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Novel phthalimide derivatives with TNF-α and IL-1β expression inhibitory and apoptotic inducing properties

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Modulation of the immune system is an emerging concept in the control of tumor growth. Bearing in mind the pharmacological properties of thalidomide and its phthalimide derivatives, we describe here the structural design, synthesis and pharmacological evaluation of *N*-acylhydrazones derived from phthalimide. The ability of these *N*-acylhydrazones in inhibiting the secretion of TNF-α in stimulated cells as well as in inhibiting the transcription of the TNF-α gene was evaluated. We identified *N*-acylhydrazones **6b** and **9c**, which substantially impaired TNF-α secretion, expression and reduced IL-1β production similar to thalidomide or Revlimid. *N*-Acylhydrazone **9c** was also able to induce apoptosis in Jurkat cells, however it does not have either antiproliferative properties or cytotoxicity for mouse splenocytes. Beyond that, we have assayed the ability of these compounds to induce cell death and a number of them are able to induce apoptosis.

Introduction

The immune system's role in cancer involves many different mechanisms, such as minimizing metastasis by attenuating the expression of pro-angiogenic factors. Another aspect is the enhancement of antitumor immunity facilitated by interferons and interleukins. Overall, reducing inflammation by modulating the immune response is important over the course of cancer. In light of these possibilities, the identification of immunomodulating agents is a task that is receiving a great deal of attention. In this way, a variety of cytokines, including IL-1 and tumor necrosis factor (TNF), play different roles as regulators of immune, inflammatory, and growth responses.[1] IL-1 ligands (IL-1α and IL-1β, collectively referred to as IL-1) are pluripotent, proinflammatory cytokines that orchestrate inflammatory and host defense responses in the body.[2] IL-1 increases T-cell responses to mitogens (and indirectly activates B cells), increases expression of vascular adhesion molecules, and induces a number of other proinflammatory cytokines, chemokines, and inflammation-associated molecules that form an amplifying cascade to stimulate an immune response.[2] Tumor necrosis factor (TNF) can kill cells by apoptosis, and this ability to induce apoptosis is presently the most intensely studied area of TNF research.[3]

A series of recent papers has provided new hope that TNF could yet be useful as an anti-tumor cytokine.[4–8] The manipulation of signaling pathways downstream of TNF receptor (TNF-R) activation showed that TNF negatively regulates its own ability to induce apoptosis. However, despite considerable incentives, viable leads for analogous small-molecule inhibitors of TNF have not been reported.[8]

Phthalimide derivatives have been attracting considerable attention since 1979 when Chapman Jr *et al.* showed that *N*-substituted phthalimides possess hypolipidemic activity.[9] Two years later, Hall *et al.* examined 12 imide analogs and proposed that phthalimides are able to inhibit acetyl CoA carboxylase activity.[10] The same group investigated 10 *N*-aryl-phthalimides and found that *o*-(*N*-phthalimido)acetophenone lowered both serum cholesterol and triglyceride levels by 57% and 44% after 16 and 14 days of treatment in Swiss white mice.[11] This class of compounds is also endowed with anti-

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inflammatory and immunomodulatory properties, with thalidomide as the best representative of bioactive phthalimides.

Furthermore, a biochemical strategy for derivatization of carboxylic acid-containing non-steroidal anti-inflammatory drugs (NSAIDs) to esters or amides was able to produce molecules capable of binding tightly to COX-2, but not COX-1. This single chemical derivatization (amidation or esterification) of the carboxylic acid moiety-containing NSAIDs generates an impressive array of potent and highly selective COX-2 inhibitors. Several of them exhibited anti-inflammatory and anti-angiogenic activities.[12]

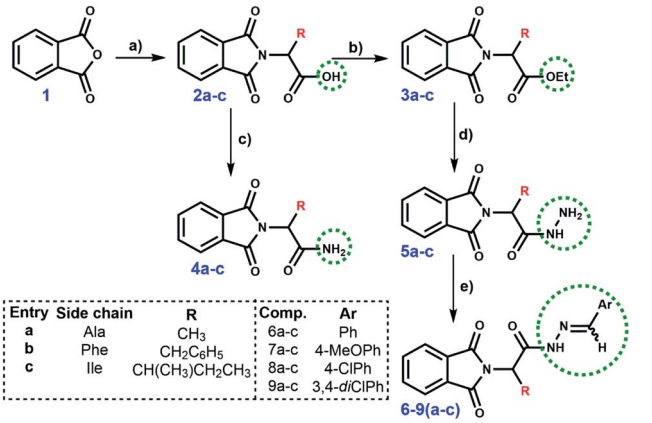
Similarly, a number of hydrazones have been evaluated for new drug development, and some of them have been shown to exhibit anti-cancer properties.[13] Some have demonstrated qualities of an apoptotic inducer with anti-proliferative chemopreventive activity in tongue cancer cells, such as the compound [bromomethyl(phenyl)methyl]-2-(2,4-dinitrophenyl)hydrazine.[14] In fact, because of their functional properties, the attachment of *N*-acylhydrazones has been employed in the design of new anti-inflammatory and immunomodulatory agents.

In view of this, we describe the synthesis of *N*-phthaloyl amino acids **2a–c** and their chemical derivatization to their respective esters **3a–c**, amides **4a–c**, hydrazides **5a–c** and *N*-acylhydrazone derivatives **6a–c**, **7a–c**, **8a–c** and **9a–c**. All these compounds are phthalimide derivatives designed to compare the bioisosteric relationship between a carboxylic acid and an amide; moreover, we aimed to compare the bioisosteric interplay between an amide and *N*-acylhydrazone. This examination also reports the pharmacological evaluation of their properties for inhibiting the secretion and expression of TNF and IL-1β in cells. Beyond that, we have assayed the ability of these compounds in inducing cell death and some of them are indeed apoptosis inducers.

Results and discussion

Chemistry

A single strategy for the production of new chemical entities is the chemical derivatization of acid or amine compounds to esters, amides or hydrazines. Indeed, *N*-phthaloyl amino acids **2a–c** were synthesized following a known procedure[15] that condenses phthalic anhydride **1** with the respective amino acids. Then *N*-phthaloyl amino acids **2a–c** were converted into ethyl esters **3a–c** and amides **4a–c** (Scheme 1). The reactions of amidification to prepare amides **4a–c** proceed well by using urea upon refluxing with ethanol (24 h), but we adapted them under ultrasound conditions using NH_4OH and K_2CO_3 as bases which produced excellent yields in general (50–85%) and in shorter time periods (30 min in most of the cases). The hydrazides **5a–c** were prepared by reacting commercially available hydrazine hydrate with the appropriate ethyl esters **3a–c** under magnetic stirring in EtOH with mild heating. Once hydrazides **5a–c** were isolated, we reacted them with aromatic aldehydes. These reactions were carried out in anhydrous EtOH in reflux under the presence of catalytic amounts of H_2SO_4, affording *N*-acylhydrazones **6a–c**, **7a–c**, **8a–c** and **9a–c** in low to moderate yields (38–84%). It is worth mentioning that only the desired



Scheme 1 Synthesis of the compounds. *Reagents and conditions*: (a) Ala, Phe or Ile, 135 °C, 30 min;[15] (b) ethanol, H_2SO_4 (4 drops), magnetic stirring, 60 °C, 4 h; (c) THF, imidazole, magnetic stirring, 60 °C, 4 h; (d) ethanol, hydrazine hydrate (4 drops), magnetic stirring, 60 °C, 4 h; and (e) ethanol, aromatic aldehyde substituted, reflux, 4 h.

S-enantiomers were obtained in all cases as verified by the addition of the chiral shift reagent $Eu(hfc)_3$ in ^1H NMR spectra.

Biological activities

To evaluate the levels of TNF and IL-1β, mouse macrophages were incubated with phthalimide derivatives over a 24 h time course. These cells were then stimulated with lipopolysaccharide (LPS). The purpose of this test was to evaluate the biological capacity of the molecules that were found to inhibit the synthesis of these extremely important cytokines in inflammatory processes and cancers. Data presented in Fig. 1 show that twelve compounds were able to inhibit TNF production at 50 μM, better than Thl. Among all series of compounds, the hydrazide and hydrazone series were the most promising. The hydrazide derivative **5b**, the phenyl-hydrazone derivative **6c** and the 4-methoxy-phenyl-hydrazone derivative **7c** all inhibit secretion of TNF by macrophages at 50 μM. Concerning IL-1β, in general, each series of compounds was able to inhibit IL-1β at the same concentration (50 μM). The ester derivative **3b** was the least potent. Compounds **5b**, **6c**, **7b** and **8c** were the most active of the series. These preliminary results indicate that, in general, the chemical derivatization to acylhydrazides and hydrazones and apolar aminoacyl moieties produces new derivatives that are most effective in modulating TNF and IL-1β expression.

To effectively measure the inhibition of the expression of TNF, the compounds were tested and evaluated for inhibition of TNF transcriptional activity *via* the NFkB signaling pathway. For this purpose, a T cell line containing a transcriptional reporter was used (FRT-Jurkat TNF) as previously described.[16,17] The green fluorescent protein (GFP) reporter gene is under the control of the TNF promoter sequence which contains an NFkB binding site. As a measure of TNF expression, the activity of GFP was quantified by flow cytometry as previously described.[18] First, it was determined whether the compounds inhibited the expression of TNF at 10 μM and 100 μM doses (Fig. 2). Thalidomide was used as a standard for comparing the levels of

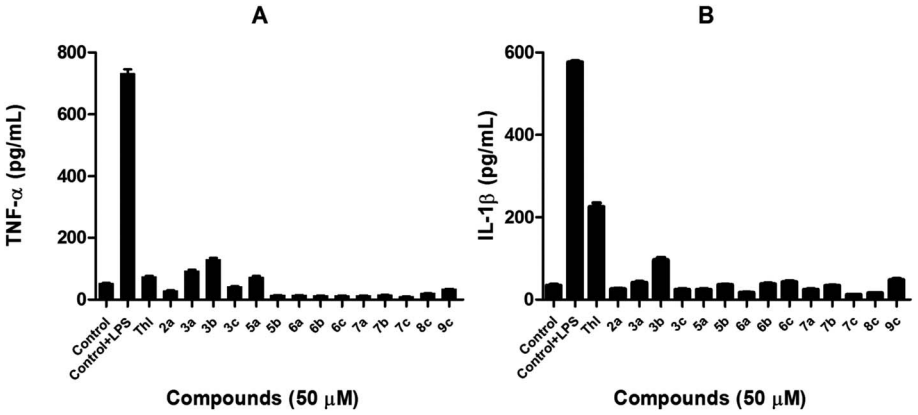


Fig. 1 (a) Effect of phthalimides and Thl on the production of TNF-α in mouse macrophages (2×10^6) stimulated with LPS (2 μg mL^{-1}). TNF-α was measured after 24 h of incubation with compounds (50 μM) by sandwich ELISA (eBioscience kit). Data are mean ± S.D. obtained in triplicate. (b) Effect of phthalimides and Thl on the production of interleukin-1β (IL-1β) in mouse macrophages (2×10^6) stimulated with LPS (2 μg mL^{-1}). IL-1β was measured after 24 h of incubation with compounds (50 μM) by sandwich ELISA (eBioscience kit). Data are mean ± S.D. (error bars) obtained in triplicate. Thl, thalidomide; S.D., standard deviation.

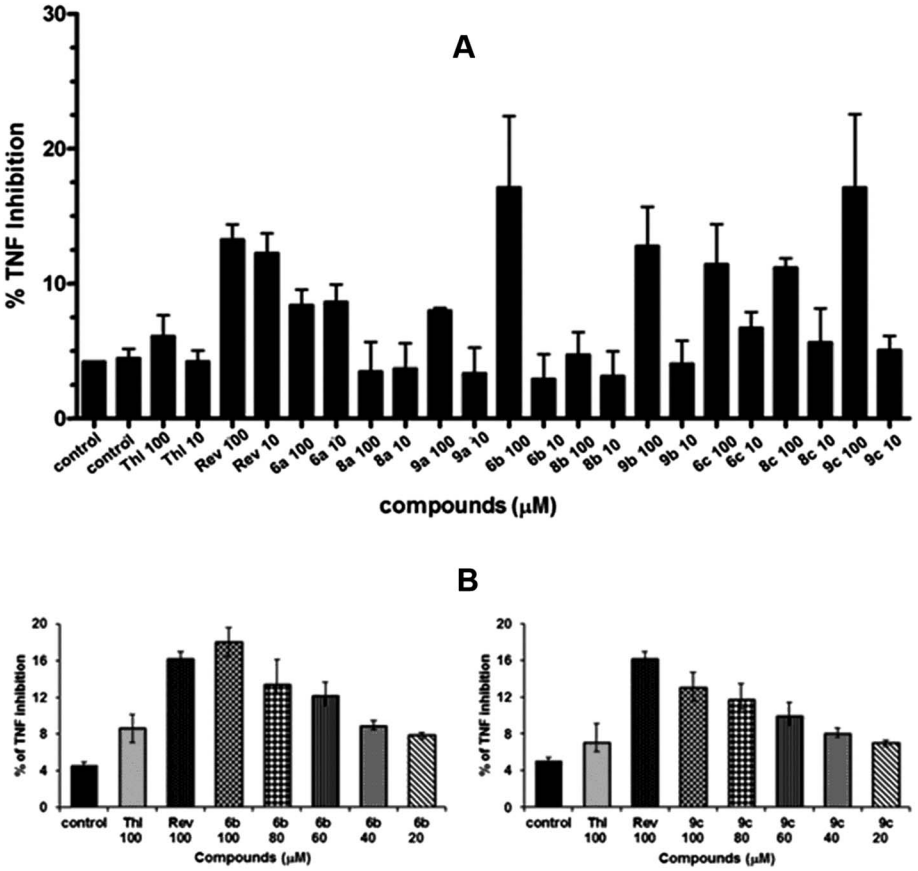


Fig. 2 (a) Effect of phthalimides and Thl on the expression of TNF in the human T cell line. Jurkat cells containing GFP-gene report under control of the TNF gene promoter (FRT-Jurkat TNF) were incubated for 24 h with the compounds (100 or 10 μM). Inhibition of TNF expression was measured as the GFP expression. Data are mean ± S.D. obtained in duplicate; one single experiment. (b) Dose–response curve of selected phthalimides in the expression of TNF in the human T cell line (Jurkat); two independent experiments. Controls have only buffer and DMSO (0.5%). Thl, thalidomide; Rev, Revlimid; S.D., standard deviation expressed in error bars.

inhibition. Revlimid (pomalidomide), a thalidomide derivative, which has been clinically shown to be 1000 times more powerful in relation to inhibition of TNF, was also used as a standard compound. In this model, Revlimid shows only twice the potency of thalidomide, therefore it suggests that if a compound shows a level of inhibition in this model similar to

Revlimid, in a clinical context, it will be a significant improvement over thalidomide.

The *N*-acyl-hydrazone **6a** derivative showed a percentage inhibition of TNF-α of 9.8% at both concentrations 100 μM and 10 μM, which is about 3% more than thalidomide (around 5–6% at 100 μM and 10 μM). The **6b** (phenyl-hydrazone derivative) and **9c** (3,4-dichloro-phenyl-hydrazone derivative) compounds were the most effective with inhibition around 17% at 100 μM, which is three times more effective than thalidomide and equipotent to Revlimid (Fig. 2A). No significant inhibitory activity was verified at 10 μM. As we can see in Fig. 2B, compounds **6b** and **9c** inhibit TNF-α in a dose-dependent manner. The TNF expression inhibition was more effective for the **6**, **8** and **9** series. These series have in common an *N*-phenyl-acyl-hydrazone moiety. In previous studies, it was observed that improvements in thalidomide derivatives bearing hydrophobic groups (phenyl or alkyl) have been found to provide highly potent inhibitors of TNF expression.[19]

As shown in Fig. 3, we analyzed the induction of total cell death by flow cytometry. Observing the DMSO control it is clear that the solvent does not interfere with the test and does not lead to cell death. Therefore, any toxicity observed would be due solely to the presence of the compounds. By adding thalidomide (10 μM) it is possible to observe a rate of 2–3% of cell death, however Revlimid is the less toxic option. In other words, Revlimid is the most effective in inhibiting TNF without killing

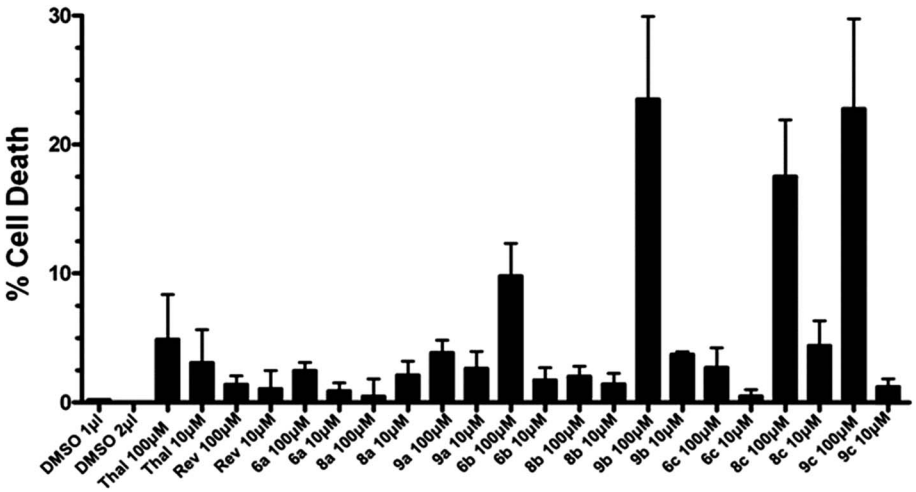


Fig. 3 Induction of total cell death. The effect of each compound on cellular viability was assessed using the FRT-Jurkat TNF reporter cell line using a FACSCalibur 4 colour flow cytometer. Viable and non-viable cells present following either solvent (DMSO) alone or compound treatment for 24 h were assayed. Cells were counted by gating on each cell population using a forward scatter (FSC) *versus* side scatter (SSC) plot. The percentage of viable cells was determined as the percentage of cells inside the FSC/SSC gate that encompassed the major population in solvent-only treated control cells. Viability was confirmed following propidium iodide staining and detection of fluorescence at 570 nm. Data are mean ± S.E. (standard error of the mean) obtained in triplicate. Thal, thalidomide, Rev, Revlimid.

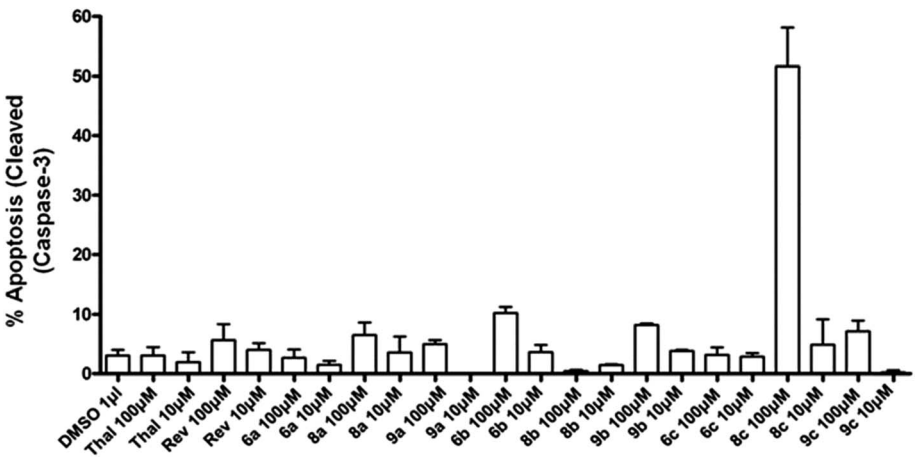


Fig. 4 Induction of apoptosis. Treatments were carried out as in Fig. 3 using the parental Jurkat line. Apoptosis was measured following treatment with a FITC-labelled caspase 3 substrate. The degree of apoptosis was assessed as the percentage of the cleaved caspase 3 substrate measured as an increase in fluorescence at 515 nm due to FITC release. Data are mean ± S.E. (standard error of the mean) obtained in triplicate. Thal, thalidomide, Rev, Revlimid.

cells. In general, all compounds showed acceptable levels of cell death comparable to thalidomide and even less than the Revlimid like acyl-hydrazones **6a**, **6c** and **9c** (all at 10 μM). The compounds **6b**, **9b**, **8c** and **9c** have shown a high rate of cell death at 100 μM (Fig. 3). However, it was not previously possible to say whether this was through programmed cell death.

It is interesting to note that the compound **6a** (alanyl-hydrazone derivative), at 10 μM, induced cell death at a lower percentage than Revlimid, also at 10 μM, and showed a percentage of inhibition of TNF similar to Revlimid (9% and 14% respectively, both at 10 μM). To determine whether cell death was *via* apoptosis (programmed cell death), we used the same T cell line, Jurkat, however without the GFP reporter (Fig. 4). Cells were treated with compounds and after 24 hours, the degree of caspase 3 cleavage was assessed as a measure of apoptosis. Cleavage could be measured as the release of the added fluorescein isothiocyanate (FITC)-labeled substrate against cleaved caspase 3.

In Fig. 4 it can be noted that the compound **6a** in this trial had a greater activity than the leading parent compound Revlimid. At both 100 μM and 10 μM concentrations, our synthesized compound **6a** showed a lower apoptotic action against the test cells. It is also observed that the compound **8c** (4-Cl-phenyl-hydrazone derivative) has a high rate of apoptosis. From the first test (Fig. 2) it appears that this compound inhibits TNF-α around 13% while Revlimid inhibits 14% at 100 μM. Although the compound **8c** does not show more efficient inhibition against TNF, it is equivalent to a drug for clinical use. However, this compound is far more effective in promoting programmed cell death.

Conclusion

In conclusion, new analogues of thalidomide and immunomodulatory activity were obtained. Compounds inhibited the production of TNF-α and IL-1 also *in vitro*. The profiles of compounds **6a**, **6b** and **9c** showed good inhibition of TNF-α as compared to the standards used (thalidomide and Revlimid). The compounds **6b**, **8c**, **9b** and **9c** showed high levels of cytotoxicity. In addition, compound **8c** showed a high rate of induction of apoptosis. These results indicate that, in general the chemical derivatization to acylhydrazones and hydrazones, instead of esters or amides is most effective in modulating TNF and IL-1 expression and also is effective in promoting programmed cell death. These select compounds are important lead candidates to act as immunomodulatory agents.

Experimental

General

Melting points which were measured with a Fisatom (Mod. 430D, 60 Hz) melting point apparatus are uncorrected. ^{1}H NMR spectra were recorded on a 300 MHz spectrometer in appropriate solvents using TMS as an internal standard or the solvent signals as secondary standards, and the chemical shifts are shown in the δ (ppm) scale. Multiplicities of NMR signals are designated as s (singlet), d (doublet), br (broad) and m (multiplet, for unresolved lines). ^{13}C NMR spectra were recorded on a 75.5 MHz spectrometer. All the experiments were monitored by analytical thin layer chromatography (TLC) performed on silica gel GF254 precoated plates. After elution, the plate was visualized under UV illumination at 254 nm for UV active materials.

Chemistry

General procedure for the synthesis of 3a–c. The respective acid **2a–c** (4.56 mmol), 15 mL of ethanol and H_2SO_4 (4 drops) were added to a 50 mL round bottom flask under magnetic stirring and warmed to 60 °C for 4 h. After cooling back to rt, the precipitate was filtered off and the solvent was evaporated. A white solid was obtained, filtered in a Buchner funnel with a sintered disc filter, washed with cold water, and then dried over SiO_2.

Ethyl 2-(1,3-dioxoisoindolin-2-yl)propanoate (3a). Chemical formula: $C_{13}H_{13}NO_4$. MW: 247.25 g. Yield: 70.09%. R_f: 0.6 (Hex/AcEt: 8/2). M.p.: liquid product. IR (KBr, cm^{-1}): 3008 (C–H), 1711 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 0.98 (M, 3H, CH_3), 1.12 (d, 3H, CH_3), 2.49 (m, 3H, CH and CH_2), 7.85–8.05 (m, 4H, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 10.8 (CH_3), 15.4 (CH_3), 20.8 (CH), 125.5 (Ar), 127.6 (Ar), 132.9 (Ar), 152.2 (C=O), 170.1 (C=O). Elemental analysis: liquid product.

Ethyl 2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanoate (3b). Chemical formula: $C_{19}H_{17}NO_4$. MW: 323.34 g. Yield: 74.89%. R_f: 0.5 (Hex/AcEt: 8/2). M.p.: liquid product. IR (KBr, cm^{-1}): 3447 (NH_2), 3018 (N–H), 1679 (C=O) cm^{-1}, 2918 (C–H), 1679 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 1.09 (m, 3H, CH_3), 3.50 (m, 4H, 2 × CH_2), 4.91 (m, 1H, CH), 7.08–7.14 (m, 5H, Ar), 7.12 (br, 2H, NH_2), 7.78–7.91 (m, 4H, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 10.0 (CH_3), 37.34 (CH_2), 55.8 (CH), 123.6 (Ar), 126.8 (Ar), 128.6 (Ar), 129.1 (Ar), 134.9 (Ar), 168.1 (C=O). Elemental analysis: liquid product.

Ethyl 2-(1,3-dioxoisoindolin-2-yl)-3-methylpentanoate (3c). Chemical formula: $C_{16}H_{19}NO_4$. MW: 289.33 g. Yield: 72.46%. R_f: 0.6 (Hex/AcEt: 8/2). M.p.: liquid product. IR (KBr, cm^{-1}): 3062 (C–H), 1776 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 0.77 (m, 6H, 2 × CH_3), 0.91 (m, 2H, CH_2), 0.98 (m, 3H, CH_3), 2.51 (m, 1H, CH), 4.34 (m, 1H, CH), 7.87–7.86 (m, 4H, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 10.5, 11.2 (CH_3), 17.1 (CH_3), 25.8 (CH_2), 33.3 (CH), 58.5 (CH), 123.6 (Ar), 135.0 (Ar), 168.1 (C=O), 170.1 (C=O). Elemental analysis: liquid product.

General procedure for the synthesis of 4a–c. The respective acid (**2a–c**, 4.56 mmol), 15 mL of THF and imidazole (0.31 g, 4.56 mmol) were added to a 50 mL round bottom flask under magnetic stirring and warmed to 60 °C for 4 h. After cooling back to rt, the precipitate was filtered off and the solvent was evaporated. A white solid was obtained, filtered in a Buchner funnel with a sintered disc filter, washed with cold water, and then dried over SiO_2.

2-(1,3-Dioxoisoindolin-2-yl)propanamide (4a). Chemical formula: $C_{11}H_{10}N_2O_3$. MW: 218.21 g. Yield: 58.31%. R_f: 0.2 (Hex/AcEt: 3/7). M.p.: 187–189 °C. IR (KBr, cm^{-1}): 3439 (NH); 1683 (C=O); 1467 (C–N–C); 1387 (C–N–C). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 1.12 (d, 3H, CH_3), 2.49 (m, 1H, CH), 6.91 (br, 2H, NH_2), 7.85–7.91 (m, 2H, Ar), 8.05 (m, 2H, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 15.4 (CH_3), 20.9 (CH), 125.5 (Ar), 127.6

(Ar), 132.9 (Ar), 152.2 (C=O), 170.1 (C=O). Elemental analysis – EA_{theor}: N 12.84%; C 60.55%; H 4.62%; EA_{exp}: N 12.74%; C 60.79%; H 4.71%.

2-(1,3-Dioxoisoindolin-2-yl)-3-phenylpropanamide (4b). Chemical formula: $C_{17}H_{14}N_2O_3$. MW: 294.30 g. Yield: 59.05%. R_f: 0.5 (Hex/AcEt: 3/7). M.p.: 233–234 °C. IR (KBr, cm^{-1}): 3387 (NH); 1687 (C=O); 1468 (C–N–C); 1384 (C–N–C). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 3.50 (m, 2H, CH_2), 4.91 (m, 1H, CH), 7.08–7.14 (m, 5H, Ar), 7.12 (br, 2H, NH_2), 7.78–7.91 (m, 4H, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 37.3 (CH_2), 54.7 (CH), 123.6 (Ar), 126.8, 128.6, 129.1 (Ar), 134.9 (Ar), 168.1 (C=O), 170.1 (C=O). Elemental analysis – EA_{theor}: N 9.52%; C 69.38%; H 4.79%; EA_{exp}: N 9.36%; C 69.49%; H 4.87%.

2-(1,3-Dioxoisoindolin-2-yl)-3-methylpentanamide (4c). Chemical formula: $C_{14}H_{16}N_2O_3$. MW: 260.29 g. Yield: 56.39%. R_f: 0.6 (Hex/AcEt: 3/7). M.p.: 218–219 °C. IR (KBr, cm^{-1}): 3402 (NH); 1650 (C=O); 1458 (C–N–C); 1389 (C–N–C). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 0.77 (m, 3H, CH_3), 0.91 (m, 2H, CH_2), 0.98 (m, 2H, CH_3), 2.51 (m, 1H, CH), 4.34 (m, 1H, CH), 7.12 and 7.49 (2 br, 2H, NH_2), 7.87–7.86 (m, 4H, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 11.2 (CH_3), 17.1 (CH_3), 25.8 (CH_2), 33.3 (CH), 58.5 (CH), 123.6 (Ar), 135.0 (Ar), 168.1 (C=O), 170.1 (C=O). Elemental analysis – EA_{theor}: N 10.76%; C 64.60%; H 6.20%; EA_{exp}: N 10.57%; C: 64.74%; H: 6.36%.

General procedure for the synthesis of 5a–c. The respective ester (**3a–c**, 4.04 mmol), 15 mL of ethanol and hydrazine hydrate (4 drops) were added to a 50 mL round bottom flask under magnetic stirring and warmed to 60 °C for 4 h. After cooling back to rt, the precipitate was filtered off and the solvent was evaporated. A white solid was obtained, filtered in a Buchner funnel with a sintered disc filter, washed with cold water, and then dried over SiO_2.

2-(1,3-Dioxoisoindolin-2-yl)propanehydrazide (5a). Chemical formula: $C_{11}H_{11}N_3O_3$. MW: 233.22 g. Yield: 61.45%. IR (KBr, cm^{-1}): 3018 (NH); 1661 (C=O); 1458 (C–N–C); 1377 (C–N–C). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 1.12 (d, 3H, CH_3), 2.49 (m, 1H, CH), 4.11 (br, 2H, NH_2), 7.85–8.05 (m, 4H, Ar). The signal in 4.11 disappears after the addition of 3 drops of D_2O. ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 15.4 (CH_3), 20.8 (CH), 125.5 (Ar), 127.6 (Ar), 132.9 (Ar), 152.2 (C=O), 170.1 (C=O). EA_{theor}: N 18.02%; C 56.65%; H 4.75%. Elemental analysis – EA_{exp}: N 17.99%; C 56.68%; H 4.68%.

2-(1,3-Dioxoisoindolin-2-yl)-3-phenylpropanehydrazide (5b). Chemical formula: $C_{17}H_{15}N_3O_3$. MW: 309.32 g. Yield: 69.96%. IR (KBr, cm^{-1}): 3026 (NH); 1662 (C=O); 1493 (C–N–C); 1378 (C–N–C). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 3.50 (m, 2H, CH_2), 4.91 (m, 1H, CH), 7.08–7.14 (m, 5H, Ar), 7.12 (br, 2H, NH), 7.46 (NH_2), 7.78–7.91 (m, 4H, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 37.3 (CH_2), 54.7 (CH), 126.8 (Ar), 128.6 (Ar), 129.1 (Ar), 123.6 (Ar), 134.9 (Ar), 168.1 (C=O), 170.1 (C=O). EA_{theor}: N 13.58%; C 66.01%; H 4.89%; elemental analysis – EA_{exp}: N 13.43%; C 66.17%; H 5.06%.

2-(1,3-Dioxoisoindolin-2-yl)-3-methylpentanehydrazide (5c). Chemical formula: $C_{14}H_{17}N_3O_3$. MW: 275.30 g. Yield: 75.17%. IR (KBr, cm^{-1}): 3018 (NH); 1662 (C=O); 1494 (C–N–C); 1378 (C–N–C). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 0.77 (m, 3H, CH_3), 0.91 (m, 2H, CH_2), 0.98 (m, 2H, CH_3), 2.51 (m, 1H, CH), 4.34 (m, 1H, CH), 7.12–7.49 (2 br, 2H, NH_2), 7.87–7.86 (m, 4H, Ar), 7.99 (s, 1H, NH). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 11.2 (CH_3), 17.1 (CH_3), 25.8 (CH_2), 33.3 (CH), 58.5 (CH), 123.6 (Ar), 135.0 (Ar), 168.1 (C=O), 170.1 (C=O). Elemental analysis – EA_{theor}: N 15.26%; C 61.08%; H 6.22%; EA_{exp}: N 15.13%; C 61.21%; H 6.31%.

General procedure for the synthesis of 6a–9c. The respective hydrazide (**5a–c**, 4.22 mmol), 15 mL of ethanol and aromatic aldehyde *p*-substituted (4.22 mmol) were added to a 50 mL round bottom flask, under magnetic stirring and reflux for 4 h. After cooling back to rt, the precipitate was filtered off and the solvent was evaporated. A white solid was obtained, filtered in a Buchner funnel with a sintered disc filter, washed with cold water, and then dried over SiO_2.

(*Z*)-*N*′-Benzylidene-2-(1,3-dioxoisoindolin-2-yl)propanehydrazide (6a). Chemical formula: $C_{18}H_{15}N_3O_3$. MW: 321.33 g. Yield: 44.77%. R_f: 0.6 (Hex/AcEt: 9/1). M.p.: 86–88 °C. IR (KBr, cm^{-1}): 3018 (NH_2), 2897 (C–H), 1661 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6), δ: 1.11 (d, 3H, CH_3), 2.49 (m, 1H, CH), 4.15 (s, 3H, CH_3), 7.51–7.72 (m, 4H, Ar), 7.85–8.05 (m, 4H, Ar), 8.09 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 15.4 (CH_3), 20.8 (CH), 123.6 (Ar), 125.5 (Ar), 128.6 (Ar), 129.1 (Ar), 132.9 (Ar), 134.9 (Ar), 152.2 (C=O), 159.5 (CH=N), 170.1 (C=O). Elemental analysis – EA_{theor}: N 13.08%; C 67.28%; H 4.71%; EA_{exp}: N 12.98%; C 67.17%; H 4.76%.

(*Z*)-*N*′-Benzylidene-2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanehydrazide (6b). Chemical formula: $C_{24}H_{19}N_3O_3$. MW: 397.43 g. Yield: 42.22%. R_f: 0.6 (Hex/AcEt: 9/1). M.p.: 90–92 °C. IR (KBr, cm^{-1}): 1664 (C=N); 1624 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 0.77 (m, 3H, CH_3), 0.91 (m, 2H, CH_2), 0.98 (m, 2H, CH_3), 2.51 (m, 1H, CH), 4.34 (m, 1H, CH), 7.10–7.52 (m, 8H, Ar), 7.87–7.86 (m, 4H, Ar), 7.99 (s, 1H, NH), 8.01 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 25.8 (CH_2), 33.3 (CH), 58.5 (CH), 123.6 (Ar), 125.0 (Ar), 126.5 (Ar), 131.0 (Ar), 135.0 (Ar), 168.1 (C=O), 170.1 (C=O). Elemental analysis – EA_{theor}: N 10.57%; C 72.53%; H 4.82%; EA_{exp}: N 10.52%; C 72.38%; H 4.77%.

(*Z*)-*N*′-Benzylidene-2-(1,3-dioxoisoindolin-2-yl)-3-methylpentanehydrazide (6c). Chemical formula: $C_{21}H_{21}N_3O_3$. MW: 363.41 g. Yield: 42.84%. R_f: 0.6 (Hex/AcEt: 9/1). M.p.: 93–95 °C. IR (KBr, cm^{-1}): 1664 (C=N); 1623 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 0.77 (m, 3H, CH_3), 0.91 (m, 2H, CH_2), 0.98 (m, 2H, CH_3), 2.51 (m, 1H, CH), 4.34 (m, 1H, CH), 7.19–7.32 (m, 5H, Ar), 7.87–7.86 (m, 4H, Ar), 7.99 (s, 1H, NH), 8.01 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 11.2 (CH_3), 17.1 (CH_3), 25.8 (CH_2), 33.3 (CH), 58.5 (CH), 123.6 (Ar), 135.0 (Ar), 168.1 (C=O), 170.1 (C=O). Elemental analysis – EA_{theor}: N 11.56%; C 69.41%; H 5.82%; EA_{exp}: N 11.49%; C 69.58%; H 5.73%.

(*Z*)-2-(1,3-Dioxoisoindolin-2-yl)-*N*′-(4-methoxybenzylidenepropanehydrazide) (7a). Chemical formula: $C_{19}H_{17}N_3O_4$. MW: 351.36 g. Yield: 47.95%. R_f: 0 : 3 (Hex/AcEt: 9/1). M.p.: 169–170 °C. IR (KBr, cm^{-1}): 3018 (NH_2), 2897 (C–H), 1661 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 1.12 (d, 3H, CH_3), 2.49 (m, 1H, CH), 4.11 (s, 3H, CH_3), 7.21–7.31 (m, 4H, Ar), 7.85–8.05 (m, 4H, Ar), 8.09 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 15.4 (CH_3), 20.8 (CH), 123.6 (Ar), 125.5 (Ar), 128.6

(Ar), 129.1 (Ar), 132.9 (Ar), 134.9 (Ar), 152.2 (C=O), 159.5 (CH=N), 170.1 (C=O). Elemental analysis – EA_{theor}: N 11.96%; C 64.95%; H 4.88%; EA_{exp}: N 11.83%; C 64.90%; H 5.04%.

(*Z*)-2-(1,3-Dioxoisoindolin-2-yl)-*N*′-(4-methoxybenzylidene)-3-phenylpropanehydrazide (7b). Chemical formula: $C_{25}H_{21}N_3O_4$. MW: 427.45 g. Yield: 49.12%. R_f: 0.3 (Hex/AcEt: 9/1). M.p.: 169–170 °C. IR (KBr, cm^{-1}): 1620 (C=N); 1602 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 2.61 (m, 1H, CH), 4.11 (s, 3H, OCH_3), 4.34 (m, 1H, CH), 7.12 and 7.49 (2 br, 2H, NH_2), 7.10–7.52 (m, 8H, Ar), 7.86–7.87 (m, 4H, Ar), 7.99 (s, 1H, NH), 8.01 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 25.8 (CH_2), 33.3 (CH), 45.9 (CH_3O), 58.5 (CH), 123.6 (Ar), 125.0 (Ar), 126.5 (Ar), 131.0 (Ar), 135.0 (Ar), 168.1 (C=O), 170.1 (C=O). **7b** – elemental analysis – EA_{theor}: N 9.83%; C 70.25%; H 4.95%; EA_{exp}: N 10.02%; C 70.13%; H 4.98%.

(*Z*)-2-(1,3-Dioxoisoindolin-2-yl)-*N*′-(4-methoxybenzylidene)-3-methylpentanehydrazide (7c). Chemical formula: $C_{22}H_{23}N_3O_4$. MW: 393.44 g. Yield: 48.75%. R_f: 0.2 (Hex/AcEt: 9/1). M.p.: 169–171 °C. IR (KBr, cm^{-1}): 1661 (C=N); 1623 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 0.77 (m, 3H, CH_3), 0.91 (m, 2H, CH_2), 0.98 (m, 2H, CH_3), 2.51 (m, 1H, CH), 4.11 (s, 3H, OCH_3), 4.34 (m, 1H, CH), 7.12 and 7.49 (2 br, 2H, NH_2), 7.19–7.32 (m, 4H, Ar), 7.87–7.86 (m, 4H, Ar), 7.99 (s, 1H, NH), 8.01 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 11.2 (CH_3), 17.1 (CH_3), 25.8 (CH_2), 33.3 (CH), 45.9 (CH_3O), 58.5 (CH), 123.6 (Ar), 135.0 (Ar), 168.1 (C=O), 170.1 (C=O). Elemental analysis – EA_{theor}: N 10.68%; C 67.16%; H 5.89%. EA_{exp}: N 10.85%; C 67.30%; H 5.69%.

(*Z*)-*N*′-(4-Chlorobenzylidene)-2-(1,3-dioxoisoindolin-2-yl)propanehydrazide (8a). Chemical formula: $C_{18}H_{14}ClN_3O_3$. MW: 355.78 g. Yield: 60.04%. R_f: 0.7 (Hex/AcEt: 9/1). M.p.: 209–210 °C. IR (KBr, cm^{-1}): 1660 (C=N); 1624 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 1.11 (d, 3H, CH_3), 2.49 (m, 1H, CH), 4.11 (s, 3H, CH_3), 7.51–7.72 (m, 4H, Ar), 7.85–8.05 (m, 4H, Ar), 8.09 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 15.4 (CH_3), 20.8 (CH), 123.6 (Ar), 125.5 (Ar), 128.6, 129.1 (Ar), 132.9 (Ar), 134.9 (Ar), 147.9 (Ar, C–Cl), 152.2 (C=O), 159.5 (CH=N), 170.1 (C=O). Elemental analysis – EA_{theor}: N 11.81%; C 60.77%; H 3.97%; EA_{exp}: N 11.72%; C 60.68%; H 3.89%.

(*Z*)-*N*′-(4-Chlorobenzylidene)-2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanehydrazide (8b). Chemical formula: $C_{24}H_{18}ClN_3O_3$. MW: 431.87 g. Yield: 50.25%. R_f: 0.7 (Hex/AcEt: 9/1). M.p.: 210–211 °C. IR (KBr, cm^{-1}): 1658 (C=N); 1624 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 2.51 (m, 2H, CH_2), 4.34 (m, 1H, CH), 7.10–7.52 (m, 8H, Ar), 7.87–7.86 (m, 4H, Ar), 7.99 (s, 1H, NH), 8.01 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 25.8 (CH_2), 33.3 (CH), 45.9 (CH_3O), 58.5 (CH), 123.6 (Ar), 125.0 (Ar), 126.5 (Ar), 131.0 (Ar), 135.0 (Ar), 168.1 (C=O), 170.1 (C=O). Elemental analysis – AE_{teor}: N 9.73%; C 66.75%; H 4.20%; AE_{exp}: N 9.92%; C 66.83%; H 4.47%.

(*Z*)-*N*′-(4-Chlorobenzylidene)-2-(1,3-dioxoisoindolin-2-yl)-3-methylpentanehydrazide (8c). Chemical formula: $C_{21}H_{20}ClN_3O_3$. MW: 397.86 g. Yield: 55.54%. R_f: 0.7 (Hex/AcEt: 9/1). M.p.: 210–211 °C. IR (KBr, cm^{-1}): 1660 (C=N); 1624 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 0.77 (m, 3H, CH_3), 0.91 (m, 2H, CH_2), 0.98 (m, 2H, CH_3), 2.51 (m, 1H, CH), 4.11 (s, 3H, OCH_3), 4.34 (m, 1H, CH), 7.19–7.32 (m, 4H, Ar), 7.87–7.86 (m, 4H, Ar), 7.99 (s, 1H, NH), 8.01 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 11.2 (CH_3), 17.1 (CH_3), 25.8 (CH_2), 33.3 (CH), 45.9 (CH_3O), 58.5 (CH), 135.0 (Ar), 123.6 (Ar), 168.1 (C=O), 170.1 (C=O). Elemental analysis – EA_{theor}: N 10.56%; C 63.40%; H 5.07%; EA_{exp}: N 10.72%; C 63.51%; H 5.11%.

(*Z*)-*N*′-(3,4-Dichlorobenzylidene)-2-(1,3-dioxoisoindolin-2-yl)-propanehydrazide (9a). Chemical formula: $C_{18}H_{13}Cl_2N_3O_3$. MW: 390.22 g. Yield: 45.76%. R_f: 0.7 (Hex/AcEt: 9/1). M.p.: 170–172 °C. IR (KBr, cm^{-1}): 3442 (NH_2), 2997 (C–H), 1710 (C=O), 1627 (C=N) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 1.11 (d, 3H, CH_3), 2.49 (m, 1H, CH), 4.11 (s, 3H, CH_3), 7.51–7.63 (4H, Ar), 7.85–8.05 (m, 4H, Ar), 8.19 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 15.4 (CH_3), 20.8 (CH), 125.5 (Ar), 128.6 (Ar), 129.1 (Ar), 132.9 (Ar), 134.9 (Ar), 147.9 (Ar, C–Cl), 152.2 (C=O), 159.5 (CH=N), 170.1 (C=O). Elemental analysis – EA_{theor}: N 10.77%; C 55.40%; H 3.36%; EA_{exp}: N 10.67%; C 55.46%; H 3.25%.

(*Z*)-*N*′-(3,4-Dichlorobenzylidene)-2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanehydrazide (9b). Chemical formula: $C_{24}H_{17}Cl_2N_3O_3$. MW: 466.32 g. Yield: 46.82%. R_f: 0.7 (Hex/AcEt: 9/1). M.p.: 171–173 °C. IR (KBr, cm^{-1}): 3442 (NH_2), 2997 (C–H), 1700 (C=O), 1687 (C=N) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 2.49 (m, 1H, CH), 4.11 (s, 3H, CH_3), 7.10–7.52 (m, 8H, Ar), 7.85–8.05 (m, 4H, Ar), 8.19 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 25.8 (CH_2), 33.3 (CH), 58.5 (CH), 125.0 (Ar), 126.5 (Ar), 131.0 (Ar), 135.0 (Ar), 143.6 (Ar), 168.1 (C=O), 170.1 (C=O). Elemental analysis – EA_{theor}: N 9.01%; C 61.82%; H 3.67%; EA_{exp}: N 9.24%; C 61.74%; H 3.46%.

(*Z*)-*N*′-(3,4-Dichlorobenzylidene)-2-(1,3-dioxoisoindolin-2-yl)-propanehydrazide (9c). Chemical formula: $C_{21}H_{19}Cl_2N_3O_3$. MW: 432.30 g. Yield: 50.02%. R_f: 0.7 (Hex/AcEt: 9/1). M.p.: 178–180 °C. IR (KBr, cm^{-1}): 3442 (NH_2), 2997 (C–H), 1700 (C=O), 1687 (C=N) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 1.11 (d, 3H, CH_3), 2.49 (m, 1H, CH), 4.11 (s, 3H, CH_3), 7.51–7.63 (m, 2H, Ar), 7.85–8.05 (m, 4H, Ar), 8.19 (s, 1H, CH=N). ^{13}C NMR (75.5 MHz, DMSO-d_6), δ (ppm): 15.4 (CH_3), 20.8 (CH), 125.5 (Ar), 128.6 (Ar), 129.1 (Ar), 132.9 (Ar), 134.9 (Ar), 147.9 (Ar, C–Cl), 152.2 (C=O), 159.5 (CH=N), 170.1 (C=O). Elemental analysis – EA_{theor}: N 9.72%; C 58.35%; H 4.43%; EA_{exp}: N 9.60%; C 58.27%; H 4.67%.

Biological *in vitro* evaluation

TNF-α and IL-1 levels. Mice peritoneal macrophages were placed into 96 well plates at a cell density of 2×10^6 cells per mL and incubated for 2 h at 37 °C and 5% CO_2. Cells at 2×10^6 concentration were suspended in RPMI 1640 with 5% FBS, 100 UI mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 50 mM 2-mercaptoethanol. 100 μL of suspension and 100 μL of samples were incubated with LPS 2 μg mL^{-1} (positive control) or with the test compounds in different concentrations. After 24 h, the supernatants were removed and kept at −80 °C until the evaluation of cytokine levels (TNF and IL-1). The doses of cytokines in the exudates were assayed by sandwich ELISA, using monoclonal antibodies specific to the detection of cytokines.

TNF-α, cell death and apoptosis inhibition. The effect of each compound on inhibiting TNF expression and decreasing cell viability was determined using the cell line FRT-Jurkat TNF.

The analysis was carried out using a flow cytometer FACSCalibur 4-Colour (Becton, Dickinson and Company, New Jersey, USA). The data analysis was obtained with FlowJo software (Tree-Star, Ashland, OR, USA). The study of cell viability with DMSO and with the compounds, for 24 h, was carried out by flow cytometry. The compounds were suspended and diluted to 100 μM and 10 μM in dimethyl sulfoxide. TNF inhibition was determined as a decrease in GFP fluorescence, which was detected at a wavelength of 515 nm, in channel FL3. Viable and non-viable cells, present following either the solvent alone or compound treatment, were counted by gating on each cell population using a forward scatter (FSC) *versus* side scatter (SSC) plot. Viability was also assessed following propidium iodide staining and detection of fluorescence at 570 nm in the FL2 channel. For detection of apoptosis following treatment with each compound, parental Jurkat cells were treated with the substrate, labelled with fluorescein isothiocyanate (FITC), against cleaved caspase 3 by the Nucview-488 caspase 3 assay (Biotium Inc., Hayward, USA). The resulting fluorescence was quantitated in channel FL3.

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