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Synthesis and evaluation of 3-(benzylthio)-5-(1*H*-indol-3-yl)-1,2,4-triazol-4-amines as Bcl-2 inhibitory anticancer agents

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ABSTRACT

A series of substituted 3-(benzylthio)-5-(1*H*-indol-3-yl)-4*H*-1,2,4-triazol-4-amines has been synthesised and tested in vitro as potential pro-apoptotic Bcl-2-inhibitory anticancer agents. Synthesis of the target compounds was readily accomplished in good yields through a cyclisation reaction between indole-3-carboxylic acid hydrazide and carbon disulfide under basic conditions, followed by S-benzylation. Active compounds, such as the nitrobenzyl analogue **6c**, were found to exhibit sub-micromolar IC_{50} values in Bcl-2 expressing human cancer cell lines. Molecular modelling and ELISA studies further implicated anti-apoptotic Bcl-2 as a candidate molecular target underpinning anticancer activity.

The intracellular balance between pro- and anti-apoptotic Bcl-2 family proteins is critical for regulation between programmed cell death and cell survival respectively.[1] For example in cancer cells, where resistance to apoptotic cell death is a well documented hallmark,[2] over-expression of anti-apoptotic family members such as the prototypical Bcl-2 protein is well known.[3] Hence inhibition of anti-apoptotic Bcl-2 family members has emerged in recent years as an exciting anticancer drug discovery strategy, since Bcl-2 inhibition should lead to a selective pro-apoptotic cascade from which cancer cells cannot easily recover.

Cancer drug design strategies towards the Bcl-2 family have tended to focus on sites of protein–protein interaction between anti-apoptotic Bcl-2 members (e.g., Bcl-2, Bcl-xL, Mcl-1) and their pro-apoptotic binding partners (e.g., Bax, Bak, Bid).[4] Pioneering Bcl-2 inhibitory drug candidates within this class include the potent structural analogues ABT-737 (**1**) and ABT-263 (**2**, Navitoclax) from Abbott Laboratories that have progressed to Phase I/II clinical evaluation in cancer (Fig. 1).[5] The relatively large size of leading Bcl-2 inhibitors as drug candidates (M_r = 975 in the case of ABT-263), arising from the requirement to make interactions across a protein–protein interface,[6] has driven efforts to develop selective Bcl-2 inhibitors of lower molecular weight in recent years. The evolution of small molecules such as the pan-Bcl-2 inhibitory indole-bipyrrole drug candidate Obatoclax mesylate (**3**, GX15-070), currently in Phase I/II clinical trials,[7] suggests that structurally simple drug-like molecules may have the necessary attributes as candidate Bcl-2 family inhibitors. Our own previous work in the design of indole-based pro-apoptotic heterocycles[8] has included the recent identification of antitumour indolyl-oxadiazoles[9] and -isoxazoles.[10] In this Letter we extend our previous studies on pro-apoptotic indole-based heterocycles to the synthesis and antitumour evaluation of a series of novel 3-(benzylthio)-5-(1*H*-indol-3-yl)-4*H*-1,2,4-triazol-4-amines as Bcl-2 inhibitory anticancer agents.

The synthesis of 3-(benzylthio)-5-(1*H*-indol-3-yl)-1,2,4-triazol-4-amines was accomplished in two steps from commercially available indole 3-carboxylic acid hydrazide (**4**) according to literature precedent.[11] Briefly, commercially available compound **4** was cyclised by reaction with carbon disulfide under basic conditions, followed by hydrazine hydrate under reflux. This one-pot reaction produced the required 4-amino-5-(1*H*-indol-3-yl)-4*H*-[1,2,4]triazole-3-thiol (**5**) in 75% yield following recrystallisation from ethanol.[12] Reaction of intermediate compound **5** with a series of substituted benzyl bromides under basic conditions afforded the 3-(benzylthio)-5-(1*H*-indol-3-yl)-1,2,4-triazol-4-amine products in good yields (69–95%) following recrystallisation from ethanol (Scheme 1).[13]

Evaluation of newly synthesized 3-(benzylthio)-5-(1*H*-indol-3-yl)-1,2,4-triazol-4-amine products[14] was carried out in the established human MDA-MB-231 (breast) and HeLa (cervical) cancer cell lines using the colorimetric MTT cell proliferation assay to

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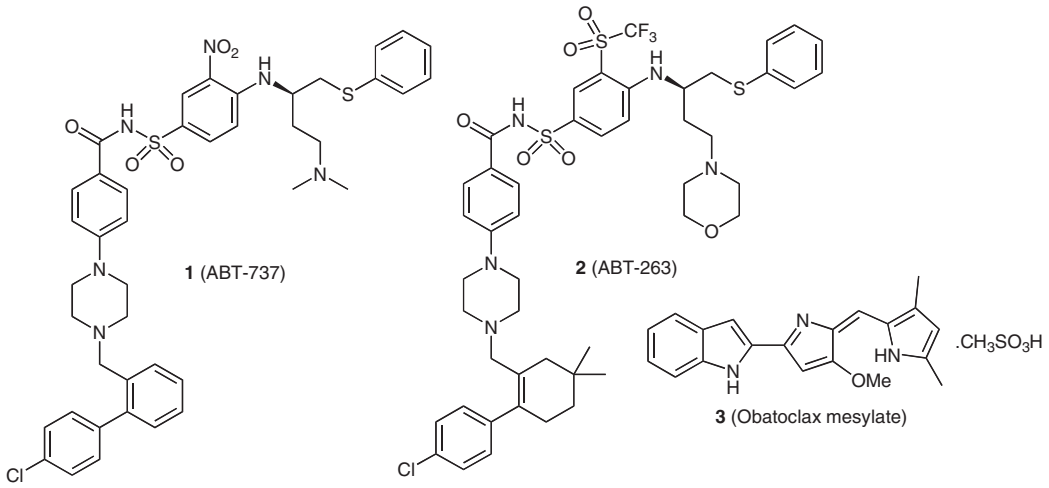
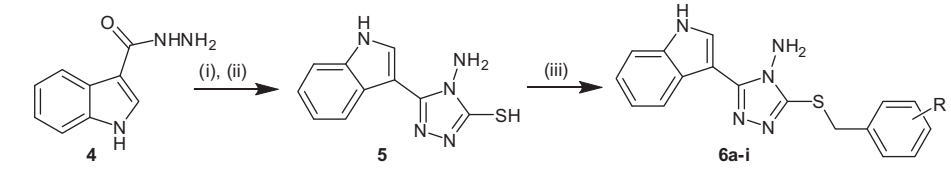


Figure 1. Examples of small molecule Bcl-2 inhibitors in development.



[a]Reagents and conditions: (i) CS_2, KOH, EtOH, room temp., 16h; (ii) $N_2H_4.H_2O$, reflux, 3h; (iii) subs. benzyl bromide, KOH, EtOH, room temp., 16h

6	R	6	R
a	H	f	4-Br
b	2-NO_2	g	3-OMe
c	3-NO_2	h	4-OMe
d	4-NO_2	i	4-CH_3
e	4-F		

Scheme 1. Synthesis of 3-(benzylthio)-5-(1*H*-indol-3-yl)-1,2,4-triazol-4-amines. [a]Reagents and conditions: (i) CS_2, KOH, EtOH, room temp, 16 h; (ii) $N_2H_4{\cdot}H_2O$, reflux, 3 h; (iii) subs. benzyl bromide, KOH, EtOH, room temp, 16 h.

assess viability, as previously described (see Supplementary data).[15] The MDA-MB-231 and HeLa human cancer cell lines are well established in our laboratory for the initial screening for growth inhibitory activity of newly synthesized compounds. The MDA-MB-231 cell line is known to express Bcl-2 and previous studies have shown down-regulation of Bcl-2 in these cells by Western blot analysis following inhibitor treatment.[16] Stable expression of Bcl-2 in the HeLa cell line is also well known.[17] The results, expressed as mean values following testing on at least three separate occasions, are presented in Table 1.

To test the effect of differing Bcl-2 status within the human cancer cell environment, further evaluation of new compounds was carried out in the leukaemic cell lines KG1a (acute myelogenous leukaemia) and Jurkat (T-cell leukaemia). The Jurkat cell line has previously been characterized as Bcl-2 negative, by our group[18] and others,[19] unlike the Bcl-2-expressing KG1a cell line.[20] For these studies we used the CellTiter-Blue® cell viability assay (see Supplementary data), appropriate for endpoint determinations in these non-adherent cell lines. The results, expressed as mean values following testing on at least three separate occasions, are presented in Table 2.

The results presented in Tables 1 and 2 indicate some clear structure–activity relationship trends. In general the HeLa cell line was the most sensitive to the effects of test compounds with IC_{50} values in the sub- to low-micromolar range (0.25–8.0 μM). The MDA-MB-231 breast cancer cell line also gave sub- to low-micro-

Table 1
Growth inhibitory activity (IC_{50}, μM) values for 3-(benzylthio)-5-(1*H*-indol-3-yl)-1,2,4-triazol-4-amine products **6a–i** in human cancer cell lines MDA-MB-231 (breast) and HeLa (cervical), using the MTT assay. Results are expressed as triplicate mean values (SEM)

Compound	MDA-MB-231	HeLa
6a	26.5 (0.3)	2.30 (0.07)
6b	9.52 (0.13)	2.52 (0.19)
6c	0.31 (0.03)	0.40 (0.07)
6d	0.74 (0.03)	0.25 (0.09)
6e	1.35 (0.09)	0.29 (0.02)
6f	71.4 (0.68)	8.00 (0.06)
6g	15.56 (0.36)	1.73 (0.07)
6h	1.70 (0.07)	0.75 (0.02)
6i	10.49 (0.59)	5.38 (0.04)

Table 2
Growth inhibitory activity (IC_{50}, μM) values for 3-(benzylthio)-5-(1*H*-indol-3-yl)-1,2,4-triazol-4-amine products **6a–i** in human leukaemia KG1a and Jurkat cell lines, using the CellTitre-Blue® assay as readout for anti-proliferative activity. Results are expressed as triplicate mean values (SEM)

Compound	KG1a	Jurkat
6a	17.2 (0.23)	>100
6b	20.82 (0.69)	>100
6c	0.65 (0.21)	>100
6d	6.90 (0.18)	>100
6e	13.2 (0.07)	>100
6f	55.80 (0.68)	>100
6g	12.86 (0.14)	>100
6h	>100	>100
6i	>100	>100

molar IC_{50} values for the majority of test compounds. Although the Bcl-2-expressing KG1a acute myelogenous leukaemia cell line was generally less responsive to test agents, IC_{50} values in the low micromolar range were observed, particularly for compounds **6a–e** and **6g**. Particularly noteworthy (Table 2) was that anti-proliferative activity was concentrated within the Bcl-2-expressing KG1a leukaemic cell line, whereas all compounds were found to be inactive in the Bcl-2 negative human T-cell leukaemia Jurkat cells. The data from Table 2 suggests that Bcl-2 may play a role in mediating the observed anticancer activity.

The most active compound overall was the 3-nitrobenzyl derivative **6c** that shows potent (sub-micromolar IC_{50}) values across the three Bcl-2-expressing cancer cell lines (MDA-MB-231, HeLa and KG1a). Also active was the isomeric 4-nitrobenzyl analogue **6d**, however a marked drop in activity was observed for the 2-nitrobenzyl derivative **6b**. A further notable compound showing sub- to low-micromolar activity across the three Bcl-2 expressing cell lines was the 4-fluorobenzyl analogue **6e**.

Further evidence that Bcl-2 might be responsible for the observed anticancer activity of the new triazole-amines such as the 3-nitrobenzylthio derivative **6c** was gained through molecular modelling studies. A comparison of interactions between compound **6c** and several less active analogues (e.g., **6b**, **6d**, **6f**, **6g** and **6i**) with a published ligand-bound Bcl-2 crystal structure[21] (PDB code: 4AQ3; BH3 domain)[22] were studied using MOE[23] and the Leadit molecular docking software.[24] The BH3 domain of the Bcl-2 model consists of one hydrophobic and one hydrophilic groove connected by hydrophobic amino acid residues. Compound **6c** was found to bind within the pro-apoptotic BH3-only peptide hydrophobic groove of Bcl-2, making a number of key binding interactions particularly between the Bcl-2 side chain residue Tyr-67 and the 4-amino and the sulfur atoms of **6c**. The 2-nitrobenzyl substituted compound **6b** (and other less active analogues) adopted a less favourable binding conformation in the Bcl-2 active site. Compound **6b** docked further away from the active site and was unable to make key binding interactions with important active site residues such as Tyr-67. Figure 2 shows the comparative binding interactions between compounds **6c** and **6b** within the Bcl-2 active site.

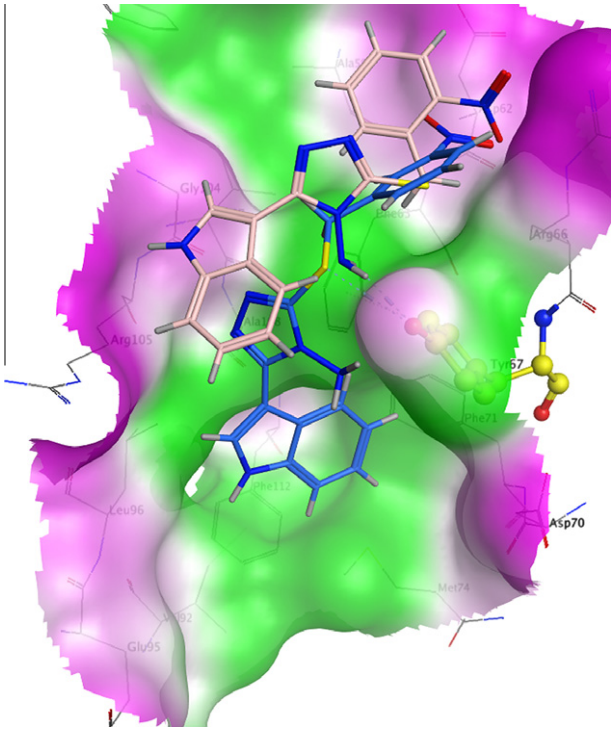


Figure 2. 3-(3-Nitrobenzylthio)-5-(1*H*-indol-3-yl)-1,2,4-triazol-4-amine (**6c**, blue) and the corresponding less active 2-nitrobenzyl analogue (**6b**, salmon) binding in the Bcl-2 active site.

The ability of compound **6c** to compete with the pro-apoptotic BH3 (Bcl-2 homology domain 3) Bim peptide for binding to Bcl-2 was evaluated using an ELISA assay, adapted from a previously published protocol (see Supplementary data for details).[25] In brief, biotinylated Bim peptide was attached to streptavidin-coated microtitre plates. Mixtures of test compound **6c** at varying concentrations and His-tagged Bcl-2 were then added to the plates, allowing competitive binding between **6c** and Bim peptide for His-tagged Bcl-2. Following further washing, anti-His secondary antibody conjugated to the enzyme horse-radish peroxidase (HRP) was added. Addition of *o*-phenylenediamine, a chromogenic substrate that produces a soluble coloured end-product (2,3-diaminophenazine) in the presence of peroxidase enzyme, was followed by spectrophotometric reading at 450 nm. Reduction of the signal at 450 nm by competitive binding of compound **6c** to Bcl-2, is then plotted against concentration to derive an IC_{50} value for **6c**. Triplicate measurement of the ELISA IC_{50} gave a mean value of 1.43 μM (SEM = 0.19), compared with an IC_{50} of 2.11 μM for the standard Bcl-2 inhibitor gossypol, a natural product-based agent currently in clinical trials against cancer.[26]

In summary, the design and synthesis of a series 3-(benzylthio)-5-(*1H*-indol-3-yl)-*4H*-1,2,4-triazol-4-amines as potential anticancer agents targeting Bcl-2 has lead to the discovery of a number of compounds with sub-micromolar IC_{50} activity in Bcl-2 expressing human cancer cell lines. The 3-nitrobenzyl derivative **6c** was identified as the most active compound of the series. Compound **6c** was further found to bind within the BH3 domain of Bcl-2, according to molecular modelling and ELISA studies, providing support for the prototypical anti-apoptotic protein Bcl-2 as a potential mechanistic target underpinning in vitro anticancer activity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.02.029.

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12. *Synthesis of 4-amino-5-(1H-indol-3-yl)-4H-[1,2,4]triazole-3-thiol (***5***).* Carbon disulfide (12 mL) was added dropwise to a mixture of indole-3-carboxylic acid hydrazide (10 mmol) and potassium hydroxide (15 mmol) in absolute ethanol (20 mL), following by stirring at room temperature for 16 h. Diethyl ether (30 mL) was added and the precipitate collected by vacuum filtration. Hydrazine hydrate (20 mmol) and water (20 mL) were then added and the reaction mixture heated under reflux for 3 h. After cooling, the mixture was diluted with water (10 mL) and neutralized using 1 M HCl (aq). The crude product precipitate was collected by vacuum filtration and washed with cold water. Recrystallisation from ethanol gave the required 4-amino-5-(1*H*-indol-3-yl)-4H-[1,2,4]triazole-3-thiol product in 75% yield, which was used immediately in the next step.
13. *General method for S-benzylation of 4-amino-5-(1H-indol-3-yl)-4H-[1,2,4]triazole-3-thiol.* A solution of substituted benzyl bromide (10 mmol) in absolute ethanol (10 mL) was added to a solution of intermediate thiol (**5**, 10 mmol) and potassium hydroxide (1 mmol) in ethanol (10 mL). The reaction mixture was stirred for 16 h at room temperature, then the resulting precipitate was collected by vacuum filtration and recrystallised from ethanol to give the required 3-(benzylthio)-5-(1*H*-indol-3-yl)-1,2,4-triazol-4-amines (**6a–i**) in 69–95% yield.
14. *Representative characterization data: (**a**) 5-(1H-Indol-3-yl)-3-(benzylthio)-4H-1,2,4-triazol-4-amine (***6a***):* (76% yield). Mp 235–237 °C. ^{1}H NMR (DMSO-d_6) δ 4.43 (2H, s, CH_2), 6.06 (2H, s, NH_2), 7.16 (1H, t, *J* 8.0, ArH), 7.20 (1H, t, *J* 8.0, ArH), 7.27 (1H, t, *J* 8.0, ArH), 7.34 (2H, m, ArH), 7.48 (3H, m, ArH), 8.26 (2H, d, *J* 7.1, ArH), 11.65 (1H, s, NH indole). ^{13}C NMR (DMSO-d_6) δ 35.13 (CH_2), 101.71 (C-3), 111.62 (ArCH), 120.08 (ArCH), 121.33 (ArCH), 122.12 (ArCH), 125.35 (ArC), 125.63 (ArCH), 127.26 (ArCH), 128.38 (ArCH), 129.00 (ArCH), 135.63 (ArC), 137.71 (ArC), 150.71 (ArC), 151.77 (ArC). MS (ESI$^+$) 322 (M^++1). Anal. Calcd for $C_{17}H_{15}N_5S$: C, 63.53; H, 4.70; N, 21.78. Found: C, 63.37; H, 4.71; N, 22.00. (***b***) *5-(1H-Indol-3-yl)-3-(3-nitrobenzylthio)-4H-1,2,4-triazol-4-amine (***6c***)* (85% yield). Mp 209–211 °C. ^{1}H NMR (DMSO-d_6) δ 4.58 (2H, s, CH_2), 6.12 (2H, s, NH_2), 7.16 (1H, t, *J* 8.0, ArH), 7.20 (1H, t, *J* 8.0, ArH), 7.49 (1H, d, *J* 7.9, ArH), 7.63 (1H, t, ArH), 7.95 (1H, d, *J* 6.3, ArH), 8.13 (1H, d, *J* 6.7, ArH), 8.25 (2H, m, ArH), 8.39 (1H, d, *J* 2.2, H-2′), 11.65 (1H, s, NH indole). ^{13}C NMR (DMSO-d_6) δ 33.84 (CH_2), 101.62 (C-3), 111.64 (ArCH), 120.12 (ArCH), 121.30 (ArCH), 122.13 (ArCH), 122.15 (ArCH), 123.61 (ArCH), 125.33 (ArC), 125.70 (ArCH), 129.75 (ArCH), 135.64 (ArC), 135.79 (ArCH), 140.75 (ArC), 147.66 (ArC), 150.35 (ArC), 151.98 (ArC). MS (ESI$^-$) 365 (M^+−1). Anal. Calcd for $C_{17}H_{15}N_5S$: C, 55.73; H, 3.85; N, 22.94. Found: C, 55.23; H, 3.85; N, 22.88.
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