as described in Figure 1 legend. After 48 h incubation, the cell-free supernatants from THP-1 cell cultures were transferred to the wells containing SH-SY5Y cells. Viability of SH-SY5Y cells was assessed after 72 h by measuring LDH activity in the supernatants (A) and by the MTT assay (B). Data (means ± SEM) from 3 to 7 independent experiments are presented. The concentration-dependent effects of **5c** were assessed by randomized block design ANOVA.

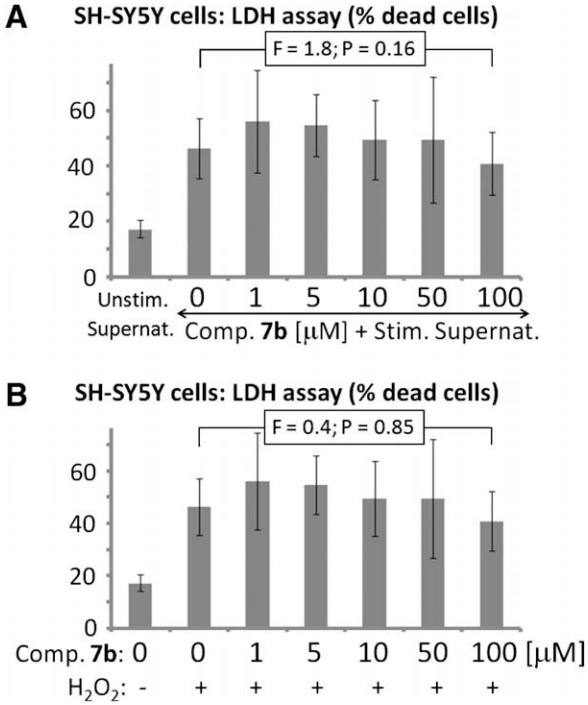


Figure 4. Compound **7b** does not have direct neuroprotective effect on human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were seeded into 24-well plates at a concentration of 2×10^5 cells per mL in 0.4 mL of DMEM-F12 medium containing 5% FBS. Various concentrations of compound **7b** were added at the time of transfer of supernatants from THP-1 cells that had been stimulated with LPS + IFN-γ (A) or culture media containing toxic concentration of H_2O_2 (250 μM). Cell death was assessed by the LDH assay 72 h later. Data (means ± SEM) from 4 to 6 independent experiments are presented. The concentration-dependent effects of compound **7b** were assessed by randomized block design ANOVA.

observed (Fig. 4A shows LDH assay data for **7b**). Furthermore, both of these compounds also failed to rescue SH-SY5Y cells killed by hydrogen peroxide (H_2O_2), which is often used to model oxidative stress in cell culture experiments (Fig. 4B shows LDH assay data for **7b**). Therefore compounds **5c** and **7b** were ineffective as neuroprotective agents when added directly to SH-SY5Y cells independent of the agent used to induce neuronal death. Thus it could be concluded that **5c** and **7b** possess anti-neurotoxic activity by inhibiting THP-1 toxic secretions.

2.2.5. Inhibition of COX enzymatic activity

We have previously reported that COX inhibitors diminish THP-1 cell-induced toxicity towards SH-SY5Y cells and that several NSAIDs were effective when added before THP-1 cell stimulation and were significantly less effective when added at the time of transfer of supernatants.[25,26] Table 2 shows data obtained by a cyclooxygenase (COX) inhibitor screening assay. At 10 μM, **5c** and **7b** partially inhibited both COX isoforms, while celecoxib, as expected, was active mainly against the COX-2 enzyme (Table 2). Therefore it appears that the anti-neurotoxic in vitro activity of **5c** and **7b** could be due to their ability to inhibit prostaglandin production by THP-1 cells.[26]

Table 2
Inhibition of cyclooxygenase (COX-1 and -2) enzymatic activity

Compound	Concentration (μM) (number of experiments)	COX-1 activity (% inhibition)	COX-2 activity (% inhibition)
5c	10 (3)	28.4 ± 11.6	19.4 ± 8.2
7b	10 (3)	26.5 ± 6.4	13.6 ± 1.1
Celecoxib	10 (3)	0.3 ± 2.5	30.8 ± 5.9

2.3. In vivo assays

The anti-inflammatory activity of those analogs that were effective in vitro, namely **5a–c** and **7b**, were also studied in vivo. Two screening protocols were used: the formalin-induced paw edema and turpentine oil-induced granuloma pouch bioassays. Celecoxib (20 mg/kg) was used as a reference anti-inflammatory agent. The study of rat paw edema was employed as a model for acute and sub-acute inflammation, while the turpentine oil-induced granuloma pouch assay was utilized as a model for sub-acute inflammatory condition (Tables 3–5).

2.3.1. Formalin-induced paw edema bioassay (acute inflammatory model)

Each test compound was administered orally (po, 20 mg/kg body weight) 1 h prior to induction of inflammation by formalin injection. Celecoxib was utilized as a reference anti-inflammatory drug[8] at a dose of 20 mg/kg. The anti-inflammatory activity was assessed 1, 2, 3 and 4 h after induction and data are presented in Table 3 as the mean paw volume (mL). Percent anti-inflammatory activity was also calculated.

All of the test compounds, including celecoxib, significantly reduced paw edema volume at all assessed time points. A comparison of the percentage of anti-inflammatory activities of the test compounds relative to the control at different time intervals indicated a distinctive pharmacokinetic profile for compound **7b** after 1 h, as revealed by its potent and rapid onset of action. Its anti-inflammatory activity (53%) was higher than that of celecoxib (41%) at a dose of 20 mg/kg, po. Compound **5b** showed activity comparable to celecoxib (41%), while compounds **5a** and **5c** had somewhat lower inhibitory activity (35% and 24%, respectively).

Table 3
Anti-inflammatory activity of select compounds in formalin-induced rat paw edema bioassay (acute inflammatory model)

Compound[a]	Volume of edema (mL)[b]				
	0 h	1 h	2 h	3 h	4 h
Control	0.31 ± 0.01	0.48 ± 0.01	0.55 ± 0.01	0.78 ± 0.01	0.78 ± 0.005
5a	0.31 ± 0.003	0.42 ± 0.01* (35)[c]	0.46 ± 0.01* (38)	0.58 ± 0.01* (43)	0.58 ± 0.02* (43)
5b	0.30 ± 0.01	0.40 ± 0.005* (41)	0.42 ± 0.01* (50)	0.42 ± 0.01* (74)	0.42 ± 0.01* (74)
5c	0.31 ± 0.001	0.44 ± 0.01 (24)	0.44 ± 0.01* (46)	0.46 ± 0.003* (68)	0.46 ± 0.02* (68)
7b	0.33 ± 0.02	0.41 ± 0.01* (53)	0.42 ± 0.01* (63)	0.46 ± 0.01* (72)	0.49 ± 0.01* (66)
Celecoxib	0.31 ± 0.01	0.41 ± 0.005* (41)	0.43 ± 0.02* (50)	0.50 ± 0.005* (60)	0.46 ± 0.03* (68)

* Significantly different compared to respective control values, $P < 0.05$.
[a] Dose levels of 20 mg/kg (po) were used for all test compounds including celecoxib.
[b] Values are expressed as means ± SEM ($N = 5$ rats per experimental group).
[c] Values in parentheses represent percentage anti-inflammatory activity.

Table 4
Anti-inflammatory activity of select compounds in formalin-induced rat paw edema bioassay (sub-acute inflammatory model)

Compound[a]	Paw edema on day 8[b] (g)	% inhibition
Control	2.65 ± 0.08	—
5a	1.23 ± 0.04*	53
5b	1.53 ± 0.02*	42
5c	1.29 ± 0.09*	51
7b	1.39 ± 0.04*	47
Celecoxib	1.33 ± 0.08*	50

* Significantly different compared to respective control values, $P < 0.05$.
[a] Dose levels of 20 mg/kg (po) were used for all test compounds including celecoxib.
[b] Values are expressed as means ± SEM ($N = 5$ rats per experimental group).

Table 5
Anti-inflammatory activity of select compounds in turpentine oil-induced granuloma pouch bioassay in rats

Compound[a]	Volume of exudates (mL)[b]	% inhibition
Control	2.28 ± 0.07	—
5a	1.07 ± 0.06*	53
5b	1.27 ± 0.12*	44
5c	1.12 ± 0.06*	51
7b	1.12 ± 0.04*	50
Celecoxib	1.05 ± 0.10*	54

* Significantly different compared to respective control values, $P < 0.05$.
[a] Dose levels of 20 mg/kg (po) were used for all test compounds including celecoxib.
[b] Values are expressed as means ± SEM ($N = 5$ rats per experimental group).

At the 2 h interval, compound **7b** again showed the highest anti-inflammatory activity (63%) whereas compounds **5b** (50%) and **5c** (46%) were nearly or as effective as celecoxib (50%). After 3 h compounds **5b**, **5c** and **7b** showed anti-inflammatory activity (74%, 68% and 72%, respectively) even higher than celecoxib (60%). At the conclusion of the experiment (4 h), compound **5b** had the highest anti-inflammatory activity (74%), whereas compounds **5c** (68%) and **7b** (66%) were nearly equipotent to the reference drug celecoxib (68%).

2.3.2. Formalin-induced paw edema bioassay (sub-acute inflammatory model)

For this sub-acute model, inflammation was induced by formalin injection in the first and third days, and test compounds were administered orally at 20 mg/kg daily for seven days. Again, celecoxib was used as a reference anti-inflammatory agent. The anti-inflammatory activity was calculated at the 8th day after induction and presented in Table 4 as the mean weight of paw edema and the percentage anti-inflammatory activity. The obtained data revealed that all of the studied compounds significantly reduced paw edema. Compounds **5a**, **5c** and **7b** displayed anti-inflammatory activity (47–53%) nearly equal to celecoxib (50%), while compound **5b** showed moderate anti-inflammatory activity (42%).

2.3.3. Turpentine oil-induced granuloma pouch bioassay (sub-acute inflammatory model)

In this bioassay, each test compound was administered orally (20 mg/kg) 1 h prior to turpentine oil injection and the administration was continued daily for seven days. At the 8th day, the exudate volume (mL) was measured and the percentage of granuloma inhibition was calculated. Celecoxib (20 mg/kg) was used as a reference drug. Table 5 illustrates that all of the studied compounds significantly reduced the exudate volume when compared to the control group of animals. Compounds **5a**, **5c** and **7b** were nearly as potent (50–53%) as celecoxib (54%), while compound **5b** showed moderate anti-inflammatory activity in this bioassay with percentage of granuloma inhibition of 44%.

The in vivo experiments (Tables 3–5) revealed that **5b** had the highest inhibitory activity among all the compounds tested in the acute formalin paw edema model. **5a** had the highest inhibitory activity of the newly synthesized compounds in formalin-induced paw edema and turpentine oil-induced granuloma pouch assays (sub-acute inflammatory models). Compounds **5c** and **7b** appeared to perform equally well in all three assays. These observations indicate that compound **5b** might be more effective in managing acute inflammation, while compound **5a** might be more effective in controlling chronic inflammatory conditions. Compounds **5c** and **7b** appeared to be equally effective in suppressing acute and chronic inflammatory conditions.

2.3.4. Ulcerogenic activity

Compounds that were selected for in vivo experiments were further evaluated for their ulcerogenic potential in rats. Gross observation of the isolated rat stomachs showed a normal stomach texture for all of the tested compounds with no observable hyperemia. This indicates a superior gastrointestinal safety profile (0% ulceration) in the population of the test animals at an oral dose of 300 mg/kg, when administered twice at 2 h interval in fasting rats. It is worth mentioning that celecoxib and indomethacin, two standard anti-inflammatory drugs; were found to cause 0% and 100% ulceration, respectively, under the same experimental conditions.

2.3.5. Acute toxicity

All of the selected compounds were further evaluated for their acute lethal dose (ALD_{50}) in mice. All of the tested compounds proved to be non-toxic and were well tolerated by the experimental animals. The compounds showed a high safety margin when screened at graded doses (0–300 mg/kg, po). ALD_{50} values for all of the newly synthesized compounds were found to be >300 mg/kg.

2.3.6. Effective dose 50 (ED_{50})

The test compounds were further evaluated in rats for their ED_{50}; they were tested at 5, 10, 20, 40 and 50 mg/kg body weight. The following ED_{50} values were obtained: **5a** (21.3 mg/kg); **5b** (13.0 mg/kg); **5c** (15.2 mg/kg); **7b** (15.4 mg/kg); and celecoxib (14.3 mg/kg). Thus, compounds **5b**, **5c** and **7b** had an ED_{50} similar to celecoxib, which indicated that they were nearly equipotent to the reference drug.

3. Conclusion

A series of pyrazolyl-2,4-thiazolidinediones was synthesized and initially tested in vitro for their cytotoxic, anti-inflammatory and anti-neurotoxic properties. Compounds **5c** and **7b** showed anti-neurotoxic activity at concentrations below their cytotoxic range; such activity has been reported before for several other compounds with different chemical structures.[3,21] We demonstrated that **5c** and **7b** were effective as COX-1 and COX-2 inhibitors, which have previously been shown to be anti-neurotoxic.[25] However other mechanisms of action for these drugs cannot be ruled out and will require further investigation. Our observations that none of the compounds inhibited MCP-1 secretion by THP-1 cell may indicate that two of the mitogen-activated protein kinases, extracellular signal-regulated kinases 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK), as well as nuclear factor-kappaB (NF-kappaB) were not affected by the newly synthesized compounds.[22]

Compounds **5a–c** inhibited mononuclear phagocyte respiratory burst at concentrations that were not toxic to cells. Phagocyte NADPH oxidase, which is responsible for the respiratory burst, generates high concentrations of reactive oxygen species, such as the superoxide anion. These highly reactive intermediates have been shown to be directly toxic to different cell types[27] and also to amplify inflammatory processes.[28] Therefore NADPH oxidase inhibitors might have a potential as anti-inflammatory agents.

Four of the drugs that showed beneficial in vitro activity (**5a–c**, **7b**) were selected for in vivo testing. Experiments conducted with rats and mice revealed a pronounced anti-inflammatory activity of the new compounds, which was comparable to that of celecoxib in both the acute and sub-acute inflammatory models. In addition, all of the selected compounds possessed excellent gastrointestinal safety profile and were well tolerated by experimental animals with high safety margins (ALD_{50} >300 mg/kg).

Compound **5b** showed higher activity in the animal model of acute inflammation while compound **5a** was more effective in controlling chronic inflammation. Compounds **5c** and **7b** were equipotent in all of the in vivo assays performed. It can be concluded that compounds that contain 2,4-thiazolidinediones exhibit better anti-neurotoxic and anti-inflammatory activity than compounds that have substituted benzyl 2,4-thiazolidinediones. The latter compounds also showed increased toxicity towards THP-1 cells. Compounds **5a–c** and **7b** contain 2,4-thiazolidinediones with smaller ring structures compared to substituted benzyl 2,4-thiazolidinediones; therefore, less steric hindrance may account for the higher biological activity of these compounds.

We have identified four novel compounds with potential anti-inflammatory and neuroprotective properties. These four compounds represent a fruitful matrix for the future development of a new class of anti-inflammatory and neuroprotective agents that deserves further investigation and derivatization. Future experiments will evaluate neuroprotective properties of the biologically active compounds. We are planning in vivo studies by using animal models of Alzheimer's and Parkinson's diseases.

4. Experimental

4.1. Chemistry

Melting points were determined in open glass capillaries on a Stuart melting point apparatus and are uncorrected. IR spectra ν cm^{-1} (KBr) were recorded on a FTIR Nicolet IR 200 Spectrophotometer. ^1H and ^{13}C NMR spectra were run on an AS 400 MHz NMR Oxford Spectrophotometer, using tetramethylsilane (TMS) as the internal standard and DMSO-d_6 as the solvent.

Chemical shifts were recorded as δ (ppm). Elemental analyses for all the synthesized compounds were within ±0.4% of the theoretical values.

4.1.1. 5-((1,3-Aryl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-diones (5a–c)

Compounds **3a–c** (1.31 mmol) and thiazolidine-2,4-dione (0.153 g, 1.31 mmol) were added to 20 mL of anhydrous toluene. A few drops of glacial acetic acid and piperidine were added and refluxed for 18 h while removing water using a Dean-Stark trap. The reaction was cooled to room temperature and stirred for 6 h. The resultant solid was filtered, washed with diethyl ether (3 × 10 mL) and recrystallized from the appropriate solvent. IR (cm^{-1}) for compounds **5a–c**: 3399–3369 (NH), 1741–1736, 1689–1683 (C=O).

4.1.1.1. 5-((1,3-Diphenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-dione (5a). Ethanol, 80% yield, mp: 263–265 °C. ^1H NMR (δ ppm): 7.41 (s, 1H, –CH=), 7.52–8.02 (m, 10H, Ar–H), 8.68 (s, 1H, pyrazole C-5 H), 12.28 (br, IH, NH, D_2O exchangeable). ^{13}C NMR (δ ppm): 171.87, 169.12, 153.64, 139.75, 133.59, 130.37, 130.16, 129.41, 129.14, 128.55, 127.88, 126.93, 119.68, 118.59, 117.21. Elemental Anal. Calcd for $C_{19}H_{13}N_3O_2S$ (347.39): C, 65.69; H, 3.77; N, 12.10. Found: C, 66.10; H, 3.52; N, 11.98.

4.1.1.2. 5-((3-(4-Chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-dione (5b). $CHCl_3$, 82% yield, mp: 280–282 °C. ^1H NMR (δ ppm): 7.43 (s, 1H, –CH=), 7.54- 8.01 (m, 9H, Ar–H), 8.69 (s, 1H, pyrazole C-5 H), 12.28 (br, IH, NH, D_2O exchangeable). ^{13}C NMR (δ ppm): 171.87, 169.12, 153.73, 139.75, 135.34, 130.26, 130.14, 129.83, 129.29, 127.79, 127.31, 125.93, 119.98, 118.99, 117.51. Elemental Anal. Calcd for $C_{19}H_{12}ClN_3O_2S$ (381.84): C, 59.76; H, 3.17; N, 11.00. Found: C, 59.45; H, 3.02; N, 11.35.

4.1.1.3. 5-((3-(4-Chlorophenyl)-1-(4-nitrophenyl)-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-dione (5c). Dioxane–water, 72% yield, mp: 275–277 °C. ^1H NMR (δ ppm): 7.34 (s, 1H, –CH=), 7.64 (d, 2H, J = 8.8 Hz, *p*-chlorophenyl C-2,6 H), 7.69 (d, 2H, J = 8.8 Hz, *p*-chlorophenyl C-3,5 H), 8.30 (d, 2H, J = 9.2 Hz, *p*-nitrophenyl C-2,6 H), 8.40 (d, 2H, J = 9.2 Hz, *p*-nitrophenyl C-3,5 H), 8.77 (s, 1H, pyrazole C-5 H), 12.53 (br, IH, NH, D_2O exchangeable). ^{13}C NMR (δ ppm): 171.87, 169.12, 153.73, 145.51, 144.81, 135.34, 130.26, 130.14, 129.89, 129.41, 127.79, 124.56, 120..31, 118.54, 117.44. Elemental Anal. Calcd for $C_{19}H_{11}ClN_4O_4S$ (426.83): C, 53.46; H, 2.60; N, 13.13. Found: C, 53.04; H, 2.72; N, 12.75.

4.1.2. 5-((3-Substituted-1-phenyl-1*H*-pyrazol-4-yl)methylene)-3-propylthiazolidine-2,4-diones (6a,b)

Compounds **5a**,**b** (0.21 mmol) were dissolved in 1.0 mL of anhydrous DMF. Potassium carbonate (0.035 g, 0.25 mmol) was added under a N_2 atmosphere and iodopropane (0.054 g, 0.32 mmol) was injected 10 min later. The mixture was stirred for 2 h at room temperature. Water (5 mL) was added and the resultant solid was washed with water (3 × 10 mL). The solid was dried and recrystallized from the appropriate solvent. IR (cm^{-1}) for compounds **6**,**a**,**b**: 1741–1736, 1689–1683 (C=O).

4.1.2.1. 5-((1,3-Diphenyl-1*H*-pyrazol-4-yl)methylene)-3-propylthiazolidine-2,4-dione (6a). $CHCl_3$–hexane, 85% yield, mp: 170–172 °C. ^1H NMR (δ ppm): 0.93 (t, 3H, J = 7.4 Hz, CH_3), 1.68–1.70 (m, 2H, CH_2), 3.69 (t, 2H, J = 7.2 Hz, CH_2), 7.24 (s, 1H, –CH=), 7.37–7.91 (m, 10H, Ar–H), 8.18 (s, 1H, pyrazole C-5 H). Elemental Anal. Calcd for $C_{22}H_{19}N_3O_2S$ (389.47): C, 67.84; H, 4.92; N, 10.79. Found: C, 68.25; H, 5.12; N, 11.18.

4.1.2.2. 5-((3-(4-Chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)-3-propylthiazolidine-2,4-dione (6b). $CHCl_3$, 87% yield, mp: 202–204 °C. ^{1}H NMR (δ ppm): 0.94 (t, 3H, J = 7.4 Hz, CH_3), 1.54–1.72 (m, 2H, CH_2), 3.70 (t, 2H, J = 7.2 Hz, CH_2), 7.24 (s, 1H, –CH=), 7.37–7.91 (m, 9H, Ar–H), 8.16 (s, 1H, pyrazole C-5 H). Elemental Anal. Calcd for $C_{22}H_{18}ClN_3O_2S$ (423.92): C, 62.33; H, 4.28; N, 9.91. Found: C, 61.89; H, 4.12; N, 10.18.

4.1.3. 3-Allyl-5-((3-substituted-1-phenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-diones (7a,b)

Compounds **5a**,**b** (0.21 mmol) were dissolved in 1.0 mL of anhydrous DMF. Potassium carbonate (0.035 g, 0.25 mmol) was added under a N_2 atmosphere and allyl bromide (0.039 g, 0.32 mmol) was added 10 min later. The mixture was stirred for 2 h at room temperature. Water (5 mL) was added and the resultant solid was washed with water (3 × 10 mL). The solid was dried and recrystallized from $CHCl_3$. IR (cm^{-1}) for compounds **7a**,**b**: 1740–1736, 1689–1681 (C=O).

4.1.3.1. 3-Allyl-5-((1,3-diphenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-dione (7a). Yield: 83%, mp: 175–177 °C. ^{1}H NMR (δ ppm): 4.33 (d, 2H, J = 5.9 Hz, N–CH_2), 5.23–5.31 (m, 2H, =CH_2), 5.81–5.88 (m, 1H, CH), 7.24 (s, 1H, –CH=), 7.38–7.86 (m, 10H, Ar–H), 8.17 (s, 1H, pyrazole C-5 H). Elemental Anal. Calcd for $C_{22}H_{17}N_3O_2S$ (387.45): C, 68.20; H, 4.42; N, 10.85. Found: C, 67.85; H, 4.22; N, 10.58.

4.1.3.2. 3-Allyl-5-((3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-dione (7b). Yield: 81%, mp: 210–212 °C. ^{1}H NMR (δ ppm): 4.32 (d, 2H, J = 5.9 Hz, N–CH_2), 5.20–5.31 (m, 2H, =CH_2), 5.81–5.88 (m, 1H, CH), 7.24 (s, 1H, –CH=), 7.39–7.89 (m, 9H, Ar–H), 8.15 (s, 1H, pyrazole C-5 H). ^{13}C NMR (δ ppm): 166.45, 165.48, 153.72, 139.18, 135.34, 130.42, 130.22, 130.03, 129.83, 129.29, 127.97, 127.51, 124.43, 120.31, 119.74, 119.19, 116.25, 44.09. Elemental Anal. Calcd for $C_{22}H_{16}ClN_3O_2S$ (421.9): C, 62.63; H, 3.82; N, 9.96. Found: C, 62.95; H, 4.20; N, 10.23.

4.1.4. 3-Substituted benzylthiazolidine-2,4-diones (9a–c)

A mixture of thiazolidine-2,4-dione (2.34 g, 0.02 mol), substituted benzyl halide (0.02 mol) and sodium hydroxide (0.8 g, 0.02 mol) in 50% ethanol (20 mL) was refluxed for 18 h. The crude product was crystallized from ethanol. Compound **9a**, mp: 62–63 °C (Ref. 29, mp: 61 °C), **9b** mp: 96 °C (Ref. 29, mp: 97–98 °C), **9c** mp: 75–77 °C (Ref. 30).

4.1.5. 3-Substituted benzyl-5-((3-substituted-1-phenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-diones (10a–f)

Compounds **4a–c** (1.31 mmol) and 3-substituted benzylthiazolidine-2,4-dione **9a–c** (1.31 mmol) were added to 20 mL of anhydrous toluene. A few drops of glacial acetic acid and piperidine were added and refluxed for 20 h while removing water using a Dean-Stark trap. After cooling down to room temperature, stirring was performed for 6 h. The resultant solid was filtered and washed with diethyl ether (3 × 10 mL). The filtered solid was dried and recrystallized from the appropriate solvent. IR (cm^{-1}) for compounds **10a–f**: 1736–1730, 1689–1683 (C=O).

4.1.5.1. 3-Benzyl-5-((1,3-diphenyl-1*H*-pyrazol-4-yl)methylene)-thiazolidine-2,4-dione (10a). $CHCl_3$, 82% yield, mp: 208–210 °C. ^{1}H NMR (δ ppm): 4.83 (s, 2H, CH_2), 7.24 (s, 1H, –CH=), 7.29–7.92 (m, 15H, Ar–H), 8.16 (s, 1H, pyrazole C-5 H). Elemental Anal. Calcd for $C_{26}H_{19}N_3O_2S$ (437.51): C, 71.38; H, 4.38; N, 9.60. Found: C, 70.97; H, 4.52; N, 9.98.

4.1.5.2. 3-(4-Chlorobenzyl)-5-((1,3-diphenyl-1*H*-pyrazol-4-yl)-methylene)thiazolidine-2,4-dione (10b). $CHCl_3$, 79% yield, mp: 196–198 °C. ^{1}H NMR (δ ppm): 4.83 (s, 2H, CH_2), 7.24 (s, 1H, –CH=), 7.28–7.92 (m, 14H, Ar–H), 8.16 (s, 1H, pyrazole C-5 H). Elemental Anal. Calcd for $C_{26}H_{18}ClN_3O_2S$ (471.96): C, 66.17; H, 3.84; N, 8.90. Found: C, 65.91; H, 3.50; N, 9.18.

4.1.5.3. 3-(3-Bromobenzyl)-5-((1,3-diphenyl-1*H*-pyrazol-4-yl)-methylene)thiazolidine-2,4-dione (10c). $CHCl_3$, 81% yield, mp: 198–200 °C. ^{1}H NMR (δ ppm): 4.82 (s, 2H, CH_2), 7.24 (s, 1H, –CH=), 7.18–7.90 (m, 14H, Ar–H), 8.16 (s, 1H, pyrazole C-5 H). Elemental Anal. Calcd for $C_{26}H_{18}BrN_3O_2S$ (516.41): C, 60.47; H, 3.51; N, 8.14. Found: C, 60.81; H, 3.65; N, 7.86.

4.1.5.4. 3-Benzyl-5-((3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-dione (10d). $CHCl_3$, 82% yield, mp: 218–220 °C. ^{1}H NMR (δ ppm): 4.66 (s, 2H, CH_2), 7.15–7.87 (m, 15H, Ar–H, –CH=), 8.63 (s, 1H, pyrazole C-5 H). Elemental Anal. Calcd for $C_{26}H_{18}ClN_3O_2S$ (471.96): C, 66.17; H, 3.84; N, 8.90. Found: C, 65.98; H, 3.52; N, 8.99.

4.1.5.5. 3-(4-Chlorobenzyl)-5-((3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-dione (10e). $CHCl_3$–hexane, 85% yield, mp: 178–180 °C. ^{1}H NMR (δ ppm): 4.32 (s, 2H, CH_2), 6.83–7.53 (m, 14H, Ar–H, –CH=), 8.29 (s, 1H, pyrazole C-5 H). Elemental Anal. Calcd for $C_{26}H_{17}Cl_2N_3O_2S$ (506.4): C, 61.67; H, 3.38; N, 8.30. Found: C, 61.38; H, 3.72; N, 8.69.

4.1.5.6. 3-(3-Bromobenzyl)-5-((3-(4-chlorophenyl)-1-phenyl-1*H*-pyrazol-4-yl)methylene)thiazolidine-2,4-dione (10f). $CHCl_3$–hexane, 80% yield, mp: 188–190 °C. ^{1}H NMR (δ ppm): 4.82 (s, 2H, CH_2), 7.31–8.04 (m, 14H, Ar–H, –CH=), 8.80 (s, 1H, pyrazole C-5 H). Elemental Anal. Calcd for $C_{26}H_{17}BrClN_3O_2S$ (550.85): C, 56.69; H, 3.11; N, 7.63. Found: C, 56.38; H, 3.42; N, 7.97.

4.2. In vitro assays

4.2.1. Reagents

The following substances were used in various assays and were obtained from Sigma (St. Louis, MO, USA): hydrogen peroxide, bacterial lipopolysaccharide (LPS, from E.coli 055:B5), diaphorase (EC 1.8.1.4, from Clostridium kluyveri, 5.8 Units mg^{-1} solid), DMSO, *p*-iodonitrotetrazolium violet, NAD^+, and MTT. Human recombinant IFN-γ, MCP-1 and antibodies used in MCP-1 enzyme linked immunoabsorbent assay (ELISA) were purchased from Peprotech (Rocky Hill, NJ, USA).

4.2.2. Cell culture

The human monocytic THP-1 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The human neuroblastoma SH-SY5Y cell line was a gift from Dr. R. Ross, Fordham University, NY. Cells were grown in Dulbecco's modified Eagle's medium-nutrient mixture F12 ham (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) supplied by Thermo Scientific HyClone (Logan, UT, USA). Both cell lines were used without initial differentiation.

4.2.3. Effects of compounds on THP-1 cells viability and MCP-1 secretion

Human monocytic THP-1 cells were seeded into 24-well plates at a concentration of 5×10^5 cells mL^{-1} in 0.9 mL of DMEM-F12 medium containing 5% FBS. The cells were incubated in the presence or absence of various compounds or their vehicle solution (DMSO) for 15 min prior to the addition of an activating stimulus (0.5 μg/mL LPS with 150 Units/mL IFN-γ). After 24 h incubation, 100 μL of THP-1 culture media was sampled for LDH to determine the percentage of dead cells, while the evaluation of surviving cells was performed by the MTT assay. In addition, the concentration of

MCP-1 (ng/mL) was measured in 100 μL of cell-free culture medium by ELISA, according to the protocol provided by the supplier of the antibodies (Peprotec).

4.2.4. Cytotoxicity of THP-1 cells toward SH-SY5Y neuroblastoma cells

The experiments were performed as previously described.[21] THP-1 cells were seeded into 24-well plates and stimulated in the presence and absence of various compounds as described above. After 24 h or 48 h incubation, 0.4 mL of cell-free supernatant was transferred to each well containing SH-SY5Y cells. The cells had been plated 24 h earlier at a concentration of 2×10^5 cells mL^{-1} in 0.4 mL of DMEM-F12 medium containing 5% FBS. After 72 h incubation, the neuronal culture media was sampled for LDH to determine leakage from dead cells, while the evaluation of surviving cells was performed by the MTT assay.

4.2.5. Neuroprotective effects

SH-SY5Y cells were seeded into 24-well plates at a concentration of 2×10^5 mL^{-1} in 0.4 mL of DMEM-F12 medium containing 5% FBS. After 24 h incubation cell culture medium was replaced by either supernatants from THP-1 cells that had been stimulated for 48 h with LPS + IFN-γ or culture media containing 500 μM H_2O_2. Various concentrations of the compounds were added directly to the SH-SY5Y cells at the time of the media transfer. Cell death was assessed by the LDH assay 72 h later.

4.2.6. Cell viability assays: LDH release

Cell death was evaluated by LDH release. LDH activity in cell culture supernatants was measured by an enzymatic test as described by Decker and Lohmann-Matthes,[31] in which formation of the formazan product of iodonitrotetrazolium dye was followed colorimetrically. Briefly, 100 μL of cell culture supernatants were pipetted into the wells of 96-well plates, followed by addition of 15 μL lactate solution (36 mg/mL) and 15 μL *p*-iodonitrotetrazolium violet solution (2 mg/mL). The enzymatic reaction was started by addition of 15 μL of NAD^+/diaphorase solution (3 mg/mL NAD^+; 2.3 mg solid/mL diaphorase). After a 15–30 min incubation period, the reaction was terminated by the addition of 15 μL of oxamate (16.6 mg/mL). Optical densities at 490 nm were measured by a microplate reader; the amount of LDH which had been released, was expressed as a fraction of the value obtained in comparative wells where the remaining cells were totally lysed by 1% Triton X 100.

4.2.7. Cell viability assays: reduction of formazan dye (MTT)

The MTT assay was performed as described by Mosmann[32] and by Hansen et al.[33] This method is based on the ability of viable, but not dead cells, to convert the tetrazolium salt (MTT) to colored formazan. The viability of SH-SY5Y cells was determined by adding MTT to the SH-SY5Y cell cultures to reach a final concentration of 0.5 mg/mL. Following a 1 h incubation period at 37 °C, the dark crystals which had formed were dissolved by adding to the wells an equal volume of SDS/DMF extraction buffer (20% sodium dodecyl sulfate, 50% *N*,*N*-dimethyl formamide, pH 4.7). Subsequently, the plates were placed overnight at 37 °C; optical densities at 570 nm were then measured by transferring 100 μL aliquots to 96-well plates and using a platereader. The viable cell value was calculated as a fraction of the value obtained from cells incubated with fresh medium only.

4.2.8. Phagocyte respiratory burst

Differentiated human promyelocytic HL-60 cells were used to study the effects of the compounds on phagocyte respiratory burst. These cells have been shown to express all subunits of phagocyte NADPH oxidase. Real-time chemiluminescence measurements were performed as described by Muranaka et al.[23] and Kopprasch et al.[34] First, HL-60 cells were differentiated by plating into 10 cm tissue culture plates at a density of 2×10^5 cells mL^{-1} in DMEM-F12 medium containing 10% FBS and 1.3% DMSO. After a 6–7 days differentiation period, which was confirmed in preliminary studies to enhance the respiratory burst activity, HL-60 cells were washed and transferred into DMEM-F12 medium without phenol red in the absence of serum. They were seeded into 96-well plates at a concentration of 1×10^6 mL^{-1} in 80 μL per well. Plates were incubated for 30 min at 37 °C, followed by addition of various concentrations of drugs or the solvent (DMSO) and inserted into FLUOstar Omega platereader (BMG Labtech, Offenburg, Germany). During the experiment plates were maintained at 37 °C. First, each well was injected with luminol solution and following 7.5 min of baseline recording FMLP solution was injected. The final reaction volume was 100 μL, luminol concentration 5 mM, FMLP 1 μM and DMSO solvent 0.5%. Chemiluminescence response of HL-60 cells in the presence or absence of drugs was recorded for 30 min and expressed as the area under the curve in Relative Light Units.

4.2.9. Cyclooxygenase (COX) enzymatic assay

Compounds were tested for their ability to inhibit the two COX isoforms by using COX inhibitor screening assay kit supplied by the Cayman Chemical Company (Ann Arbor, MI, USA) according to the protocols provided by the manufacturer. This kit includes ovine COX-1 and human recombinant COX-2 enzymes, and it measures percent inhibition of prostaglandin production by the various compounds for the two COX isoforms.

4.3. In vivo anti-inflammatory activity

4.3.1. Animal experiments

Male Wistar strain albino rats weighing 180–200 g and Swiss albino mice (20–30 g) were used; they were acquired from a closed random bred colony at Faculty of Veterinary Medicine, University of Alexandria, Egypt. The animals were given *ad libitum* access to food and water and housed in groups of four in isolated cages under standard conditions of light and temperature. The animals were acclimatized for two weeks prior to experiments. The investigation confirmed to the guide for the Care and Use of Laboratory Animals published by US National Institute of Health (NIH publication no. 83–23, revised 1996). The local ethical committee approved the study.

4.3.2. Formalin-induced paw edema bioassay (acute inflammatory model)

This acute inflammatory model was performed as previously described.[35,36] Male albino rats weighing 180–200 g were used. The animals were randomly divided into groups of five. One group was kept as a control and another group received the standard anti-inflammatory drug celecoxib at a dose of 20 mg/kg body weight, po. A solution of formalin (2%, 0.1 mL) was injected into the subplantar region of the left hind paw under light ether anesthesia 1 h after oral administration of the test compound at a dose of 20 mg/kg body weight. The paw volume (mL) was measured by means of a water plethysmometer and re-measured again 1, 2, 3, and 4 h after the administration of formalin. The edema was expressed as an increase in the volume of paw, and the percentage of edema inhibition for each rat and each group was obtained as follows:

$$\%\ \text{Inhibition} = ((V_t - V_0)\text{control} - (V_t - V_0)\text{tested compound})/(V_t - V_0)\text{control} \times 100$$

where V_t = volume of edema at specific time interval and V_0 = volume of edema at time zero.

4.3.3. Formalin-induced paw edema bioassay (sub-acute inflammatory model)

This sub-acute inflammatory model was performed as previously described.[35,36] Rats in the first experiment were given the same test compounds at a dose of 20 mg/kg body weight daily for 7 consecutive days. A solution of formalin (2%, 0.1 mL) was injected into the subplantar region of the left hind paw under light ether anesthesia 1 h after oral administration of the test compound. A second injection of formalin (2%, 0.1 mL) was given on the third day. The changes in the weight (g) of paw edema were measured at the eighth day. Percentage inhibition of inflammation was calculated according to the following equation:

$$\%\ \text{Inhibition} = (\text{weight of paw edema of control} - \text{weight of paw edema of treated})/\text{weight of paw edema of control} \times 100$$

4.3.4. Turpentine oil-induced granuloma pouch bioassay (sub-acute inflammatory model)

This sub-acute inflammatory model was performed as previously described.[36,37] Male albino rats weighing 120–150 g were used throughout this assay. One group of five rats was kept as a control and another group received the standard drug celecoxib at a dose of 20 mg/kg body weight, po. Subcutaneous dorsal granuloma pouch was made in ether-anesthetized rats by injecting 2 mL of air, followed by injection of 0.5 mL of turpentine oil into it. All of the test compounds were administered orally at a dose of 20 mg/kg body weight 1 h prior to turpentine oil injection and continued for seven consecutive days. On the eighth day, the paw was opened under anesthesia and the exudates were taken out with a syringe. The volume (mL) of the exudates was measured and the percentage inhibition of inflammation was determined as follows:

$$\%\text{Inhibition} = (V_{\text{control}} - V_{\text{treated}})/V_{\text{control}} \times 100$$

4.3.5. Ulcerogenic activity

This assay was performed as previously described.[36,38] Male albino rats (180–200 g) were divided into groups of five animals and were fasted for 12 h prior to the administration of the test compounds. Water was given *ad libitum*. Control group received 1% gum acacia orally. Other groups received celecoxib, indomethacin or the test compounds orally in two equal doses at 0 and 12 h for three successive days at a dose of 100 mg/kg per day. Animals were sacrificed by diethyl ether 6 h after the last dose and their stomachs were removed. An opening at the greater curvature was made and the stomach was cleaned by washing with cold saline and inspected with a 3× magnifying lens for any evidence of hyperemia, hemorrhage, hemorrhagic erosion or ulcers.

4.3.6. Acute toxicity

This assay was performed as previously described.[36] Four groups of Swiss albino mice (20–30 g) each consisting of five animals, were used in this test. The animals were fasted for 24 h prior to administration of the test compounds. The compounds were given orally in graded doses of 0–300 mg/kg body weight, po. The compounds were screened at graded doses for their acute LD_{50} and the mortalities were recorded at each dose level after 24 h.

4.3.7. Determination of ED_{50}

The selected compounds were further tested in rats at 5, 10, 20, 40 and 50 mg/kg body weight and the ED_{50} was determined by measuring the inhibition of edema volume 3 h after formalin injection (2%, 0.1 mL).

4.3.8. Statistical analysis

Data are presented as means ± standard error of the mean (SEM). The concentration-dependent effects of various drugs in vitro were evaluated statistically by the randomized blocks design analysis of variance (ANOVA). Statistical differences between various in vivo data sets were estimated by using the Analysis of Variance (ANOVA) followed by Student–Newman–Keuls Multiple Comparison Test. Statistical Analysis System (SAS) and SPSS analytical software were used. The difference in results was considered significant when $P < 0.05$.

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