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New thalidomide analogues derived through Sonogashira or Suzuki reactions and their TNF expression inhibition profiles

Scott G. Stewart *, Carlos J. Braun, Sze-Ling Ng, Marta E. Polomska, Mahdad Karimi, Lawrence J. Abraham

The School of Biomedical, Biomolecular and Chemical Sciences, The University of Western Australia Crawley, WA 6009, Australia

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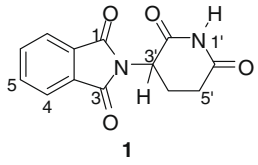
ABSTRACT

A library of new thalidomide C4/5 analogues containing either a phenyl or alkyne tether were synthesized using Sonogashira or Suzuki cross coupling reactions from their aryl halogenated precursors. All thalidomide analogues were tested for their ability to inhibit the expression of the proinflammatory cytokine Tumor Necrosis Factor (TNF). More explicitly the use of a novel reporter system utilizing the promoter region of the TNF gene in a human T-cell line provided a rapid and effective measure of NFκB transcriptional activity. Several compounds either containing either an aryl-isobutyl or aryl-isopropoxy group were the most effective in inhibiting TNF expression, and were several times more active than thalidomide itself. Five of the more active derivatives indicated an apoptotic response while one of these compounds, containing an aldehyde tether, showed possible influence of cell cycling effects.

1. Introduction

The racemic drug thalidomide, [(*R,S*)-2-(2,6-dioxo-3-piperidinyl)-1*H*-isoindole-1,3(2*H*)-dione (**1**) (Fig. 1)] tragically was administered to pregnant woman in the 1950's as a treatment for insomnia and as an antiemetic agent. This market release, prior to a full understanding of the pharmacological profile of both enantiomers, was later found to be premature as the C3' *R*-stereoisomer, (*R*)-**1** was responsible for a sedative response and the *S*-isomer (*S*)-**1** had teratogenic properties.[1–4] As a result, in 1962 this popular commercial drug was withdrawn, although not before 10,000 infants with various limb malformations were born.

Currently, with the knowledge of the previous side effects, there has been a resurgence in the use of thalidomide as a pharmaceutical agent. In 1998, Celgene received FDA approval to use thalidomide (**1**), (Thalomid™), for the treatment of erythema nodosum leprosum (ENL) based on results from earlier serendipitous treatment of leprosy patients in 1962.[5,6] The efficacy of thalidomide (**1**) in the treatment of various proinflammatory and autoimmune conditions has lead to greater interest in therapeutic applications. At present, this reborn drug is being evaluated in the treatment of many disease states such as rheumatoid arthritis,[7,8] Behçet's disease,[8] Crohn's disease,[9] HIV/AIDS related illnesses,[9] mesothleoma,[10] tuberculosis[11] and sarcoidosis.[12] However, one of the most promising avenues of investigation is the use of thalidomide



1

Figure 1. Thalidomide **1**.

(**1**) to treat the as-yet-incurable form of bone marrow cancer, multiple myeloma.[3]

At a cellular level thalidomide (**1**) influences several processes including peptidase inhibition, glucosidase inhibition, androgen receptor antagonism and (cyclooxygenase) COX inhibition.[13] This assortment of cellular processes indicates that thalidomide (**1**) may have many distinct downstream cellular targets. However, the precise molecular mechanism of action of thalidomide (**1**) has been difficult to determine primarily due to its differing in vitro versus in vivo activities. These discrepancies are thought to result from the formation of up to 100 in vivo metabolic breakdown products.[14] One of the most studied biological activities influenced by thalidomide (**1**), is the inhibition of the pro-inflammatory cytokine, tumour necrosis factor (TNF) expression.[3,15] TNF is a central regulator of the inflammatory cascade controlling many effector pathways, the main ones being anti-angiogenic, anti-inflammatory and immuno-modulatory. At a molecular level the mode of action of thalidomide (**1**) in suppressing TNF expression is thought to involve the inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling

* Corresponding author. Tel.: +61 8 6488 3180; fax: +61 8 6488 1005.
E-mail address: sgs@cyllene.uwa.edu.au (S.G. Stewart).

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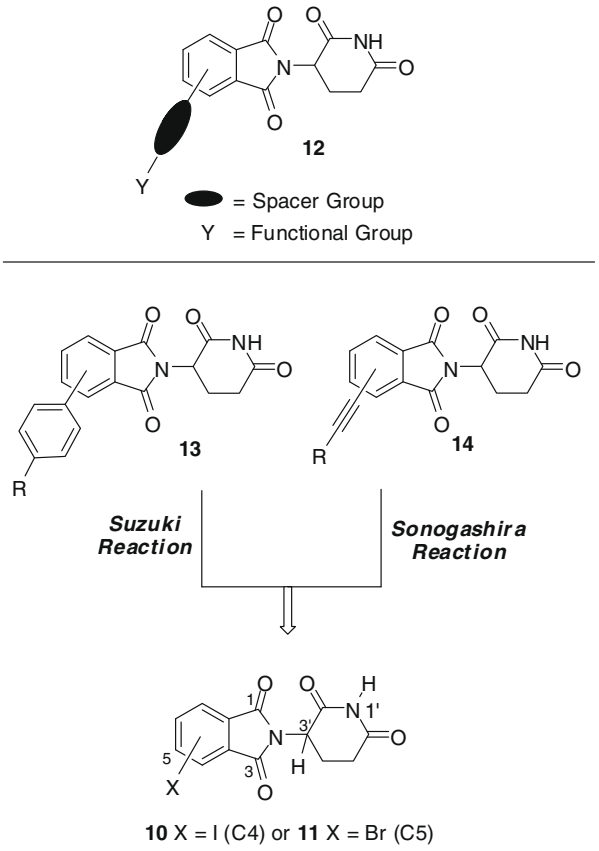
pathway[16] and more specifically by inhibiting the activity of the IκB kinase, IKKα.[17] As part of our ongoing studies into the relationship between thalidomide (**1**) and TNF expression, we decided to screen new analogues for enhanced ability to specifically inhibit the NFκB pathway.[18]

With the growing attention this compound has received, it is expected that the number of thalidomide analogues will grow in the coming years. Currently in the literature several classes of thalidomide derivatives have been described. Thalidomide analogues which greatly improve water solubility, for example CC3052[19] **2** and the valine ammonium salt derivative **3** (Fig. 2) have an improved ability to inhibit TNF.[20] Structural modifications to the left hand phthalimide ring system have also seen great improvements in activity, for example the two marketed drugs lenalidomide (Revlimid™) **4** and Actimid™, **5**,[6,21] containing amino groups at C4, have shown excellent activity in several assays including TNF expression inhibition. As such, these two compounds, have been marketed as immunomodulatory drugs (iMiD's).[3] The C5-hydroxy-thalidomide **6**, listed as a potential metabolite, caused a loss of TNF inhibiting activity when compared to the inhibition level of thalidomide (**1**) itself.[22] In other biological evaluations the C5-carboxylic acid analogue **7** displayed improvements in basic fibroblast growth factor (bFGF) inhibition along with an improved water solubility.

In view of the fact that these highlighted simple analogues functionalised in the C4 and C5 position have been shown to improve the inhibitory activity of TNF expression, we have focussed attention on preparing more complex analogues which could enhance potential protein interactions and in turn inhibitory activity.

These requirements led us to investigate the production of new thalidomide olefinic analogues via a Heck cross coupling reaction.[18] The bioactivity of each analogue was determined using a TNF inhibition assay.[18,23] From this initial study two key analogues, the furan derivative **8** and the vinyl butoxy derivative **9** (Fig. 2) showed a marked improvement in repression of TNF expression. Unfortunately, in the analysis of these compounds an accurate structure–activity relationship (SAR) was difficult to achieve due to the complexity of the attached groups at C4 and C5. Synthetically, the Heck cross coupling procedure allowed for ease of analogue production in good to high yields (ca. 55–90%) depending on the electronic nature of the coupling partner. Considering these marked improvements in TNF expression inhibition by these C4 and C5 substituted derivatives synthesized within our group and the previous illustrated cases, we sought to investigate this region of thalidomide further. We also looked to exploit the synthetic utility of our halothalidomides **10** and **11** (Scheme 1) with other cross coupling reactions at these two carbon centres. Our focus turned to



Scheme 1. Introduction of phenyl and alkyne motifs to the thalidomide backbone via Sonogashira and Suzuki cross coupling reactions.

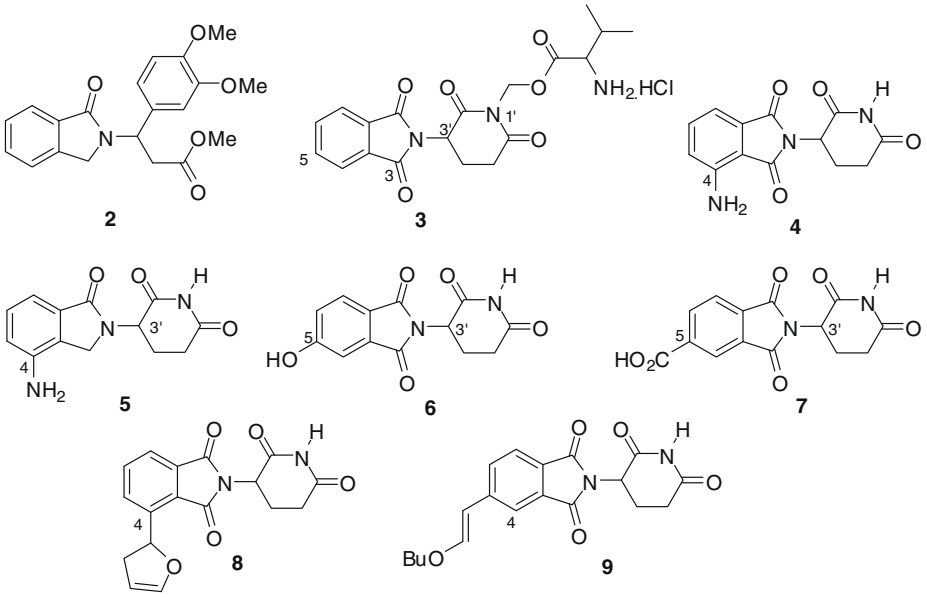


Figure 2. Thalidomide analogues in the literature and produced by the Stewart group.

two of the most reliable and chemoselective cross coupling reactions, the Sonogashira and Suzuki transformations.[24,25] Such cross coupling methodologies are frequently used in large scales in chemical industry and in new medicinal chemistry research programs.[26,27]

2. Results and discussion

2.1. Synthetic approaches

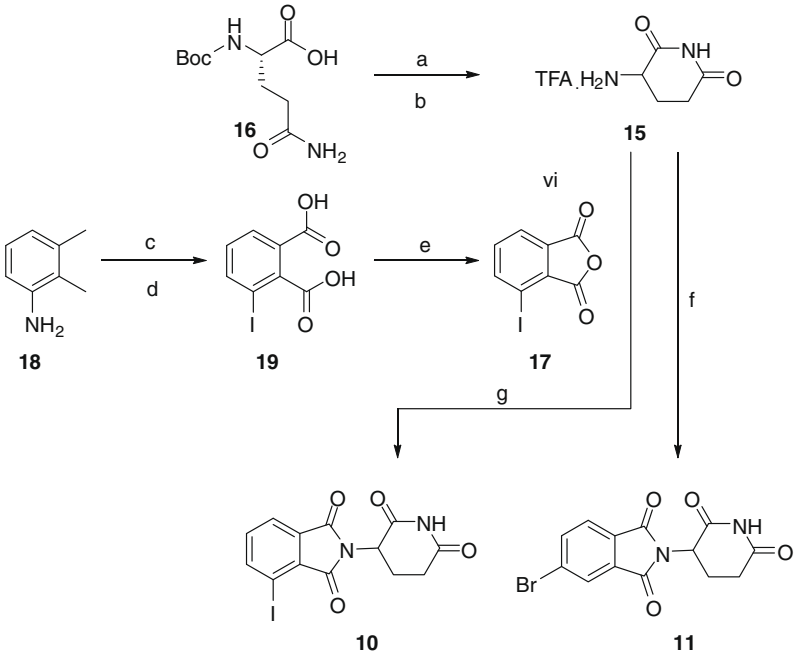
In this investigation we set out to identify reliable structural improvements to the thalidomide skeleton and in the process construct a SAR for the inhibition of TNF expression. In further probing the region of space around C4 and C5 and taking our initial active analogues into account, we initially looked at a system which contained a hydrocarbon spacer unit and a heteroatom containing functional group (i.e., generic derivative **12**). Initially we conceived that a *para*-aryl substitution pattern meets this prerequisite. Synthetically, the Suzuki cross coupling has been proven to efficiently introduce new aryl groups through transmetallation of boronic acids and esters.[25] In this study we also envisaged an alkyne spacer to also explore this region of space (Scheme 1). This fragment could also be introduced through a second cross coupling reaction, the Sonogashira reaction (a modified Castro–Stevens reaction).[24]

The piperidine-2,6-dione ring fragment of the thalidomide core was prepared by first synthesising the trifluoroacetic acid salt of aminoglutaramide **15** (Scheme 2). Thus, following the procedure of Muller or Brown, the commercially available *tert*-butoxycarbonyl-L-glutamide **16** was cyclised through a condensation reaction and subsequently deprotected to afford compound **15** in good yields ca. 72%.[28] The first halogented left hand ring fragment of thalidomide, iodinated phthalic anhydride **17**, was prepared from 2,3-dimethylaniline (**18**). Accordingly, an initial Sandmeyer type iodination to the corresponding dimethyl iodobenzene (49%)[29] followed by a potassium permanganate mediated dibenzylic oxidation furnished the diacid **19** (41%).[30] Treatment of this latter compound with acetic anhydride gave the desired cyclised iodoanhydride **17** in 68% yield. The second left hand ring fragment, 5-bromophthalic anhydride was purchased from Wako chemicals.[31] Condensation of either iodoanhydride **17** or bromophthalic anhydride with the salt of aminoglutaramide **15** produced the required halogenated cross coupling partners **10** and **11**.

3. Cross-coupling reactions

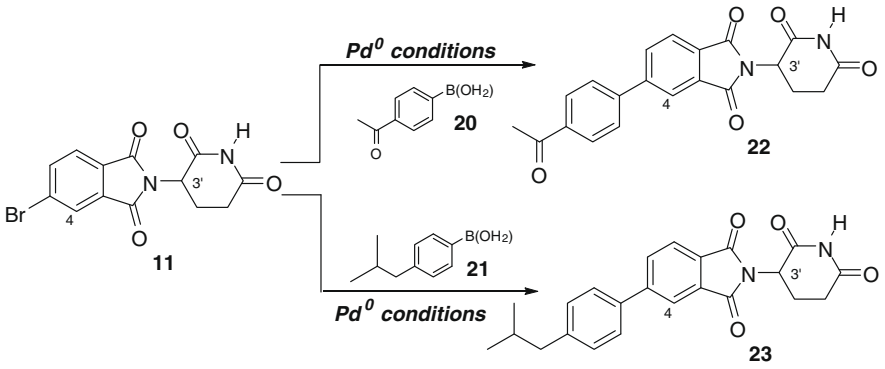
To optimise the conditions of Suzuki reaction for the brominated thalidomide **11**, seven sets of catalytic conditions using the 4-acetylphenyl boronic acid **20** or the 4-isobutyl boronic acid **21** were attempted (Table 1). Reasonable results were achieved using $Pd(dppf)Cl_2$ or the more traditional Suzuki cross coupling coupling catalysts, that is, $Pd(PPh_3)_4$. However, under a $Pd_2(dba)_3$, $[(tBu)_3PH]BF_4$ and two base catalytic system (NaOH and dicyclohexyl-*N*-methyl amine) the cross coupling was found to be the most efficient in producing compounds **22** and **23**.[32] The two catalytic systems, (Table 1, entries 5 and 8) were clearly the most suited to the thalidomide scaffold and provided a range of thalidomide analogues in mostly in good to excellent yields. The only exception in these cases was the coupling of the *p*-phenol boronic acid which proceeded in only 51%, possibly due to the solubility and reactivity of the phenoxide ion. In these initial sets of conditions no homo coupling product was observed and thus exploration of the boronic ester equivalent reagents was not required.

Following the clean conversion of the Suzuki reaction we focused our attention to installing an alkyne through the Sonogashira reaction. This $Pd(PPh_3)_2Cl_2$ mediated cross coupling proceeded in good yields (48–78%) for most reactions where an electron donating group in the α-position of the alkyne was used (Scheme 3). These isolated yields agree with the literature findings where high yields are normally observed with terminal alkynes of similar electronics and not with substrates containing an electron withdrawing group. Unfortunately, we also required a substrate with an electron withdrawing substituent in this position for SAR investi-



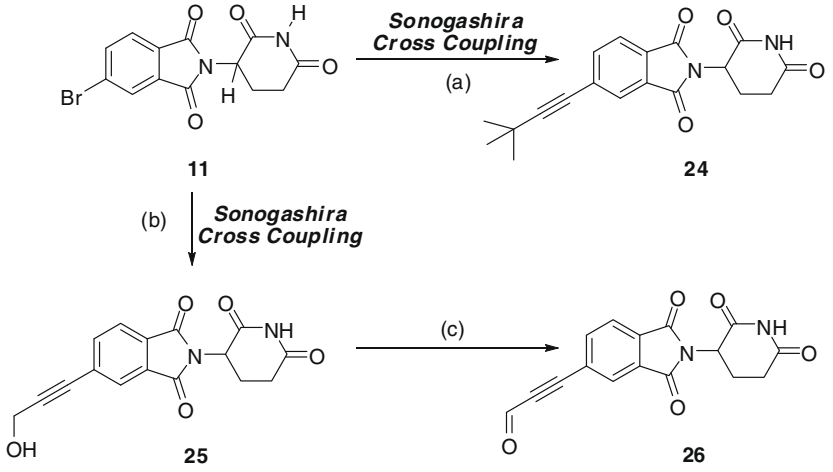
Scheme 2. Reagents and conditions: (a) CDI, DMAP, THF, reflux, 24 h, 76%; (b) 1:1 TFA/CH_2Cl_2, 22 °C, 1 h, 95%; (c) H_2O, HCl (concd), $NaNO_2$, −15 °C, KI (aq), then 55 °C, 5 min then 16 h, rt, 66%; (d) H_2O, $KMnO_4$, 80 °C, 4 days, 41%; (e) Ac_2O, reflux, 3 h, 68%; (f) NEt_3, **15**, 5-bromophthalic anhydride, THF, reflux, 48 h, 63%; (g) NEt_3, **15**, **17**, THF, reflux, 24 h, 56%. CDI: 1,1-carbonyldiimidazole; DMAP: 4-dimethylaminopyridine; TFA: trifluoroacetic acid.

Table 1
Optimisation of Suzuki reaction between 5-bromothalidomide **11** and boronic acids **20** and **21**



Entry	Product and boronic acid	Catalyst (mol %)	Phosphine (mol %)	Base(s)	Yield (%)
1	**22** and **20**	$Pd(PPh_3)_4$, 10 mol %		CsF_2	17
2	**22** and **20**	$Pd_2(dba)_3$,[c] 5 mol %	(t-Bu)$_3$PHBF$_4$, 10 mol %	CsF_2	33
3	**22** and **20**	$Pd_2(dba)_3$,[c] 5 mol %	(t-Bu)$_3$PHBF$_4$, 10 mol %	NaOH, Cy_2NMe	70
4	**22** and **20**	$Pd(dppf)Cl_2$,[a] 10 mol %		NaOH, Cy_2NMe[b]	54
5	**22** and **20**	$Pd_2(dba)_3$,[c] 5 mol%	(t-Bu)$_3$PHBF$_4$, 10 mol %		72
6	**23** and **21**	$Pd(OAc)_2$, 10 mol %	dppf, 20 mol %	NaOH	19
7	**23** and **21**	$Pd_2(dba)_3$, 10 mol %	dppf, 20 mol %	NaOH	9
8	**23** and **21**	$Pd(dppf)Cl_2$,[a] 10 mol %		NaOH, Cy_2NMe[b]	90

[a] dppf = 1,1′-Bis(diphenylphosphino)ferrocene.
[b] Cy_2NMe = Dicyclohexyl-*N*-methyl amine.
[c] $Pd_2(dba)_3$ = Tris(dibenzylideneacetone)dipalladium(0).



Scheme 3. Reagents and Conditions: (a) $Pd(PPh_3)_2Cl_2$ (10 mol %), CuI (10 mol %), *i*PrNEt$_2$, THF, reflux, 18 h, 76%; (b) $Pd(PPh_3)_2Cl_2$ (10 mol %), CuI (10 mol %), *i*PrNEt$_2$, THF, reflux, 4 h, 48%; (c) Dess–Martin periodinane, CH_2Cl_2, rt, 1 h, 76%.

gations. Thus, the respective coupled alcohol **25** was treated with Dess–Martin periodinane to yield the required aldehyde **26** in 76% yield.

Once again only small differences between the cross couplings of the iodo and bromo-thalidomide yields (**10** and **11**) could be observed. In the case of the Sonogashira reactions the C4 position seems to be more reactive, possibly due to the enhanced oxidative addition normally observed with aryl iodide substrates. Nevertheless, in the context of creating methodology which can be used efficiently to generate a variety of complex analogues for biological screening these two described methods seem to be highly chemoselective.

4. TNF expression inhibition studies

While many studies involving the inhibition of TNF production have been reported[22,33,34] we have developed a more specific assay to determine the effects on the NFκB activation pathway, as a measure of immunomodulatory or anti-inflammatory activity. To effectively measure inhibition of NFκB pathway signalling by each analogue, a TNF transcriptional reporter cell line was used. The green fluorescent protein (GFP) reporter gene, under the control of the NFκB-responsive human TNF promoter, was inserted into the genome of the human T cell line, Jurkat to generate the reporter line, FRT-Jurkat TNF, as previously described.[23,35] As a measure of TNF promoter activity, GFP activity was quantitated by flow cytometry. This method has the added advantage of being able to easily assess the cellular toxicity of each compound, (by comparing forward- and side-scatter as a measure of cellular size and granularity) during flow cytometry. The cell population in each assay that exhibited low granularity were considered to be dead. This was confirmed by staining with propidium iodide, a fluorescent, DNA-intercalating agent. All thalidomide derivatives were assayed in triplicate at concentrations of 100 μM and the percentage inhi-

bition of TNF expression (relative to TNF expression from solvent treated control cells) for each compound measured. As our previous study showed significant solvent effects at 1% dimethylsulfoxide (DMSO), the final concentration in the current study was lowered to 0.1%. At this concentration, the solvent has very little effect on TNF reporter gene expression.

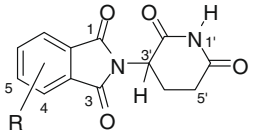
The Suzuki derived aryl based analogues were tested for their ability to inhibit TNF expression and their significance was measured verses the thalidomide (**1**) control (Table 2). Each of the derivatives was selected based on possible receptor interactions, although several derivatives were assumed to have increased water solubility, possibly also enhancing the TNF expression inhibition. Initially derivatives (**27** and **34**) containing a phenyl substituent investigated the likelihood of π-stacking or hydrophobic interactions in potential binding pockets. Each of these derivatives improved the TNF repressive activity of thalidomide by approximately twofold, however this result fell just outside the (p *<0.05*) confidence interval. Encouraged by this slight improvement we devised a series of derivatives containing a *p*-substituent considered to be a linear extension away from the thalidomide core. While the two toluyl derivatives (**28** and **35**) showed no significant improvement, the isobutyl derivatives (**23** and **29**) exploring possible interactions with a hydrophobic binding pocket showed an exceptional improvement of TNF expression inhibition. Accompanying this activity for compounds **23** and **29** was high cell death. However, dead cells were excluded and only viable cells were assayed for TNF repressive activity indicating that the two activities were separate and distinct. Of the other aryl derivatives examined ethers **32** and **38**, isosters of compounds **23** and **29**, also displayed good TNF inhibitory activity suggesting the shape of the *para* group maybe important. Analogue **38** in particular had a good cell viability count compared with each of the previously discussed derivatives.

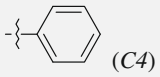
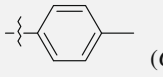
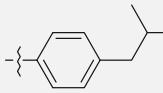
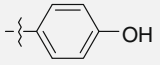
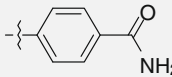
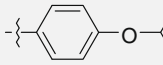
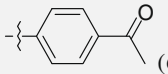
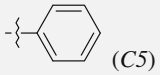
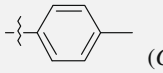
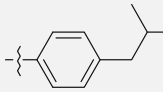
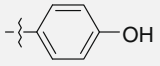
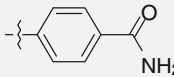
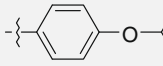
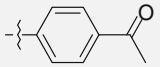
An alternative explanation to altered receptor interactions for the increased activities, may be the polarity and/or water solubility of the indicated compounds. Many of the derivatives have similar inhibition values regardless of whether the functional group tether is located at position C4 or C5. Similar observations have been reported in the literature.[19,36]

The TNF inhibition level was also determined for the alkyne based analogues (Table 3). Compounds that showed significant improvement were the two *t*Bu derivatives (**39** and **24**), surprisingly also containing non-polar fragments like the previously discussed aryl butyl compounds (**29** and **23**). Both of these derivatives exhibit approximately a fourfold increase in TNF inhibition activity when compared to thalidomide (**1**). Alcohol derivative **45** also displayed significantly increased activity compared to thalidomide (**1**). Perhaps the most interesting derivative was compound **26**, conceived to test the hypothesis that an electron withdrawing group attached to the alkyne may improve the inhibition of TNF expression. This compound displayed a percent inhibition of 85% in comparison to the thalidomide control at 5%.

The derivatives which displayed a significantly higher TNF activity in the initial study (**23**, **26**, **29**, **32** and **38**) were further examined at one tenth the initial concentration (10 μM), to determine if there was retention of activity in comparison to thalidomide (**1**) (Table 4). Each of these compounds were also examined for their influence on cell viability. As was expected compounds **23**, **29**, **32** and **38** showed a dramatic decrease in TNF inhibition, however at this concentration compound **38** was still threefold more active than thalidomide **1** with a good cell viability count. In contrast, compound **26** also indicated a high TNF inhibition at 10 μM, approximately 18 times that of thalidomide (**1**), however the rate of cell death remained high. Decreasing the concentration to 1 μM greatly improved the viable cell count. However activity was also reduced almost to the level of thalidomide (**1**).

Table 2
TNF expression inhibition of new aryl based thalidomide derivatives



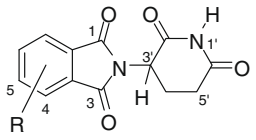
Compound[a,b]	R (carbon substitution)	% Inhibition	p Value[c]
Thalidomide **1**	H	6%	–
27	 (C4)	14	*0.0324*
28	 (C4)	10	0.2762
29	 (C4)	86	*0.0001*
30	 (C4)	19	*0.0092*
31	 (C4)	14	0.0615
32	 (C4)	47	*0.0009*
33	 (C4)	17	*0.0425*
34	 (C5)	12	0.0926
35	 (C5)	14	0.0503
23	 (C5)	90	*0.0001*
36	 (C5)	11	0.1639
37	 (C5)	10	0.0557
38	 (C5)	59	*0.0047*
22	 (C5)	26	*0.0084*

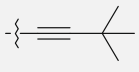
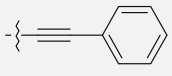
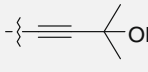
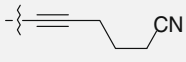
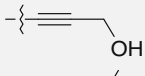
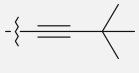
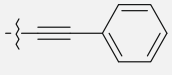
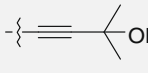
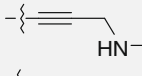
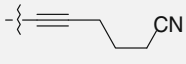
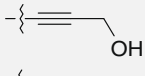
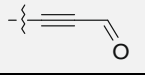
[a] Percentage inhibition was measured as the difference between GFP expression in the presence of 0.1% solvent alone, and each compound at 100 μM. The assay was carried out using the FRT-Jurkat TNF reporter cell line.
[b] All compounds were assayed in triplicate and as a racemic mixture.
[c] The p values which have a significant difference compared to thalidomide **1** (p <0.05) are italicised. Significance was estimated using the unpaired Student's t-test.

5. Characterisation of the cytotoxicity of compounds 23, 26, 29, 32 and 38

Given the high degree of cell death elicited by compounds **23**, **26**, **29**, **32** and **38** at 100 μM, we were interested in determining whether the cellular response was apoptotic in nature as this cell death pathway has been successfully targeted in a number of cancers to selectively kill the malignant cell.[37,38] As thalidomide has been shown to be effective in the treatment of a number of

Table 3
TNF expression inhibition of new alkyne based thalidomide derivatives



Compound	R (*carbon substitution*)	% Inhibition[a,b]	*p* Value[c]
Thalidomide **1**	H	5	—
39	 (C4)	24	*0.0383*
40	 (C4)	13	0.6821
41	 (C4)	6	0.6433
42	 (C4)	9	0.2230
43	 (C4)	7	0.3103
24	 (C5)	19	0.0019
44	 (C5)	6	0.0553
45	 (C5)	20	*0.0287*
46	 (C5)	10	0.2153
47	 (C5)	12	0.2130
25	 (C5)	10	*0.0344*
26	 (C5)[d]	85	*0.0002*

[a] Percentage inhibition was measured as the difference between GFP expression in the presence of 0.1% solvent alone, and each compound at 100 μM. The assay was carried out using the FRT-Jurkat TNF reporter cell line.
[b] All compounds were assayed in triplicate and as a racemic mixture.
[c] The *p* values which have a significant difference compared to thalidomide **1** ($p < 0.05$) are italicised. Significance was estimated using the unpaired Student's *t*-test.
[d] Indicated compound was produced through Dess–Martin oxidation of the corresponding alcohol **25**.

Table 4
TNF expression inhibition and cell viability for lower concentrations of compounds **23**, **26, 29**, **32** and **38**

Compound	Concentration (μM)	TNF inhibition[a,b] (%)	Viable cells[c] (%)
Thalidomide (**1**)	10	4	
23	10	5	89
29	10	7	90
32	10	8	90
38	10	10	90
26	10	86	25
26	1	5	95
26	0.1	4	96

[a] Percentage inhibition was measured as the difference between GFP expression in the presence of 0.1% solvent alone, and each compound at the indicated concentration. The assay was carried out using the FRT-Jurkat TNF reporter cell line.
[b] All compounds were assayed in triplicate and as a racemic mixture.
[c] Viable cells were counted as being indistinguishable from solvent treated cells with respect to forward and side scatter characteristics, using the flow cytometer (see gate in Fig. 3).

Table 5
Compounds **23**, **26**, **29, 32**, **38** and apoptosis

Compound	Concentration	Cell death[a,b]	Apoptosis[c] (%)
Untreated	—	7	8
DMSO	0.10%	7	8
Thalidomide	100 μM	7	9
Thalidomide	10 μM	6	7
23	100 μM	21	22
23	10 μM	6	7
29	100 μM	94	96
29	10 μM	7	7
32	100 μM	33	30
32	10 μM	8	8
38	100 μM	51	34
38	10 μM	14	12
26	10 μM	86	84
26	1 μM	7	7
26	0.1 μM	4	7

[a] The assay was carried out using the Jurkat cell line; cell death was measured as the percentage of cells showing a decrease in cell size and granularity (forward and side scatter) in flow cytometric analysis.
[b] All compounds were assayed in triplicate and as a racemic mixture.
[c] Apoptosis was measured flow cytometrically as the percentage of cells that showed an increase in green fluorescence following staining for cleaved caspase 3.

cancers, it is possible that the cytotoxicity may be used to advantage in therapeutic applications. Each of the compounds were used to treat Jurkat T cells at the concentrations shown in Table 5. The degree of apoptosis was measured and compared with cell death measured by cellular size and granularity. The results indicate that for each of the compounds, all of the cells that were undergoing cell death were in fact apoptotic, indicating that these compounds had both TNF inhibitory activity and also apoptotic activity.

Inspection of the apoptosis flow cytometry histograms for these compounds revealed that compound **26** showed unusual additional characteristics (Fig. 3). At 1 μM the majority of the cells (94%) were viable with 6% undergoing apoptosis (Fig. 3a). However, at 10 μM, 86% of the cells were apoptotic, but showed two distinct populations, one that was weakly positive and one that was strongly positive for the apoptosis phenotype (Fig. 3b). This result is consistent with a cell-cycle specific activity for compound **26,** where only cells at a particular stage of the cell cycle, are susceptible to this derivative's mode of action. The weakly positive population has entered the cell cycle later than the strongly positive population. These results further support the notion that compound **26** may be more active on cells undergoing vigorous cell division, such as malignant cancer cells, than generally quiescent normal tissue.

To investigate whether compound **26** was apoptotic when used to treat normal cells rather than a transformed leukemic cell such as Jurkat, peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers, activated and treated with compound **26** or thalidomide (**1**). The effect on expression of the endogenous TNF was measured by quantitative RT-PCR (Fig. 3c). The results show that compound **26** at 10 μM strongly repressed TNF expression and even at 1 μM, significant activity was evident and showed inhibition similar to the level for thalidomide at 100 μM. None of the treatments resulted in significant cell death compared to the DMSO control.

6. Conclusion

We have described the rapid production of several thalidomide based derivatives through two cross couplings in reasonable to

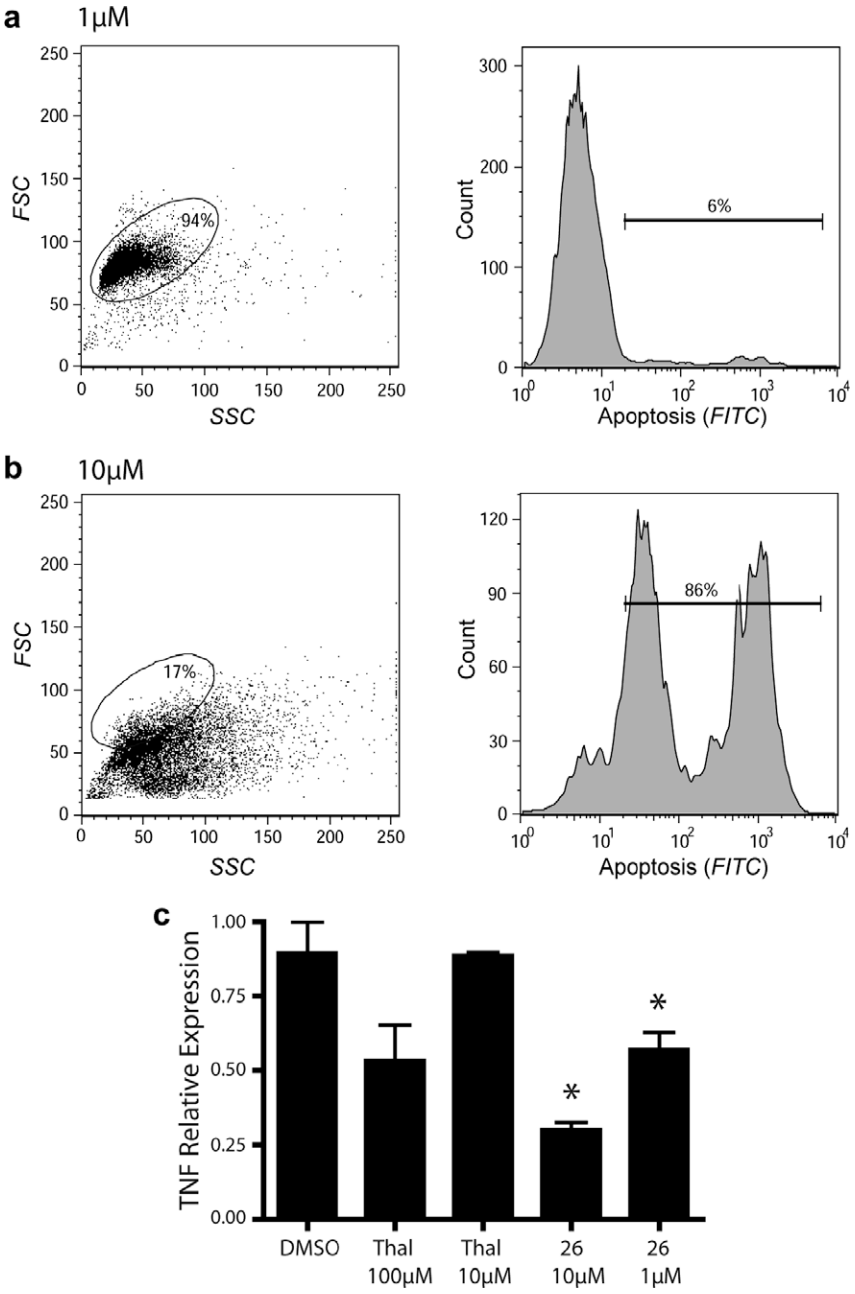


Figure 3. Treatment with compound **26** results in apoptosis and inhibition of TNF mRNA expression. (a and b) Flow cytometry of cells treated with either 1 μM (a) or 10 μM (b) compound **26** for 24 h. Viability is shown by the circled regions in the side scatter (SSC) versus forward scatter (FSC) plot (left panels). Right panels represent histograms of cell counts following treatment with the fluorescent substrate (FITC) specific for cleaved caspase-3 as a measure of the percentage of cells undergoing apoptosis. One representative of three independent experiments is shown. Horizontal gates [6%, panel (a) and 86%, panel (b)] represent the percentage of cells considered positive for apoptosis. (c) Freshly isolated PBMCs were activated and cultured in the presence of DMSO, thalidomide **1** (100 μM or 10 μM) or compound **26** (10 μM or 1 μM) for 2 h. Total RNA was extracted and assayed using TNF-specific quantitative RT-PCR. The results were normalised against the house-keeping gene β-actin (shown as TNF Relative Expression). * = significantly different to DMSO control (P <0.05). Error bars represent standard error of the mean.

excellent yields from their halogenated precursors. Biological results indicate that several thalidomide derivatives (**23**, **29**, **32** and **38**) exhibited significant increases in TNF expression inhibitory activity compared to thalidomide (**1**). Compound **38** displayed a superior TNF inhibitory activity to thalidomide (**1**) at both 100 and 10 μM. Several of these active derivatives include a short non-polar chain attached to either an aryl or alkyne spacer group, however it is not definitive whether the position of the derivatisation is important for improved TNF expression inhibition or other factors such as solubility and polarity. These factors are currently being investigated in separate studies involving the determination of the mode of action of thalidomide. The aldehyde **26**, displayed extraordinary TNF repression as well as cytotoxicity in the form of apoptosis. However, the apoptotic response to compound **26** appears to be selective for transformed or actively dividing cells as PBMCs did not undergo apoptosis following treatment. Compound **26** is being investigated further with respect to its potential selective apoptotic and cell cycle properties in other malignant cell types.

The activities of the new derivatives produced in this study support the notion that further modification of thalidomide to produce more active TNF inhibiting analogues for the treatment of disease

states where immunotherapeutic intervention is indicated, may prove clinically useful.

7. Experimental section

7.1. Chemistry

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian INOVA 300 (^{1}H at 300.14 MHz and ^{13}C at 75.47 MHz), Bruker AV 500 (^{1}H at 500.13 MHz and ^{13}C at 125.8 MHz), Varian 400 (^{1}H at 399.86 and ^{13}C at 100.54 MHz) or a Bruker AV 600 (^{1}H at 600.13 MHz and ^{13}C at 150.90 MHz) spectrometer at 25 °C. ^{1}H and ^{13}C spectra were referenced to the residual (partially) undeuterated solvents and are stated in parts per million. Assignments of ^{13}C resonances were made with the aid of DEPT experiments. Infrared (IR) spectra were recorded with a PerkinElmer Spectrum One Spectrometer FT-IR spectrometer. Samples were analysed as thin films on NaCl discs. Mass Spectra were collected using electron impact ionisation (EI-MS) on a VG AutoSpec. EI-HRMS was performed with a resolution of approximately 10,000. All air and/or moisture sensitive reactions were performed in flame dried glassware under an argon atmosphere. All solvents were distilled prior to use, and if used in air and/or moisture sensitive reactions, were degassed. The degassing procedure was carried out by three freeze–pump–thaw cycles. Anhydrous solvents were obtained by distillation from the appropriate drying agent. Thin layer chromatography (TLC) was performed on Merck Silica Gel 60 F_{254}, precoated aluminium sheets. Visualisation of developed plates was achieved through the use of a 254 nm or 365 nm UV lamp. Column chromatography was performed using Silica Gel 60 (0.063–0.200 mm) as supplied by Merck with the eluents indicated. Starting materials and reagents were generally available from Sigma–Aldrich, Fluka, Merck or Wako. The synthesis of compounds **15**, **17**, **19** and the trial cross coupling reactions for compounds **22** and **33** are described in the supporting information. Additionally, ^{1}H NMR spectra for compounds **22**, **23**, **26**, **29**, **37**, **39**, **41**, **43**, **45** and **47** are also illustrated in this section.

7.2. General protocol for the Suzuki reaction

Method A: *N,N*-Dicyclohexylmethylamine (1.1 equiv) was added to a suspension of halogenated-thalidomide **10** or **11** (1.0 equiv), phenylboronic acid/ester (1.0–1.5 equiv), $Pd_2(dba)_3{\cdot}CHCl_3$ (5–10 mol %) and tri-*t*-butylphosphonium tetrafluoroborate (10–20 mol %) in THF at ambient temperatures. The resulting mixture was degassed three times and stirred at reflux for between 2 and 22 h before the solvent was removed under reduced pressure. The ensuing mixture was subjected to column chromatography (conditions specified). The resulting product, following concentration of the appropriate fractions, was used directly for biological assay or subsequently recrystallised from ethyl acetate. The yields are specified for each individual case.

Method B: N,N-Dicyclohexylmethylamine (1.1 equiv) is added to a suspension of halogenated-thalidomide **10** or **11** (1.0 equiv), phenylboronic acid/ester (1.0–1.5 equiv), $Pd_2(dba)_3{\cdot}CHCl_3$ (5–10 mol %) and tri-*t*-butylphosphonium tetrafluoroborate (10–20 mol %), NaOH (1.0 equiv) in THF at ambient temperatures. The resulting mixture is degassed three times and stirred at reflux for between 2 and 22 h before the solvent is removed under reduced pressure. The ensuing mixture was fused to silica and subjected to column chromatography (conditions specified). The resulting product, following concentration of the appropriate fractions, was used directly for biological assay or subsequently recrystallised from ethyl acetate. The yields are specified for each individual case.

Method C: Phenylboronic acid (1.0–1.5 equiv), was added to a suspension of halogenated-thalidomide **10** or **11** (1.0 equiv), Pd(dppf)Cl_2 (10 mol %), NaOH (1.0 equiv), *N,N*-dicyclohexylmethylamine (1.0 equiv) and THF at room temperature. The mixture was then stirred at reflux for between 2 and 18 h before being fused to silica and passed through a short silica plug eluting with ethyl acetate. The crude organic eluent was then fused to silica and subjected to column chromatography (conditions specified). The resulting product, following concentration of the appropriate fractions, was used directly for biological assay or subsequently recrystallised from ethyl acetate. The yields are specified for each individual case.

7.3. General protocol for the Sonogashira reactions

The alkyne (1.2 equiv) was added in one portion to a suspension of halogenated-thalidomide **10** or **11** (1.0 equiv), $Pd(PPh_3)_2Cl_2$ (10 mol %), CuI (10 mol %) and *N,N*-diisopropylethylamine (10 equiv) in THF. The ensuing solution was stirred at room temperature (or reflux) for between 4 and 22 h. The resulting mixture was subsequently fused to silica, filtered through a silica plug eluting with ethyl acetate/hexane. The organic layer was again fused to silica and purified via flash column chromatography (conditions specified). The resulting product, following concentration of the appropriate fractions, was used directly for biological assay or subsequently recrystallised from ethyl acetate. The yields are specified for each individual case.

7.3.1. *(R,S)*-2-(2,6-Dioxopiperidin-3-yl)-4-iodoisoindole-1,3-dione (10)

Triethylamine (3 mL, 21.5 mmol) was added in one portion to a suspension of 3-iodophthalic anhydride (**17**) (1.79 g, 6.53 mmol) and TFA salt **15** (1.61 g, 6.65 mmol) in THF (14 mL) at room temperature. The resulting mixture was heated at reflux for 23 h, before being cooled to 0 °C. The ensuing precipitate was filtered, washed with cold THF and dried under reduced pressure to yield **10** as a pale grey solid (1.41 g, 56%). Mp = 304–305 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.98–2.12 (m, 1H, $H_{4'}H/_{5'}$), 2.52–2.66 (m, 2H, $H_{4'}/H_{5'}$), 2.82–2.96 (m, 1H, $H_{4'}/H_{5'}$), 5.12–5.21 (dd, *J* = 12.8 Hz, 5.4 Hz, 1H, $H_{3'}$), 7.54–7.62 (m, 1H, H_6), 7.93 (dd, *J* = 7.4, 0.8 Hz, 1H, Ar-H), 8.27 (dd, *J* = 7.9, 0.8 Hz, 1H, Ar-H), 11.15 (s, 1H, N*H*); ^{13}C NMR (75.4 MHz, DMSO-d_6): δ = 21.9 ($C_{4'}/C_{5'}$), 30.9 ($C_{4'}/C_{5'}$), 49.2 ($C_{3'}$), 90.5 (C_4), 123.3 (Ar-CH), 131.8 (Ar-C), 133.3 (Ar-C), 135.7 (Ar-CH), 145.5 (Ar-CH), 165.5 (C=O), 166.1 (C=O), 169.8 (C=O), 172.8 (C=O). IR (KBr) $\tilde{\nu}$: 3205.0 (N–H), 1726.1 (C=O), 1390.4, 1202.5, 737.9 cm^{-1}; MS EI, *m/z* (%) = 385 (6) $[M+H]^{\cdot+}$, 307 (61), 289 (24), 154 (100), 137 (55). $C_{13}H_{10}IN_2O_4I$ (384.1260); EI-HRMS: $[M+H]^{\cdot+}$; 384.9685; found $[M+H]^{\cdot+}$ 384.9663.

7.3.2. *(R,S)*-5-Bromo-2-(2,6-dioxopiperidin-3-yl)-isoindole-1,3-dione (11)

Triethylamine (12 mL, 86.1 mmol) was added to a suspension of 4-bromophthalic anhydride (4.81 g, 21.2 mmol) and TFA salt **15** (5.11 g, 21.1 mmol) in THF (45 mL) at room temperature. The resulting mixture was then heated at reflux for 48 h, before being cooled on ice. The ensuing precipitate was collected, washed with cold THF and dried under reduced pressure to yield **11** as a pale lavender solid (4.49 g, 63%). Mp >230 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 2.01–2.08 (m, 1H, $H_{5'}/H_{4'}$), 2.50–2.63 (m, 2H, $H_{5'}/H_{4'}$), 2.83–2.92 (m, 1H, $H_{4'}/H_{5'}$), 5.15 (dd, *J* = 12.9 and 5.4 Hz, 1H, $H_{3'}$), 7.85 (dd, *J* = 8.0 and 0.5 Hz, 1H, H_7), 8.08 (dd, *J* = 8.0 and 1.7 Hz, 1H, H_6), 8.14 (dd, *J* = 1.7 and 0.5 Hz, H_4); ^{13}C NMR (125.8 MHz, DMSO-d_6): δ = 21.9 ($C_{4'}/C_{5'}$), 30.9 ($C_{4'}/C_{5'}$), 49.2 ($C_{3'}$), 125.3 (Ar-CH), 126.4 (Ar-CH), 128.5 (Ar-Br), 130.2 (Ar-C), 133.2 (Ar-C), 137.6 (Ar-CH), 165.9 (C=O), 166.4 (C=O), 169.7 (C=O), 172.7 (C=O); IR (KBr) $\tilde{\nu}$: 3195 (N–H), 1724 (C=O), 1706.7 (C=O), 739.4 cm^{-1}.

7.3.3. *(R,S)*-5-(4-Acetylphenyl)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (22)

Compound **22** was prepared using the Suzuki general protocol Method C using 4-acetylphenylboronic acid **20**. Flash column chromatography (1:1 ethyl acetate/hexane) gave the desired biaryl compound **22** as a white solid (70%). R_f = 0.23 (1:1 ethyl acetate/hexane); mp = >230 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.07–2.11 (m, 1H, $H_{4'}/H_{5'}$), 2.50–2.55 (m, 2H, $H_{4'}/H_{5'}$), 2.64 (s, 3H, CH_3), 2.90 (m, 1H, $H_{4'}/H_{5'}$), 5.20 (dd, J = 12.6 and 5.1 Hz, 1H, $H_{3'}$), 8.03–8.11 (m, 5H, Ar-CH), 8.21–8.27 (m, 2H, Ar-CH), 11.10 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 22.0 ($C_{4'}/C_{5'}$), 26.8 (CH_3) 30.9 ($C_{4'}/C_{5'}$), 49.1 ($C_{3'}$), 121.8 (Ar-CH), 124.2 (Ar-CH), 127.7 (2 × Ar-CH), 129.0 (2 × Ar-CH), 130.6 (Ar-C), 132.3 (Ar-C), 133.4 (Ar-CH), 136.7 (Ar-C), 142.3 (Ar-C), 145.4 (Ar-C), 166.8 (2 × C=O), 169.8 (C=O), 172.7 (C=O), 197.6 (C=O); IR (KBr) $\tilde{\nu}$: 3442 (N–H), 1775, 1716 (C=O), 1383, 1268, 1203, 751 cm^{-1}; MS EI, *m/z* (%): 376 (27) $[M]^{\cdot+}$, 261 (98) $[M-CH_3]^{\cdot+}$; EI-HRMS calcd for $C_{21}H_{16}N_2O_5$: 376.1059; found: 376.1064.

7.3.4. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-5-(4-isobutylphenyl)isoindoline-1,3-dione (23)

Compound **23** was prepared using the Suzuki general protocol Method C using 4-isobutylphenylboronic acid **21.** Flash column chromatography (7:20 ethyl acetate/hexane) gave the title biaryl compound **23** as a yellow solid (90%); R_f = 0.31 (7:20 ethyl acetate/hexane); mp = 118–120 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.89 (d, J = 6.6 Hz, 6H, 2 × CH_3), 1.89 (septet, J = 6.9 Hz, 1H, CH), 2.06–2.10 (m, 1H, $H_{4'}/H_{5'}$), 2.49–2.54 (m, 2H, CH_2), 2.58–2.64 (m, 2H, $H_{4'}/H_{5'}$), 2.87–2.91 (m, 1H, $H_{4'}/H_{5'}$), 5.18 (dd, J = 12.6 and 5.4 Hz, 1H, $H_{3'}$), 7.31 ('d', 'J' = 8.1 Hz, 2H, $H_{3''}/H_{5''}$), 7.76 ('d', 'J' = 8.1 Hz, 2H, $H_{2''}/H_{6''}$), 7.96 (m, 1H, Ar-CH), 8.17 (m, 2H, Ar-CH), 11.15 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 22.0 ($C_{4'}/C_{5'}$, CH_2), 22.2 (2 × CH_3), 29.6 (CH), 30.9 ($C_{4'}/C_{5'}$, CH_2), 44.2 (CH_2), 49.1 ($C_{3'}$, CH), 121.1 (Ar-CH), 124.0 (Ar-CH), 127.1 (2 × Ar-CH), 129.5 (Ar-C), 129.8 (2 × Ar-CH), 132.3 (Ar-C), 132.6 (Ar-CH), 135.5 (Ar-C), 142.3 (Ar-C), 146.6 (Ar-CH), 166.9 (C=O), 167.0 (C=O), 169.8 (C=O), 172.8 (C=O); IR (KBr) $\tilde{\nu}$: 3231 (N–H), 2955, 1778 (C=O), 1716 (C=O), 1383, 1198, 749 cm^{-1}; MS EI, *m/z* (%): 390 (56) $[M]^{\cdot+}$, 348 (26), 347 (100) $[M-C_3H_7]^{\cdot+}$, 262 (18), 236 (10); EI-HRMS calcd for $C_{23}H_{22}N_2O_4$: 390.1579; found: 390.1575.

7.3.5. *(R,S)*-5-(3,3-Dimethylbut-1-ynyl)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (24)

Compound **24** was prepared using general protocol for the Sonogashira reaction using 3,3-dimethyl-1-butyne. Flash column chromatography (7:13 ethyl acetate/hexane) gave the alkyne **24**, as a yellow solid (76%); R_f = 0.25 (7:13 ethyl acetate/hexane); mp = 222–225 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.32 (s, 9H, 3 × CH_3), 2.05–2.08 (m, 1H, $H_{4'}/H_{5'}$), 2.50–2.62 (m, 2H, $H_{4'}/H_{5'}$), 2.85–2.89 (m, 1H, $H_{4'}/H_{5'}$), 5.15 (dd, J = 12.8 and 5.2 Hz, 1H, $H_{3'}$), 7.81–7.88 (m, 3H, Ar-H), 11.13 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 21.9 ($C_{4'}/C_{5'}$, CH_2), 27.8 ($C(CH_3)_3$), 30.4 (3 × CH_3), 30.9 ($C_{4'}/C_{5'}$, CH_2), 49.1 ($C_{3'}$), 77.9 (C≡C), 103.5 (C≡C), 123.7 (Ar-CH), 125.5 (Ar-CH), 129.6 (Ar-C), 129.8 (Ar-C), 131.7 (Ar-C), 137.3 (Ar-CH), 166.4 (C=O), 166.6 (C=O), 169.7 (C=O), 172.7 (C=O); IR (KBr) $\tilde{\nu}$: 3424 (N–H), 2221 (C≡C), 1775, 1725 (C=O), 1385, 1261, 1200 (C–O), 745 cm^{-1}; MS EI, *m/z* (%): 339 (17) $[M+H]^{\cdot+}$, 338 (100) $[M]^{\cdot+}$, 184 (42), 169 (33); EI-HRMS: calcd for $C_{19}H_{18}N_2O_4$: 338.1267; found: 338.1269.

7.3.6. *(R,S)*-2-(2,6-Dioxopiperidin-3-yl)-5-(3-hydroxyprop-1-ynyl)isoindoline-1,3-dione (25)

Compound **25** was prepared using general protocol for the Sonogashira reaction using propargyl alcohol. Flash column chromatography (1:1 ethyl acetate/hexane) and subsequent recrystallisation from ethyl acetate afforded the desired alkyne **25**, as a yellow solid (48%); R_f = 0.32 (1:1 ethyl acetate/hexane); mp = 232–234 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.04–2.08 (m, 1H, $H_{4'}/H_{5'}$), 2.53–2.63 (m, 2H, $H_{4'}/H_{5'}$), 2.85–2.90 (m, 1H, $H_{4'}/H_{5'}$), 4.37 (d, J = 6.0 Hz, 2H, CH_2), 5.18 (dd, J = 12.9 and 5.4 Hz, 1H, $H_{3'}$), 5.47 (t, J = 6.0 Hz, 1H, OH), 7.88–7.94 (m, 3H, Ar-H), 11.14 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 22.8 ($C_{4'}/C_{5'}$, CH_2), 31.8 ($C_{4'}/C_{5'}$, CH_2), 50.0 (CH_2), 50.4 ($C_{3'}$, CH), 83.2 (C≡C), 95.4 (C≡C), 124.8 (Ar-C), 126.5 (Ar-C), 129.7 (Ar-CH), 131.3 (Ar-CH), 132.7 (Ar-CH), 138.4 (Ar-C), 167.3 (C=O), 167.4 (C=O), 170.7 (C=O), 173.7 (C=O); IR (KBr,) $\tilde{\nu}$: 3444 (N–H), 2210 (C≡C), 1716 (C=O), 1384, 1205 (C–O), 1049 cm^{-1}; MS EI, *m/z* (%): 312 (100) $[M]^{\cdot+}$, 284 (25), 227 (70); EI-HRMS calcd for $C_{16}H_{12}N_2O_5$: 312.0746; found: 312.0747.

7.3.7. (*R,S*)-3-(2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)propiolaldehyde (26)

Dess–Martin periodinane (244 mg, 0.576 mmol) was added in one portion to a stirred solution of alcohol **25** (150 mg, 0.480 mmol) in CH_2Cl_2 (6 mL). The mixture was stirred for 1 h before being treated with a sodium bicarbonate/sodium thiosulfate aqueous solution (1:1, 1 mL) in one portion. The reaction mixture stirred for a further 1 h, transferred to a separatory funnel and extracted with CH_2Cl_2 (3 × 50 mL) and ethyl acetate (2 × 30 mL). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The resulting solid was subsequently fused to silica and subjected to flash column chromatography (2:3 ethyl acetate/hexane) to afford the aldehyde product **26** as a yellow/orange solid (112 mg, 76%); R_f = 0.29 (2:3 ethyl acetate/hexane); mp = 203–205 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.05–2.08 (m, 1H, $H_{4'}/H_{5'}$), 2.50–2.63 (m, 2H, $H_{4'}/H_{5'}$), 2.85–2.92 (m, 1H, $H_{4'}/H_{5'}$), 5.19 (dd, J = 12.8 and 5.2 Hz, 1H, $H_{3'}$), 8.03 (d, J = 8 Hz, 1H, Ar-CH), 8.04–8.21 (m, 2H, Ar-CH), 9.49 (s, 1H, CHO), 11.15 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6) δ = 21.8 ($C_{4'}/C_{5'}$), 30.8 ($C_{4'}/C_{5'}$), 49.2 ($C_{3'}$), 90.0 (C≡C), 90.5 (C≡C), 124.0, 124.8, 127.2, 131.8, 132.7, 139.2, 165.9 (C=O), 166.2 (C=O), 169.7 (C=O), 172.7 (C=O), 178.6 (CHO); IR (KBr) $\tilde{\nu}$: 3439 (N–H), 2924, 2194 (C≡C), 1722 (C=O), 1391, 1200 cm^{-1}; MS EI, *m/z* (%): 310 (41) $[M]^{\cdot+}$, 282 (30) $[M-CHO]^{\cdot+}$, 224 (100); EI-HRMS calcd for $C_{16}H_{10}N_2O_5$: 310.0589; found: 310.0582.

7.3.8. *(R,S)*-2-(2,6-Dioxopiperidin-3-yl)-4-phenylisoindole-1,3-dione (27)

Compound **27** was prepared using the Suzuki general protocol Method A using phenylboronic acid. Column chromatography (2:3 ethyl acetate/hexane) and subsequent recrystallisation from ethyl acetate gave the desired **27** as a white solid (66%); R_f = 0.33 (2:3 ethyl acetate/hexane; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.12–2.02 (m, 1H, $H_{4'}/H_{5'}$), 2.85–2.60 (m, 3H, $H_{4'}/H_{5'}$), 4.94 (dd, J = 12.0, 5.2 Hz, 1H, $H_{3'}$), 7.46–7.40 (m, 3H, Ar-H), 7.54–7.48 (m, 2H, Ar-H), 7.65 (dd, J = 7.5, 1.1 Hz, 1H, H5/H7), 7.84 (dd, J = 7.5, 1.1 Hz, 1H, H5/H7), 7.74 (dd, J = 7.5, 1.1 Hz, 1H, H6); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 22.0 ($C_{4'}/C_{5'}$, CH_2), 30.9 ($C_{4'}/C_{5'}$, CH_2), 48.9 ($C_{3'}$, CH), 122.4 (Ar-CH), 128.0 (Ar-CH), 129.4 (Ar-CH), 135.8 (Ar-CH), 136.4 (Ar-CH), 166.5 (C=O), 166.7 (C=O), 169.8 (C=O), 172.8 (C=O); IR (NaCl) $\tilde{\nu}$: 3247 (N–HN–H), 1699 (C=O), 1393, 1261, 1199, 1121, 891, 742 cm^{-1}; MS (*m/z*) = 334 (100) $[M]^{\cdot+}$, 249 (37), 248 (38), 224 (40), 222 (21) $[M-C_5H_6NO_2]^+$, 180 (27), 152 (51), 151 (24); EI-HRMS calcd for $C_{19}H_{14}N_2O_4$: 334.0954; found: 334.0959.

7.3.9. *(R,S)*-2-(2,6-Dioxopiperidin-3-yl)-4-*p*-tolylisoindole-1,3-dione (28)

Compound **28** was prepared using the Suzuki general protocol Method A using 4-toluyl pinacolboronic acid ester. Column chromatography (2:3 ethyl acetate/hexane) gave **28** as a white solid (45%). R_f = 0.3 (1:1 ethyl acetate/hexane); R_f = 0.3 (1:1 ethyl ace-

tate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.11–2.01 (m, 1H, $H_{4'}/H_{5'}$), 2.38 (s, 1H, CH_3), 2.65–2.52 (m, 2H, $H_{4'}/H_{5'}$), 2.95–2.82 (m, 1H, $H_{4'}/H_{5'}$), 5.12 (dd, J = 12.6, 5.4 Hz, 1H, $H_{3'}$), 11.10 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 48.9 ($C_{3'}$), 122.6, 128.0, 128,6, 129.3, 129.7, 131.8, 132.5, 132.9, 134.2, 136.3, 138.1, 139.4, (C4′, C5′, CH_3), 166.6 (C=O), 166.8 (C=O), 169.8 (C=O)172.8 (C=O); IR (neat): 3220 (N–HN–H), 1710 (C=O), 1612, 1348, 1262, 1198, 813, 731, 681 cm^{-1}; EI-MS (m/z) = 348 (100) [M]$^{\cdot+}$, 238 (26), 165 (33); EI-HRMS calcd for $C_{20}H_{16}N_2O_4$: 348.1110; found: 348.1113.

7.3.10. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-4-(4-isobutylphenyl)isoindoline-1,3-dione (29)

Compound **29** was prepared using Suzuki general protocol Method C using 4-isobutylphenylboronic acid **21**. Flash column chromatography (7:20 ethyl acetate/hexane) gave the title biaryl compound **29** as a light yellow solid (91%). mp = 120–122°C; R_f = 0.27 (7:20 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 0.87 (s, 6H, 2 × CH_3), 1.86–1.90 (m, 1H, CH), 2.03–2.06 (m, 1H, $H_{4'}/H_{5'}$), 2.49–2.50 (m, 2H, CH_2), 2.52–2.60 (m, 2H, $H_{4'}/H_{5'}$), 2.83–2.87 (m, 1H, $H_{4'}/H_{5'}$), 5.11 (dd, J = 12.8 and 4.4 Hz, 1H, $H_{3'}$), 7.25 ('d', 'J' = 6.4 Hz, 2H, $H_{3''}/H_{5''}$), 7.50 ('d', 'J' = 6.4 Hz, 2H, $H_{2''}/H_{6''}$), 7.80 (m, 1H, Ar-CH), 7.91 (m, 2H, Ar-CH), 11.09 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 22.4 ($C_{4'}/C_{5'}$), 22.6 (2 × CH_3), 30.0 (CH), 31.6 ($C_{4'}/C_{5'}$), 44.5 (CH_2), 49.3 ($C_{3'}$), 122.6 (Ar-CH), 127.1 (Ar-C), 129.0 (2 × Ar-CH), 129.7 (2 × Ar-CH), 132.9 (Ar-C), 133.6 (Ar-C), 135.1 (Ar-CH), 136.8 (Ar-CH), 140.7 (Ar-C), 142.2 (Ar-C), 167.1 (C=O), 167.2 (C=O), 170.3 (C=O), 173.2 (C=O); IR (KBr, cm^{-1}) $\tilde{\nu}$: 3418 (N–H), 2955, 1714 (C=O), 1393, 1198; MS EI, m/z (%): 390 (61) [M]$^{\cdot+}$, 348 (53), 347 (100) [M−C_3H_7]$^{\cdot+}$, 275 (16), 262 (15); EI-HRMS calcd for $C_{23}H_{22}N_2O_4$: 390.1580; found: 390.1584.

7.3.11. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-4-(4-hydroxyphenyl)isoindoline-1,3-dione (30)

Compound **30** was prepared using the Suzuki general protocol Method C using 4-hydroxyphenylboronic acid. In this instance the reaction mixture was then washed with HCl (1 M, 10 mL) and the organic layer dried ($MgSO_4$) and fused to silica before being purified via flash column chromatography (11:20 ethyl acetate/hexane) to afford the desired biaryl compound **30**, as a yellow solid (51%); mp = 215–217 °C; R_f = 0.25 (1:1 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.03–2.05 (m, 1H, $H_{4'}/H_{5'}$), 2.53–2.61 (m, 2H, $H_{4'}/H_{5'}$), 2.84–2.92 (m, 1H, $H_{4'}/H_{5'}$), 5.11 (dd, J = 12.8 and 5.2 Hz, 1H, $H_{3'}$), 6.84 ('d', 'J' = 8.0 Hz, 2H, $H_{3''}/H_{5''}$), 7.43 ('d', 'J' = 8.0 Hz, 2H, $H_{2''}/H_{6''}$), 7.75 ('d', 'J' = 6.8 Hz, 1H, Ar-CH), 7.85 ('d', 'J' = 5.1 Hz, 2H, Ar-CH), 9.74 (s, 1H, OH), 11.09 ppm (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 21.9 ($C_{4'}/C_{5'}$, CH_2), 30.9 ($C_{4'}/C_{5'}$, CH_2), 48.8 ($C_{3'}$, CH), 114.8 (2 × Ar-CH), 121.5, 126.1, 126.3, 130.9, 132.5, 134.6, 136.2, 140.6, 158.1 (Ar-CO), 166.7 (C=O), 166.8 (C=O), 169.8 (C=O), 172.7 (C=O); IR (KBr) $\tilde{\nu}$: 3423 (N–H), 1763, 1711 (C=O), 1396, 1199 (C–O), 750 cm^{-1}; MS EI, m/z (%): 350 (100) [M]$^{\cdot+}$, 265 (24); EI-HRMS calcd for $C_{19}H_{14}N_2O_5$: 350.0902; found: 350.0911.

7.3.12. (*R,S*)-4-(2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)benzamide (31)

Compound **31** was prepared using the Suzuki general protocol Method C using 4-aminocarbonylphenylboronic acid. Flash column chromatography (1:9 methanol/dichloromethane) and recrystallisation from acetonitrile to afford the desired biaryl compound **31** as a white solid (12% yield); mp = >230°C; R_f = 0.37 (1:1 acetone/toluene); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.06–2.08 (m, 1H, $H_{4'}/H_{5'}$), 2.48–2.61 (m, 2H, $H_{4'}/H_{5'}$), 2.84–2.88 (m, 1H, $H_{4'}/H_{5'}$), 5.12 (dd, J = 12.8 and 5.2 Hz, 1H, $H_{3'}$), 7.45 (s, 1H, NH_2), 7.66 ('d', 'J' = 8.0 Hz, 2H, $H_{2''}/H_{6''}$), 7.83–7.85 (m, 1H, Ar-CH), 7.92–7.96 (m, 4H, Ar-CH), 8.07 (s, 1H, NH_2), 11.10 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 21.9 ($C_{4'}/C_{5'}$); 30.9 ($C_{4'}/C_{5'}$), 48.9 ($CH_{3'}$), 122.7 (Ar-CH), 127.0 (Ar-C), 127.1 (2 × Ar-CH), 129.3 (2 × Ar-CH), 132.4 (Ar-C), 134.8 (Ar-CH), 134.2 (Ar-C), 136.3 (Ar-CH), 138.5 (Ar-C), 139.3 (Ar-C), 166.4 (C=O), 166.7 (C=O), 167.4 (C=O), 169.8 (C=O), 172.7 (C=O); IR (KBr) $\tilde{\nu}$: 3422 (N–H), 1710 (C=O), 1390, 1208, 746 cm^{-1}; EI-HRMS calcd for $C_{20}H_{15}N_3O_5$: 377.1011; found: 377.1005.

7.3.13. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-4-(4-isopropoxyphenyl)-isoindoline-1,3-dione (32)

Compound **32** was prepared using the Suzuki general protocol Method C using 4-isopropoxyphenylboronic acid. Flash column chromatography (7:20 ethyl acetate/hexane) afforded the title biaryl compound **32** as a bright yellow solid (71%); mp = 100–103°C; R_f = 0.62 (1:1 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.29 (d, J = 6.0 Hz, 6H, 2 × CH_3), 2.03–2.06 (m, 1H, $H_{4'}/H_{5'}$), 2.50–2.61 (m, 2H, $H_{4'}/H_{5'}$), 2.83–2.88 (m, 1H, $H_{4'}/H_{5'}$), 4.69 (septet, J = 6.0 Hz, 1H, CH(CH_3)$_2$), 5.11 (dd, J = 12.8 and 5.2 Hz, 1H, $H_{3'}$), 7.00 ('d', 'J' = 8.4 Hz, 2H, $H_{3''}/H_{5''}$), 7.52 ('d', 'J' = 8.4 Hz, 2H, $H_{2''}/H_{6''}$), 7.77 (m, 1H, Ar-CH), 7.88 (m, 2H, Ar-CH), 11.09 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 21.8 (2 × CH_3), 21.9 ($C_{4'}/C_{5'}$, CH_2), 30.9 ($C_{4'}/C_{5'}$, CH_2), 48.8 ($C_{3'}$, CH), 69.2 (CH), 114.8 (2 × Ar-CH), 121.7 (Ar-CH), 126.3 (Ar-C), 127.6 (Ar-C), 130.9 (2 × Ar-CH), 132.5 (Ar-C), 134.6 (Ar-CH), 136.3 (Ar-CH), 140.1 (Ar-C), 157.9 (Ar-CO), 166.7 (C=O), 166.8 (C=O), 169.8 (C=O), 172.7 (C=O); IR (KBr,) $\tilde{\nu}$: 3234 (N–H), 2976, 1770, 1712, 1393, 1189 (C–O), 749 cm^{-1}; MS EI, m/z (%): 392 (37) [M]$^{\cdot+}$, 350 (100) [M−C_3H_7]$^{\cdot+}$, 278 (15), 265 (24); EI-HRMS calcd for $C_{22}H_{20}N_2O_5$: 392.1372; found: 392.1364.

7.3.14. (*R,S*)-4-(4-Acetylphenyl)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (33)

Compound **33** was prepared using the Suzuki general protocol Method B using 4-acetylphenylboronic acid **20**. Flash column chromatography (2:25 methanol/dichloromethane) and additional recrystallisation from ethyl acetate to afforded the title biaryl compound **33**, as a white solid (55%); mp = >230°C; R_f = 0.3 (2:25 methanol/dichloromethane); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.04–2.08 (m, 1H, $H_{4'}/H_{5'}$), 2.48–2.56 (m, 2H, $H_{4'}/H_{5'}$), 2.64 (s, 3H, CH_3), 2.81–2.88 (m, 1H, $H_{4'}/H_{5'}$), 5.12 (dd, J = 12.9 and 5.4 Hz, 1H, $H_{3'}$), 7.73 ('d', 'J' = 8.4 Hz, 2H, $H_{2''}/H_{6''}$), 7.85 (dd, J = 6.3 and 2.7 Hz, 1H, Ar-CH), 7.95–7.99 (m, 2H, Ar-CH), 8.04 ('d', 'J' = 8.7 Hz, 2H, $H_{3''}/H_{5''}$), 11.10 (s, 1H, NH); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 21.9 ($C_{4'}/C_{5'}$), 26.8 (CH_3) 30.9 ($C_{4'}/C_{5'}$), 48.9 ($C_{3'}$), 123.0 (Ar-CH), 127.1 (Ar-C), 127.8 (2 × Ar-CH), 129.8 (2 × Ar-CH), 132.4 (Ar-C), 134.8 (Ar-CH), 136.2 (Ar-C), 136.5 (Ar-C), 138.9 (Ar-C), 140.4 (Ar-C), 166.4 (C=O), 166.6 (C=O), 169.8 (C=O), 172.8 (C=O), 197.7 (C=O); IR (KBr) $\tilde{\nu}$: 3462 (N–H), 1773, 1714 (C=O), 1393, 1268, 1200 cm^{-1}; MS EI, m/z (%): 376 (38) [M]$^{\cdot+}$, 261 (100) [M−CH_3]$^{\cdot+}$; EI-HRMS calcd for $C_{21}H_{16}N_2O_5$: 376.1059; found: 376.1066.

7.3.15. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-5-phenylisoindole-1,3-dione (34)

Compound **34** was prepared using the Suzuki general protocol Method C using phenylboronic acid. Column chromatography (2:3 ethyl acetate/hexane) afforded the desired **34** as a pale yellow solid (91%); mp; >230 °C; R_f = 0.3 (1:1 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.13–2.05 (m, 1H, $H_{4'}/H_{5'}$), 2.66–2.53 (m, 2H, $H_{4'}/H_{5'}$), 2.97–2.85 (m, 1H, $H_{4'}/H_{5'}$), 5.19 (dd, J = 13.0, 5.4 Hz, 1H, $H_{3'}$), 8.31–7.30 (Ar-H), 11.14 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 22.0 ($C_{4'}/C_{5'}$, CH_2), 31.0 ($C_{4'}/C_{5'}$, CH_2), 49.1 ($CH_{3'}$, CH), 121.4, 124.1, 127.3, 127.4, 129.4, 129.9, 130.0, 132.3, 133.0, 134.0, 138.1, 146.7, 167.0 (C=O), 167.0 (C=O), 169.9 (C=O), 172.8 (C=O); IR (neat): 3447 (N–H), 1713 (C=O), 1380, 1261, 1198, 1116, 743, 700 cm^{-1}; MS (m/z) = 334 (100)

[M]$^{\cdot+}$, 249 (59), 224 (31), 180 (26), 152 (31), 102 (32); HRMS calcd for $C_{19}H_{14}N_2O_4$: 334.0954; found: 334.0959.

7.3.16. *(R,S)*-2-(2,6-Dioxopiperidin-3-yl)-5-*p*-tolylisoindole-1,3-dione (35)

Compound **35** was prepared using the Suzuki general protocol Method C using 4-toluyl pinacolboronic acid ester. Column chromatography (2:3 ethyl acetate/hexane) and subsequently recrystallised from ethyl acetate to afford the desired biaryl compound **35** as an off-white solid (38%); R_f = 0.2 (2:5 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.13–2.03 (m, 1H, $H_{4'}/H_{5'}$), 2.37 (s, 3H, CH_3), 2.66–2.52 (m, 2H, $H_{4'}/H_{5'}$), 2.97–2.83 (m, 1H, $H_{4'}/H_{5'}$), 5.18 (dd, J = 13.0 and 5.8 Hz, 1H, $H_{3'}$), 8.15–7.12 (Ar-H), 11.14 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 31.0–20.7 (C4′, C5′, CH_3), 49.1 ($C_{3'}$, CH), 121.1, 124.1, 127.1, 128.0, 129.5, 129.8, 132.3, 132.6, 134.2, 138.7, 139.3, 146.6, 167.0 (C=O), 167.0 (C=O), 169.9 (C=O), 172.8 (C=O); IR (NaCl): 1707 (C=O), 1612, 1349, 1260, 1182, 816, 731, 681 cm^{-1}; MS (*m/z*) = 348 (100) [M]$^{\cdot+}$, 263 (47), 238 (21), 166 (21); EI-HRMS calcd for $C_{20}H_{16}N_2O_4$: 348.1110; found: 348.1118.

7.3.17. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-5-(4-hydroxyphenyl)-isoindoline-1,3-dione (36)

Compound **36** was prepared using the Suzuki general protocol Method C using 4-hydroxyphenylboronic acid. The reaction mixture was then transferred to a separatory funnel and washed with an aqueous HCl solution (1 M, 10 mL), brine (10 mL) and dried ($MgSO_4$). The organic mixture was then fused to silica before being purified via flash column chromatography (11:20 ethyl acetate/hexane) to afford the title biaryl compound **36**, as a bright yellow solid (91%); mp = >230°C; R_f = 0.31 (11:20 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.05–2.09 (m, 1H, $H_{4'}/H_{5'}$), 2.55–2.63 (m, 2H, $H_{4'}/H_{5'}$), 2.81–2.99 (m, 1H, $H_{4'}/H_{5'}$), 5.17 (dd, J = 12.6 and 5.1 Hz, 1H, $H_{3'}$), 6.90 ('d', 'J' = 8.4 Hz, 2H, $H_{3''}/H_{5''}$), 7.71 ('d', 'J' = 8.4 Hz, 1H, $H_{2''}/H_{6''}$), 7.92 ('d', 'J' = 6.3 Hz, 2H, Ar-CH), 8.08 ('d', 'J' = 5.1 Hz, 2H, Ar-CH), 9.86 (s, 1H, OH), 11.15 (s, 1H, NH); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 22.0 ($C_{4'}/C_{5'}$, CH_2), 31.0 ($C_{4'}/C_{5'}$, CH_2), 49.0 ($C_{3'}$), 116.1 (2 × Ar-CH), 120.4 (Ar-CH), 124.0 (Ar-CH), 128.7 (2 × Ar-CH), 131.8 (2 × Ar-CH), 132.4 (2 × Ar-C), 146.7 (Ar-C), 158.6 (Ar-CO), 167.0 (C=O), 167.1 (C=O), 169.9 (C=O), 172.8 (C=O); IR (KBr, cm^{-1}) $\tilde{\nu}$: 3222 (N–H), 1773, 1725 (C=O), 1607, 1333, 1200 (C–O), 828; MS EI, *m/z* (%): 351 (22) [M+H]$^{\cdot+}$, 350 (100) [M]$^{\cdot+}$, 265 (35), 239 (56) [M−$C_5H_6NO_2$]$^{\cdot+}$; EI-HRMS calcd for $C_{19}H_{14}N_2O_5$: 350.0902; found: 350.0909.

7.3.18. (*R,S*)-4-(2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin--5-yl)benzamide (37)

Compound **37** was prepared using the Suzuki general protocol Method C using 4-aminocarbonylphenylboronic acid. The crude mixture was run through a short silica plug eluting with ethyl acetate, before being recrystallised from acetonitrile to afford the title biaryl amide **37** as a tan solid (37%); mp = >230°C; R_f = 0.35 (7:20 acetone/toluene); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.07–2.11 (m, 1H, $H_{4'}/H_{5'}$), 2.50–2.65 (m, 2H, $H_{4'}/H_{5'}$), 2.82–3.00 (m, 1H, $H_{4'}/H_{5'}$), 5.20 (dd, J = 12.6 and 5.4 Hz, 1H, $H_{3'}$), 7.46 (s, 1H, NH_2), 7.95 ('d', 'J' = 8.7 Hz, 2H, $H_{2''}/H_{6''}$), 8.01–8.04 (m, 3H, Ar-CH), 8.10 (s, 1H, NH_2), 8.24 ('d', 'J' = 6.3 Hz, 2H, $H_{3''}/H_{5''}$), 11.15 (s, 1H, NH); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 22.0 ($C_{4'}/C_{5'}$), 30.9 ($C_{4'}/C_{5'}$), 49.1 ($CH_{3'}$), 121.7 (Ar-CH), 124.1 (Ar-CH), 127.3 (2 × Ar-CH), 128.3 (2 × Ar-CH), 130.3 (Ar-C), 132.3 (Ar-C), 133.2 (Ar-CH), 134.4 (Ar-C), 140.3 (Ar-C), 145.7 (Ar-C), 166.8 (C=O), 166.9 (C=O), 167.2 (C=O), 169.8 (C=O), 172.8 (C=O); IR (KBr) $\tilde{\nu}$: 3455 (N–H), 1709 (C=O), 1390, 1203, 746 cm^{-1}; MS EI, *m/z* (%): 377 (100) [M]$^{\cdot+}$, 332 (10) [M−$CONH_2$]$^{\cdot+}$, 292 (43), 265 (33); EI-HRMS calcd for $C_{20}H_{15}N_2O_5$: 377.1012; found: 377.1010.

7.3.19. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-5-(4-isopropoxyphenyl)-isoindoline-1,3-dione (38)

Compound **38** was prepared using the Suzuki general protocol Method C using 4-isopropoxyphenylboronic acid. Flash column chromatography (7:20 ethyl acetate/hexane) afforded the title biaryl compound **38** as a bright yellow solid (78%); mp = 105–108°C; R_f = 0.26 (7:20 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.30 (d, J = 6.0 Hz, 6H, 2 × CH_3), 2.07–2.09 (m, 1H, $H_{4'}/H_{5'}$), 2.50–2.64 (m, 2H, $H_{4'}/H_{5'}$), 2.82–2.99 (m, 1H, $H_{4'}/H_{5'}$), 4.71 (septet, J = 6.0 Hz, 1H, $CH(CH_3)_2$), 5.17 (dd, J = 12.8 and 5.2 Hz, 1H, $H_{3'}$), 7.05 ('d', 'J' = 8.8 Hz, 2H, $H_{3''}/H_{5''}$), 7.78 ('d', 'J' = 9.2 Hz, 2H, $H_{2''}/H_{6''}$), 7.95 ('d', 'J' = 5.2 Hz, 1H, Ar-CH), 8.12 ('d', 'J' = 5.2 Hz, 2H, Ar-CH), 11.14 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 21.8 (2 × CH_3), 22.0 ($C_{4'}/C_{5'}$, CH_2), 30.9 ($C_{4'}/C_{5'}$, CH_2), 49.0 ($C_{3'}$, CH), 69.4 (CH), 116.1 (2 × Ar-CH), 120.6 (Ar-CH), 124.0 (Ar-CH), 128.7 (2 × Ar-CH), 128.9 (Ar-C), 129.9 (Ar-C), 132.0 (Ar-CH), 132.7 (Ar-C), 146.4 (Ar-C), 158.4 (Ar-CO), 167.0 (C=O), 167.1 (C=O), 169.9 (C=O), 172.8 (C=O); IR (KBr) $\tilde{\nu}$: 3247 (N–H), 2976, 1774, 1716 (C=O), 1384, 1187 (C–O) cm^{-1}; EI MS, *m/z* (%): 392 (41) [M]$^{\cdot+}$, 349 (100), [M−C_2H_7]$^{\cdot+}$, 264 (33), 238 (58); EI-HRMS calcd for $C_{22}H_{20}N_2O_5$: 392.1372; found: 392.1378.

7.3.20. *(R,S)*-4-(3,3-Dimethylbut-1-ynyl)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (39)

Compound **39** was prepared using general protocol for the Sonogashira reaction using 3,3-dimethyl-1-butyne. Flash column chromatography (3:7 ethyl acetate/hexane) afforded the alkyne compound **39**, as a yellow solid (60%); mp = 228–230°C; R_f = 0.29 (1:1 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.34 (s, 9H, 3 × CH_3), 2.04–2.08 (m, 1H, $H_{4'}/H_{5'}$), 2.52–2.62 (m, 2H, $H_{4'}H_{5'}$), 2.85–2.88 (m, 1H, $H_{4'}H_{5'}$), 5.15 (dd, J = 12.8 and 5.2 Hz, 1H, $H_{3'}$), 7.76–7.87 (m, 3H, Ar-CH), 11.13 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 22.4 ($C_{4'}/C_{5'}$, CH_2), 28.4 ($C(CH_3)_3$), 30.7 (3 × CH_3), 31.4 ($C_{4'}/C_{5'}$, CH_2), 49.4 ($C_{3'}$, CH), 75.1 (C≡C), 106.6 (C≡C), 120.4 (Ar-C), 123.0 (Ar-H), 130.7 (Ar-C), 132.5 (Ar-C), 135.0 (Ar-H), 138.1 (Ar-H), 166.1 (C=O), 166.7 (C=O), 170.3 (C=O), 173.2 (C=O); IR (KBr) $\tilde{\nu}$: 3440 (N–H), 2222 (C≡C), 1717 (C=O), 1391, 1202, 746 cm^{-1}; MS EI, *m/z* (%): 339 (42) [M]$^{\cdot+}$, 323 (98), 250 (45), 269 (70); EI-HRMS calcd for $C_{19}H_{18}N_2O_4$: 338.1266; found: 338.1267.

7.3.21. *(R,S)*-2-(2,6-Dioxopiperidin-3-yl)-5-phenylethynyliso-indole-1,3-dione (40)

Compound **40** was prepared using general protocol for the Sonogashira reaction using phenylacetylene. Flash column chromatography (1:1 ethyl acetate/hexane) and subsequently recrystallisation from ethyl acetate afforded **40** as a yellow solid (59%); R_f = 0.3 (1:1 ethyl acetate/hexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.23–2.13 (m, 1H, $H_{4'}/H_{5'}$), 2.96–2.70 (m, 3H, $H_{4'}/H_{5'}$), 5.00 (dd, J = 12.6, 5.4 Hz, 1H, $H_{3'}$), 7.42–7.37 (m, 3H, Ar-H), 7.59–7.54 (m, 2H, Ar-H), 7.88–7.85 (m, 2H, Ar-H), 8.29 (s, 1H, NH), 8.01–7.95 (m, 1H, Ar-H); ^{13}C NMR (100.5 MHz, $CDCl_3$): δ = 22.7 ($C_{4'}/C_{5'}$, CH_2), 31.6 ($C_{4'}/C_{5'}$, CH_2), 49.6 ($C_{3'}$), 87.7 (C≡C), 94.6 (C≡C), 122.1 (Ar-C), 123.9 (Ar-CH)126.7 (Ar-CH), 128.7 (Ar-CH), 129.5 (Ar-CH), 130.4 (Ar-C), 132.0 (Ar-CH), 132.1 (Ar-C), 137.4 (Ar-CH), 166.8 (C=O), 166.8 (C=O), 168.0 (C=O),171.0 (C=O); IR (neat): 3446 (N–H), 2213 (C≡C), 1717 (C=O), 1380, 1196, 746 cm^{-1}; MS (*m/z*) = 358 (100) [M]$^{\cdot+}$, 273 (40), 176 (24), 126 (26); EI-HRMS calcd for $C_{21}H_{14}N_2O_4$: 358.0954; found: 358.0952.

7.3.22. *(R,S)*-2-(2,6-Dioxopiperidin-3-yl)-4-(3-hydroxy-3-methylbut-1-ynyl)isoindoline-1,3-dione (41)

Compound **41** was prepared using general protocol for the Sonogashira reaction using 2-methyl-3-butyn-2-ol. Flash column

chromatography (2:3 ethyl acetate/hexane) afforded the title alkyne **41**, as a straw yellow solid (64%); mp = 116–118 °C; R_f = 0.28 (2:3 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.51 (s, 6H, 2 × CH_3), 2.03–2.10 (m, 1H, $H_{4'}/H_{5'}$), 2.50–2.64 (m, 2H, $H_{4'}/H_{5'}$), 2.83–2.93 (m, 1H, $H_{4'}/H_{5'}$), 5.15 (dd, J = 12.2 and 5.6 Hz, 1H, $H_{3'}$), 5.58 (s, 1H, OH), 7.79–7.90 (m, 3H, Ar-H), 11.15 (s, 1H, N–H); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 21.9 ($C_{4'}/C_{5'}$, CH_2), 30.9 ($C_{4'}/C_{5'}$ CH_2), 21.3 (2 × CH_3), 48.9 ($C_{3'}$, CH), 63.8 (COH), 75.9 (C≡C), 103.2 (C≡C), 119.3 (Ar-C), 122.9 (Ar-CH), 130.3 (Ar-C), 132.0 (Ar-C), 134.7 (Ar-CH), 137.9 (Ar-CH), 165.6 (C=O), 166.3 (C=O), 169.8 (C=O), 172.7 (C=O); IR (KBr) $\tilde{\nu}$: 3421 (O–H), 1718 (C=O), 1394, 1198 (O–H) cm^{-1}; MS EI, m/z (%): 340 (100) [M]$^{\cdot+}$, 323 (100) [M−CH_3]$^{\cdot+}$, 262 (28), 237 (21); EI-HRMS calcd for $C_{18}H_{16}N_2O_5$: 340.1059; found: 340.1061.

7.3.23. (*R,S*)-6-(2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)hex-5-ynenitrile (42)

Compound **42** was prepared using general protocol for the Sonogashira reaction using 5-hexynylnitrile. Flash column chromatography (1:1 ethyl acetate/hexane) and recystallisation from acetonitrile afforded the title alkynyl compound **42** as a white solid (21%). mp = 191–193 °C. R_f = 0.33 (1:1 ethyl acetate/hexane); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.90 (t, J = 7.2 Hz, 2H, $H_{4''}$), 2.03–2.09 (m, 1H, $H_{4'}/H_{5'}$), 2.51–2.60 (m, 2H, $H_{4'}/H_{5'}$), 2.66 (t, J = 6.7 Hz, 2H, $H_{5''}$), 2.77 (t, J = 7.2 Hz, 2H, $H_{3''}$), 2.90 (m, 1H, $H_{4'}/H_{5'}$), 5.16 (dd, J = 12.6 and 5.1 Hz, 1H, $H_{3'}$), 7.83–7.90 (m, 3H, Ar-CH), 11.13 (s, 1H, NH); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 15.3 ($C_{4''}$, CH_2), 18.2 ($C_{3''}$, CH_2), 21.9 ($C_{4'}/C_{5'}$, CH_2), 23.8 ($C_{5''}$, CH_2), 30.9 ($C_{4'}/C_{5'}$, CH_2), 49.0 ($C_{3'}$, CH), 77.2 (C≡C), 96.5 (C≡C), 119.5 (C≡N), 120.2 (Ar-C), 122.9 (Ar-CH), 130.3 (Ar-C), 132.0 (Ar-C), 134.7 (Ar-C), 138.2 (Ar-CH), 165.9 (C=O), 166.3 (C=O), 169.8 (C=O), 172.8 (C=O); IR (KBr) $\tilde{\nu}$: 3425 (N–H), 2922, 2246, 1715 (C=O), 1391, 1203, 744 cm^{-1}; MS EI, m/z (%): 349 (12) [M]$^{\cdot+}$, 321 (100) [M−H_2CN]$^{\cdot+}$, 235 (25); EI-HRMS calcd for $C_{19}H_{15}N_3O_4$: 349.1063; found: 349.1071.

7.3.24. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-4-(3-hydroxyprop-1-ynyl)isoindoline-1,3-dione (43)

Compound **43** was prepared using general protocol for the Sonogashira reaction using propargyl alcohol. Column chromatography (1:3 ethyl acetate/hexane) afforded the title alkyne **43**, as a dark yellow solid (78%); mp = 214–217 °C; R_f = 0.21 (1:3 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.00–2.05 (m, 1H, $H_{4'}/H_{5'}$), 2.50–2.81 (m, 2H, $H_{4'}/H_{5'}$), 2.81–2.88 (m, 1H, $H_{4'}/H_{5'}$), 4.35 (d, J = 5.6 Hz, 2H, CH_2OH), 5.10 (dd, J = 12.4 and 5.2 Hz, 1H, $H_{3'}$), 5.44 (t, J = 5.6 Hz, 1H, OH), 7.83–7.87 (m, 4H, Ar-CH), 11.09 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 22.3 ($C_{4'}/C_{5'}$, CH_2), 31.3 ($C_{4'}/C_{5'}$, CH_2), 49.4 ($C_{3'}$, CH), 49.9 (CH_2OH), 79.3 (C≡CCH_2OH), 97.6 (C≡CCH_2OH), 119.5 (Ar-C), 123.6 (Ar-CH), 130.6 (Ar-C), 132.5 (Ar-C), 135.2 (Ar-CH), 138.7 (Ar-CH), 166.0 (C=O), 166.6 (C=O), 170.2 (C=O), 173.1 (C=O); IR (KBr) $\tilde{\nu}$: 3464 (O–H), 3283 (N–H), 2225 (C≡C), 1774, 1711 (C=O), 1384, 1206, 1037, 748 cm^{-1}; MS EI, m/z (%): 312 (23) [M]$^{\cdot+}$, 284 (28), 283 (100), 200 (24), 198 (33), 184 (20); EI-HRMS calcd for $C_{16}H_{11}N_2O_5$: 312.0746; found: 312.0751.

7.3.25. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-4-phenylethynylisoindole-1,3-dione (44)

Compound **44** was prepared using general protocol for the Sonogashira reaction using phenylacetylene. Column chromatography (1:10→1:1 ethyl acetate/hexane) afforded compound **44** (61%) as a tan solid; R_f = 0.2 (1:1 ethyl acetate/hexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.21–2.13 (m, 1H, $H_{4'}/H_{5'}$), 2.97–2.69 (m, 3H, $H_{4'}/H_{5'}$), 5.02 (dd, J = 12.6, 5.4 Hz, 1H, $H_{3'}$), 7.41–7.37 (m, 2H, Ar-H), 7.75–7.63 (m, 4H, Ar-H), 7.85–7.82 (m, 2H, Ar-H), 8.01 (s, 1H, NH); ^{13}C NMR (100.5 MHz, $CDCl_3$): 22.8 (C4′/C5′), 31.6 (C4′/C5′), 49.5 (C3′), 84.5 (C≡C), 97.6 (C≡C), 121.2 (Ar-C), 122.3 (Ar-C), 123.2 (Ar-CH), 128.6 (Ar-CH), 129.5 (Ar-CH), 132.3 (Ar-CH), 134.1 (Ar-CH), 138.1 (Ar-CH), 166.0 (C=O), 166.6 (C=O), 167.9 (C=O), 170.8 (C=O); IR (NaCl): 3275 (N–H), 2214 (C≡C), 1711 (C=O), 1388, 1203, 745 cm^{-1}; MS (m/z) = 358 (100) [M]$^{\cdot+}$, 273 (20), 176 (21); EI-HRMS calcd for $C_{21}H_{14}N_2O_4$: 358.0954; found: 358.0954.

7.3.26. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-5-(3-hydroxy-3-methylbut-1-ynyl)isoindoline-1,3-dione (45)

Compound **45** was prepared using general protocol for the Sonogashira reaction using 2-methyl-3-butyn-2-ol. Flash column chromatography (4:1 ethyl acetate/hexane) afforded the alkyne **45**, as a yellow solid (76%). Mp = 62–65 °C; R_f = 0.28 (4:1 ethyl acetate/hexane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.51 (s, 6H, 2 × CH_3), 2.04–2.08 (m, 1H, $H_{4'}/H_{5'}$), 2.50–2.62 (m, 2H, $H_{4'}/H_{5'}$), 2.85–2.88 (m, 1H, $H_{4'}/H_{5'}$), 5.15 (dd, J = 12.8 and 5.2 Hz, 1H, $H_{3'}$), 5.58 (s, 1H, OH), 7.79–7.90 (m, 3H, Ar-H), 11.12 (s, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 21.5 ($C_{4'}/C_{5'}$, CH_2), 30.5 ($C_{4'}/C_{5'}$, CH_2), 30.8 (2 × CH_3), 48.5 ($C_{3'}$, CH), 63.4 (COH), 75.5 (C≡C), 102.8 (C≡C), 118.9 (Ar-C), 122.5 (Ar-CH), 129.8 (Ar-C), 131.6 (Ar-C), 134.3 (Ar-CH), 137.5 (Ar-CH), 165.2 (C=O), 165.8 (C=O), 169.4 (C=O), 172.3 (C=O); IR (KBr,) $\tilde{\nu}$: 3415 (O–H), 2980 (N–H), 2226 (C≡C), 1776, 1716 (C=O), 1384, 1201 (C–O) cm^{-1}; MS EI, m/z (%): 340 (10) [M]$^{\cdot+}$, 325 (100) [M−CH_3]$^{\cdot+}$, 262 (90); EI-HRMS calcd for $C_{18}H_{16}N_2O_5$: 340.1059; found: 340.1044.

7.3.27. (*R,S*)-2-(2,6-Dioxopiperidin-3-yl)-5-(3-(methylamino)-prop-1-ynyl)isoindoline-1,3-dione (46)

Compound **46** was prepared using general protocol for the Sonogashira reaction using *N*-methylpropargylamine. Flash column chromatography (1:9 methanol/dichloromethane) afforded the alkyne **46**, as a brown solid (21%); mp = >230 °C; R_f = 0.31 (1:9 methanol/dichloromethane); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.01–2.08 (m, 1H, $H_{4'}/H_{5'}$), 2.39 (s, 3H, CH_3), 2.50–2.59 (m, 2H, $H_{4'}/H_{5'}$), 2.84–2.95 (m, 1H, $H_{4'}/H_{5'}$), 3.58 (s, 2H, CH_2), 5.16 (dd, J = 12.8 and 5.6 Hz, 1H, $H_{3'}$), 7.84–7.92 (m, 3H, Ar-H), 11.12 (brs, 1H, NH); ^{13}C NMR (100.5 MHz, DMSO-d_6): δ = 21.9 ($C_{4'}/C_{5'}$, CH_2), 30.9 ($C_{4'}/C_{5'}$, CH_2), 34.7 (CH_3), 49.1 ($C_{3'}$, CH), 81.8 (C≡C), 93.6 (C≡C), 123.8 (Ar-CH), 125.6 (Ar-CH), 129.0 (Ar-C), 130.1 (Ar-C), 132.8 (Ar-C), 137.4 (Ar-CH), 166.4 (C=O), 166.5 (C=O), 169.8 (C=O), 172.7 (C=O); IR (KBr) $\tilde{\nu}$: 3423 (N–H), 2224 (C≡C), 1776, 1718 (C=O), 1383, 1204, 1117 cm^{-1}; MS EI, m/z (%): 324 (100) [M]$^{\cdot+}$, 262 (79), 239 (19); EI-HRMS calcd for $C_{17}H_{15}N_3O_4$: 324.0984; found: 324.0989.

7.3.28. (*R,S*)-6-(2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-5-yl)hex-5-ynenitrile (47)

Compound **47** was prepared using general protocol for the Sonogashira reaction using 5-hexynylnitrile. Flash column chromatography (1:5→9:20 ethyl acetate/hexane) afforded the title alkynyl compound **47**, as a light yellow solid (64%); mp = 180–182 °C. R_f = 0.26 (9:20 ethyl acetate/hexane); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 1.89 (t, J = 6.9 Hz, 2H, $H_{4''}$), 2.04 (m, 1H, $H_{4'}/H_{5'}$), 2.52–2.72 (m, 6H, $H_{4'}/H_{5'}$, $H_{3''}$, $H_{5''}$), 2.94 (m, 1H, $H_{4'}/H_{5'}$), 5.15 (dd, J = 12.6 and 5.1 Hz, 1H, $H_{3'}$), 7.91–7.95 (m, 3H, Ar-CH), 11.14 (s, 1H, NH); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 15.6 ($C_{4''}$), 18.1 ($C_{3''}$), 21.9 ($C_{4'}/C_{5'}$), 23.7 ($C_{5''}$), 30.9 ($C_{4'}/C_{5'}$), 49.1 ($C_{3'}$), 80.3 (C≡C), 93.8 (C≡C), 120.3 (C≡N), 123.7 (Ar-CH), 125.8 (Ar-CH), 129.3 (Ar-C), 130.0 (Ar-C), 131.7 (Ar-C), 137.4 (Ar-CH), 166.4 (C=O), 166.5 (C=O), 169.7 (C=O), 172.7 (C=O); IR (KBr) $\tilde{\nu}$: 3417 (N–H), 2922, 2246 (C≡C), 1389, 1204 cm^{-1}; MS EI, m/z (%): 349 (82) [M]$^{\cdot+}$, 321 (15) [M−H_2CN]$^{\cdot+}$, 264 (100); EI-HRMS calcd for $C_{19}H_{15}N_3O_4$: 349.1063; found: 349.1068.

8. Biological activity assays

8.1. TNF reporter gene assay

The effect of each compound on the inhibition of TNF expression and cellular viability was assessed using the FRT-Jurkat TNF reporter cell line. Analysis was done using a FACSCalibur 4-colour Flow Cytometer (Becton, Dickinson and Company, New Jersey, USA). Data analysis was performed using FlowJo software (Tree-Star, Ashland, OR, USA). Viable and non-viable cells present following either solvent (DMSO) alone or compound treatment for 24 h, were assayed using flow cytometry. Cells were counted by gating on each cell population using a forward scatter (FSC) versus side scatter (SSC) plot. Inhibition was determined as a decrease in GFP fluorescence which was detected at a wavelength of 515 nm on the FL3 channel of the instrument. 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 cells. Viability was confirmed following propidium iodide staining and detection of flourescence at 570 nm on the FL2 channel. For detection of cells undergoing apoptosis, following treatment with each compound, cells were treated with fluorescein isothiocyanate (FITC)-labeled substrate against cleaved caspase 3 using the Nucview-488 caspase 3 assay (Biotium Inc., Hayward, USA). FITC fluorescence was quantitated on channel FL3 of the flow cytometer.

8.2. TNF mRNA quantitative PCR assay

PBMCs were isolated from leukocyte buffy coat (Australian Red Cross Blood Service) from healthy donors by Ficoll-Hypaque gradient centrifugation and maintained in RPMI 1640 with 10% FCS. Cells were stimulated to express TNF using PMA (20 ng/mL) and ionomycin (0.75 mg/mL). Total RNA was isolated using an RNeasy RNA isolation kit (Qiagen, Valencia CA). Reverse transcription with Superscript II (Invitrogen Inc, Carlsbad, CA) was done and the cDNA was used as a template for quantitative real-time PCR. The TNF primer pair used was 5′-CGAGTGACAAGCCTGTAG-3′ and 5′-GAGCCAGAAGAGGTTGAG-3′. Normalization was performed with the housekeeping gene β-actin as previously described.[39] The thermal cycler conditions were 50 cycles of 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 20 s. Data were analysed using Bio-Rad CFX Manager. Statistical significance was determined by student's unpaired *t*-test.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.12.001.

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