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# Synthesis of a rhodanine-based compound library targeting Bcl-XL and Mcl-1*

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*Abstract*: A small library of pyridine-based rhodanine analogues of BH3I-1 were synthesized and screened against B-cell lymphoma-extra large (Bcl-XL) and myeloid cell leukemia sequence 1 (Mcl-1) for the ability to displace 5-carboxyfluorescein-labeled Bak peptide (Flu-Bak). Differences in selectivity toward Bcl-XL and Mcl-1 were observed, and the binding modes of selected compounds were studied further. The results may be useful in designing potent small-molecule inhibitors of Bcl-XL and Mcl-1 as well as selective Mcl-1 inhibitors.

*Keywords*: docking studies; drug design; B-cell lymphoma-extra large (Bcl-XL); myeloid cell leukemia sequence 1 (Mcl-1); rhodanine.

## INTRODUCTION

The antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-XL, Mcl-1, A1) are attractive targets for cancer chemotherapy. It has been shown that cancer cells overexpress one or more of these proteins to prevent the induction of apoptosis or programmed cell death [1–4]. These proteins confer protection on cancer cells by sequestering the proapoptotic proteins Bax and Bak [5]. Neutralizing these antiapoptotic proteins using natural peptides (e.g., Bim, Bid, NOXA) releases Bax and Bak which then aggregate to form transmembrane channels that result in mitochondrial permeability [1]. The compromised mitochondria can then release apoptogenetic factors such as cytochrome C and apoptosis-inducing factor (AIF) which

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eventually leads to cell death. Over the years, several molecules such as ABT-737 (**1**) [6], epigallocatechin gallate (**2**) [7–10], and Obatoclax (**3**) [11] have been reported to inhibit the antiapoptotic Bcl-2 proteins and trigger apoptosis in cancer cells (Fig. 1). [2] The compound BH3I-1 (**4**) is also a well-known inhibitor of the Bcl-2 proteins [2,12]. It has been reported that modification of **4** can result in varied binding profiles to Bcl-XL with an increase in efficacy [13,14]. Such modifications can theoretically be exploited in the development of selective or universal inhibitors of the Bcl-2 proteins. Furthermore, studies within our own group have shown that **4** binds in close proximity to the benzo*[c]*phenanthridine sanguinarine (**5**) on Bcl-XL [15–17], and thus it was envisaged that combining key structural motifs from both compounds may result in even more potent inhibitors of Bcl-XL (Fig. 2). In this study, we report our efforts directed at the synthesis of such hybrid compounds. This study identified several structures which are selective inhibitors of Mcl-1 as well as dual inhibitors of Bcl-XL and Mcl-1, and provided some insights into structural determinants that determine binding to Bcl-XL and Mcl-1.

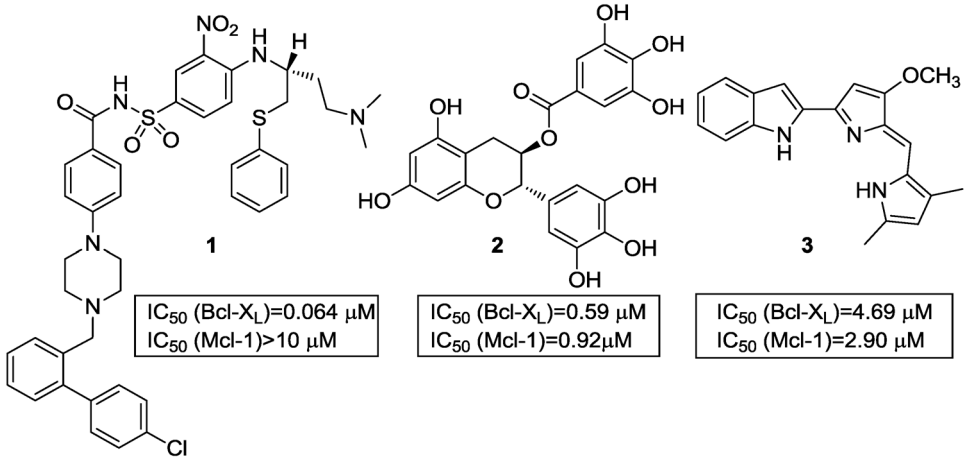


**Fig. 1** Selected inhibitors and the $IC_{50}$ values measured by FPA against the FITC-labeled Bid-BH3 peptide.

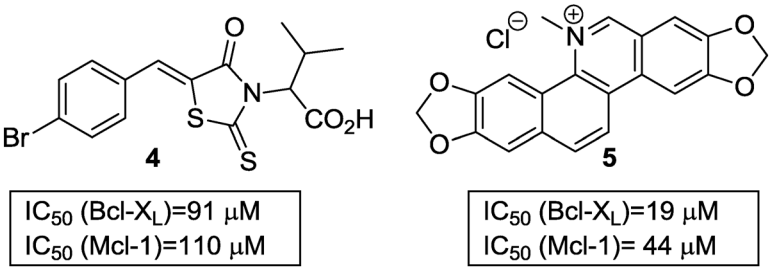


**Fig. 2** $IC_{50}$ values of BH3I-1 (**4**) and sanguinarine (**5**) measured by FPA against the Flu-Bak-BH3 peptide.

## RESULTS AND DISCUSSION

### Synthesis

The synthesis of the pyridine-based rhodanine compounds is outlined in Fig. 3. The convergent synthetic route enabled the rapid construction of a small compound library of the arylrhodanines. The rhodanines were synthesized from the natural amino acids **6a–f** (glycine, alanine, valine, leucine,

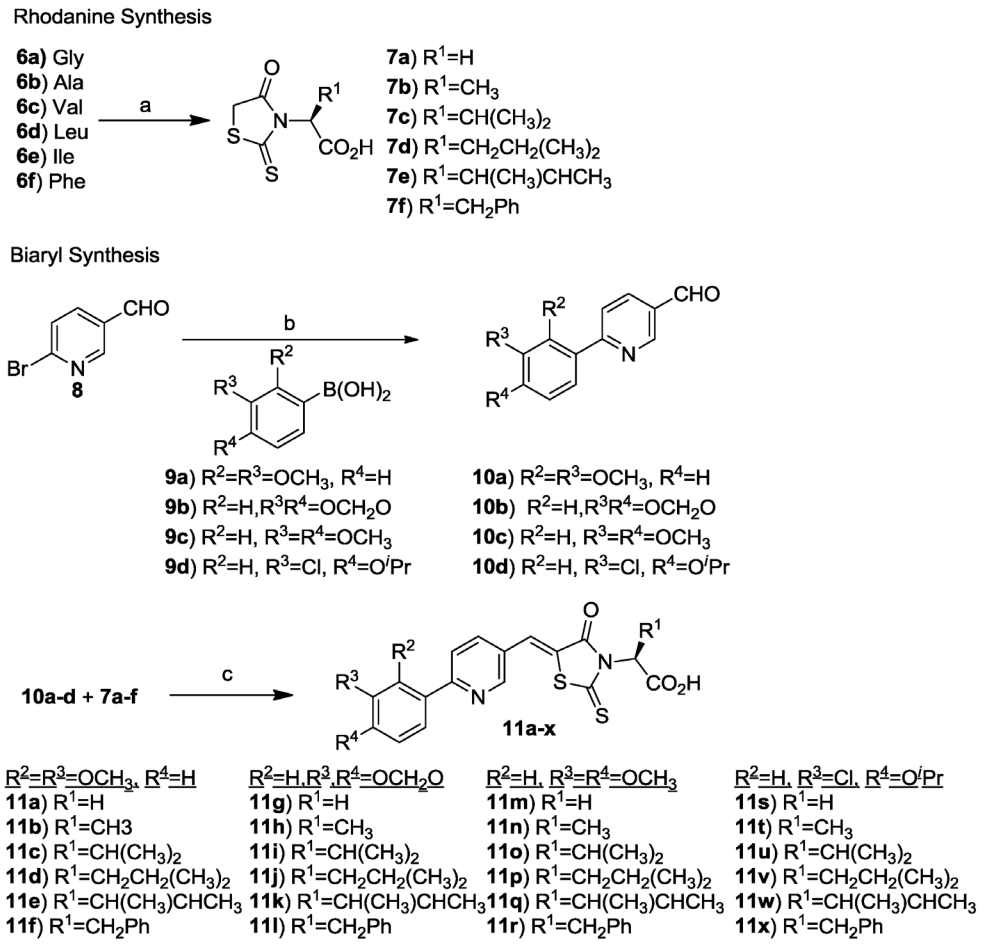


**Fig. 3** Reaction conditions: (a) 1 equiv $CS_2$, 2 equiv NaOH, EtOH, rt, 16 h then 1 equiv $ClCH_2CO_2Na$, 3 h, then 6 M HCl, reflux 16 h, 80–92 % yield. (b) 5 mol % $Pd(OAc)_2$, 10 mol % $PCy_3HBF_4$, $K_3PO_4$ (2 equiv in 1 mL $H_2O$), 1,4-dioxane, 100 °C, 16 h, 77–97 % yield. (c) 4 equiv NaOAc, glacial AcOH, 140 °C, 3 h, 65–98 % yield.

isoleucine, and phenylalanine) following a literature procedure [18]. The rhodanines **7a–f** were recrystallized from EtOH in 80–92 % yield.

The Suzuki–Miyaura reaction was employed to couple 2-bromo-5-formylpyridine (**8**) with commercially available boronic acids **9a–d**. Catalytic $Pd_2(dba)_3$ and $PCy_3HBF_4$ were utilized for the coupling reaction using a procedure reported by Handy [19]. The desired biaryls **10a–d** were obtained in 77–97 % yield.

Knoevenagel condensation of the 2-aryl-3-formylpyridines **10a–d** with the corresponding rhodanines **7a–f** in buffered acetic acid furnished the desired pyridylrhodanines **11a–x** in 65–98 % yield. Initially, column chromatography was employed to purify the compounds, but this led to poor yields and loss of material. An alternative purification method was utilized where the crude material was refluxed in 1 M HCl, then cooled and the precipitate was collected via filtration. Drying the material overnight at 80 °C under vacuum led to high yields (>90 %) of analytically pure material.

 

### Fluorescence polarization assay (FPA)

The compound library was screened using FPA against the Flu-Bak and the proteins Bcl-XL and Mcl-1. The inhibition constants ($K_i$), derived from the $IC_{50}$ values obtained by FPA, were calculated using an online calculator [20], and the values are listed in Table 1. Conversion of the $IC_{50}$ values to $K_i$ is essential as the binding of the Flu-Bak peptide differs in each protein ($K_d$ = 0.14 μM for Bcl-XL, 1.6 μM for Mcl-1). The $K_i$ values allow a direct comparison for the binding of the compounds to Bcl-XL and Mcl-1.

**Table 1** $K_i$ values with the standard error of the mean value (SEM) obtained via FPA for the interaction of the rhodanine compounds with Bcl-XL and Mcl-1. (–) indicates no binding observed within the 100 μM range.

| Compound | $K_i$ Bcl-XL μM | $K_i$ Mcl-1 μM | Compound | $K_i$ Bcl-XL μM | $K_i$ Mcl-1 μM |
|---|---|---|---|---|---|
| $R_2 = R_3 = OCH_3$, $R_4 = H$ | | | $R_2 = H$, $R_3 = R_4 = OCH_3$ | | |
| **11a)** $R_1 = H$ | – | 46 ± 2 | **11m)** $R_1 = H$ | – | – |
| **11b)** $R_1 = CH_3$ | – | 30 ± 2 | **11n)** $R_1 = CH_3$ | – | – |
| **11c)** $R_1 = CH(CH_3)_2$ | 8.1 ± 0.4 | 25 ± 1 | **11o)** $R_1 = CH(CH_3)_2$ | – | 32 ± 3 |
| **11d)** $R_1 = CH_2CH(CH_3)_2$ | 6.8 ± 0.6 | 17 ± 1 | **11p)** $R_1 = CH_2CH(CH_3)_2$ | 6.7 ± 0.5 | 27 ± 1 |
| **11e)** $R_1 = CH(CH_3)CH_2CH_3$ | 6.8 ± 0.6 | 14 ± 1 | **11q)** $R_1 = CH(CH_3)CH_2CH_3$ | 5.4 ± 0.3 | 11.7 ± 0.5 |
| **11f)** $R_1 = CH_2Ph$ | 3.7 ± 0.2 | 7.6 ± 0.2 | **11r)** $R_1 = CH_2Ph$ | – | 8.5 ± 0.4 |
| $R_2 = H$, $R_3$, $R_4 = OCH_2O$ | | | $R_2 = H$, $R_3 = Cl$, $R_4 = O^iPr$ | | |
| **11g)** $R_1 = H$ | – | – | **11s)** $R_1 = H$ | – | 25 ± 1 |
| **11h)** $R_1 = CH_3$ | – | 56 ± 7 | **11t)** $R_1 = CH_3$ | – | 24.0 ± 0.7 |
| **11i)** $R_1 = CH(CH_3)_2$ | 8 ± 1 | 36 ± 4 | **11u)** $R_1 = CH(CH_3)_2$ | 8.4 ± 0.1 | 24 ± 3 |
| **11j)** $R_1 = CH_2CH(CH_3)_2$ | 5.4 ± 0.2 | 30 ± 3 | **11v)** $R_1 = CH_2CH(CH_3)_2$ | 3.7 ± 0.2 | 9.7 ± 0.4 |
| **11k)** $R_1 = CH(CH_3)CH_2CH_3$ | 3.6 ± 0.2 | 13.8 ± 0.4 | **11w)** $R_1 = CH(CH_3)CH_2CH_3$ | 3.6 ± 0.1 | 4.8 ± 0.2 |
| **11l)** $R_1 = CH_2Ph$ | 4.8 ± 0.1 | 8.1 ± 0.3 | **11x)** $R_1 = CH_2Ph$ | 7.0 ± 0.3 | 8.5 ± 0.1 |

The results show that the glycyl and alanyl derivatives (**11a,b,g,h,m,n,s,t**) do not bind to Bcl-XL. Compounds **11a,b,h,s,t**, however, show moderate binding to Mcl-1. Compounds **11c–f,i–l,p–q**, and **11u,v** bind stronger to Bcl-XL than Mcl-1 with 2-fold selectivity or better, whereas **11w,x** binds both proteins equally well within experimental error. The compounds **11f,k,v,w** show the best binding activity to Bcl-XL with $K_i$ ~ 4 μM. With respect to Mcl-1, **11w** shows the best binding activity ($K_i$ = 4.8 μM) followed by **11f** ($K_i$ = 7.6 μM) and compounds **11l,r,x** ($K_i$ ~ 8 μM). Interestingly, **11r** does not bind to Bcl-XL, but has a strong affinity for Mcl-1 ($K_i$ = 8.5 μM).

### Docking studies

In a previous report, we used AutoDock 4.0.1 with ADT [21] to examine the binding of compounds **11f,r** to Bcl-XL (PDB ID: 1LXL) and Mcl-1 (PDB ID: 1WSX) [17]. We found that it is highly plausible that **11f** binds to Bcl-XL in its closed conformation, with the *para*-hydrogen of the 2,3-dimethoxyphenyl ring in close proximity to Y195. It is believed the steric clash between Y195 and the *para*-methoxy group of the 3,4-dimethoxyphenyl ring in **11r** prevents this compound from binding to Bcl-XL.

Using AutoDock 4.0.1 and ADT [21], we docked **11f** to the holoprotein of Bcl-XL (PDB ID: 3FDL, 95.8 % sequence identity with 1LXL) with the Bim-peptide removed to see if the perturbed BH3 binding site would have an effect on the binding of **11f,r**. To our surprise, **11f** binds almost identically in the same orientation as with the previous docking (Figs. 4A,B). The docking predicts that the benzyl group occupies the hydrophobic pocket near F54, L57, L61, V75, L79, and F95. The binding pose pre-

 

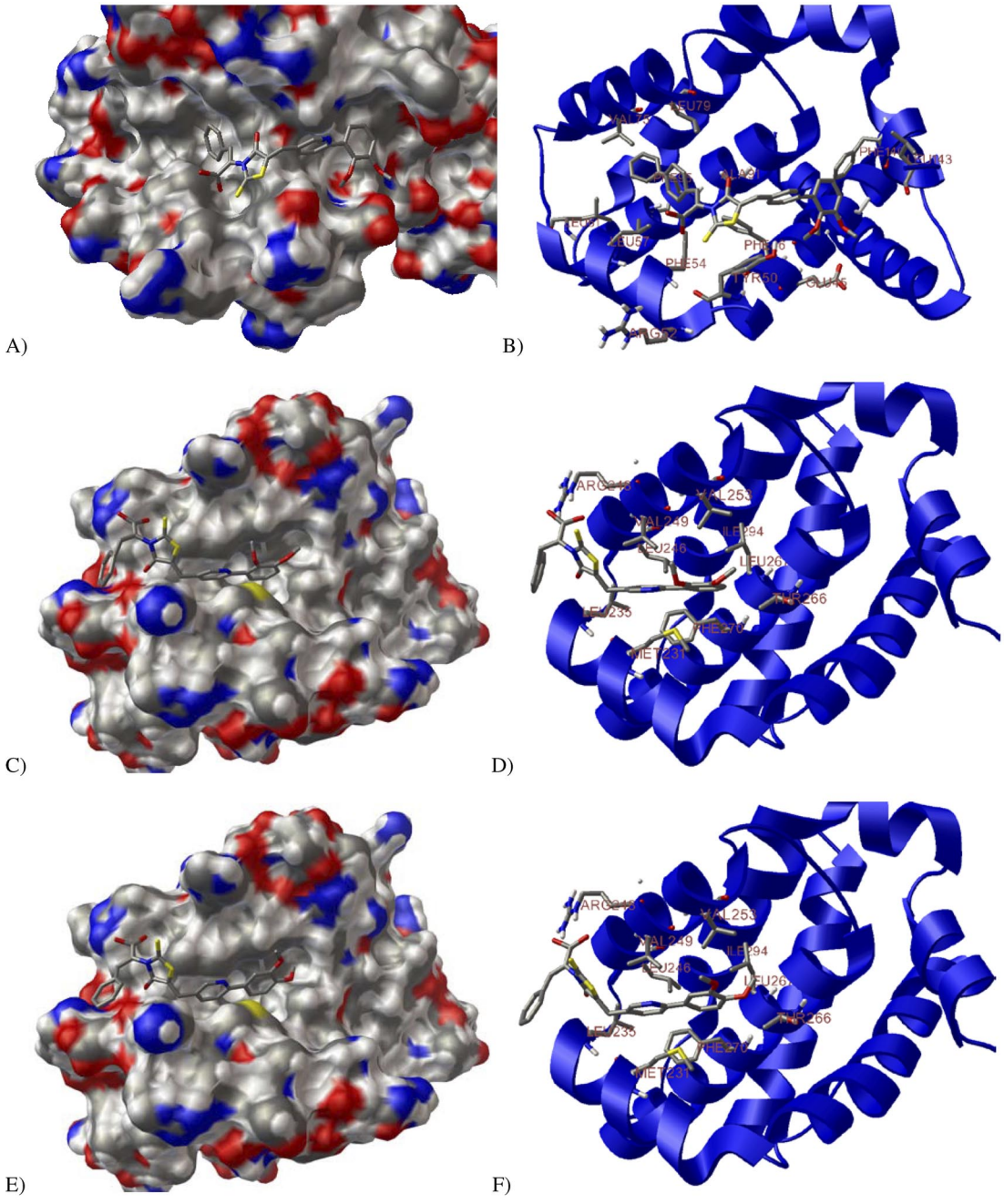


**Fig. 4** Binding of (A,B) **11f** to Bcl-XL, (C,D) **11f** to Mcl-1 and (E,F) **11r** to Mcl-1.

dicts a binding affinity of $K_i$ = 6.6 μM. This docking pose also shows the *para*-hydrogen of the 2,3-dimethoxyphenyl ring of **11f** in close proximity to where residue Y195 should be located (Fig. 4B). However, the sequence for 3DFL terminates at L134 (L194 on 1WSX) so the exact interaction with Y195 cannot be determined. However, it is highly likely that regardless of the conformation of the BH3

binding site of Bcl-XL, **11f** can bind in the predicted pose while **11r** fails to bind due to a steric clash with Y195.

With respect to Mcl-1, our previous study showed that both **11f** and **11r** bind to the BH3 binding site of the apoprotein with different orientations [17]. However, when docking to the holoprotein of Mcl-1 with the Bim-BH3 peptide removed (PDB ID: 2PQK, 90.3 % sequence identity with 1WSX), the results show both compounds bound to the binding site with identical orientations. The only difference in the binding modes is that the methoxy groups of **11f** are pointing away from the protein and the compound sits deeper in the binding site than **11r** (Figs. 4C–F). Both compounds bind with the carboxylate group interacting with R248, while the biaryl group resides in the hydrophobic BH3 pocket surrounded by the residues M231, L235, L246, V249, V253, L267, and F270. The binding energies for **11f** and **11r** in these poses are very similar (–8.11 and –8.07 kcal/mol, respectively) and the predicted $K_i$ values are also similar (1.13 μM for **11f**, 1.22 μM for **11r**).

The docking results are a fair reflection of the experimental results although the exact values of $K_i$ are not identical. However, docking studies alone cannot confirm the binding orientation of the compounds, nor can it confirm to which protein conformation binding occurs.

## CONCLUSION

In summary, a small library of rhodanine-based compounds were synthesized and screened for binding activity against Bcl-XL and Mcl-1. A number of compounds with 2-fold or better selectivity toward Bcl-XL were identified with **11k** having the best binding and selectivity. With respect to Mcl-1, **11r** was the most selective binder ($K_i$ = 8.5 μM). Furthermore, docking studies with the holoproteins of Bcl-XL and Mcl-1 were carried out, and the results obtained are in good agreement with the experimental values. However, crystal structures will be required to confirm both the conformation of the proteins and the binding orientation of the ligands.

## EXPERIMENTAL SECTION

### Chemistry: General experimental section

All $^1$H NMR and $^{13}$C NMR spectra were acquired on a Bruker 400 UltraShield Spectrometer operating at 400 and 100 MHz, respectively. The proton spectra were referenced to the respective residual solvent peaks (MeOH-$d_4$: 3.20; DMSO-$d_6$: 2.50 ppm) except for those recorded in $CDCl_3$ which were referenced to TMS. Carbon spectra were referenced to the central peak of the respective residual solvents (MeOH-$d_4$: 49.0; DMSO-$d_6$: 39.5; $CDCl_3$: 77.0 ppm). Low- and high-resolution electron impact mass spectra (EIMS) were measured using a Finnigan MAT95XP double-focusing mass spectrometer. Low-resolution electrospray ionization (ESI) mass spectra were recorded using Waters Quattro Micro$^{TM}$ API. High-resolution ESI spectra were obtained using the Agilent 6210 Time-of-Flight LC/MS. Infrared (IR) spectra were measured on a BioRad FTIR spectrophotometer with samples analyzed as KBr discs, or as thin films on a KBr plate. All elemental analyses were carried out on a EuroEA3000 series CHNS Analyzer.

### Experimental details: General procedure for the synthesis of N-substituted rhodanines (7a–f)

To a solution of the chosen amino acid (typically 5 g, 30 mmol) in water (100 mL) was added NaOH (2.4 g, 2 equiv) and $CS_2$ (1.81 mL, 30 mmol). The reaction mixture was stirred for 16 h at room temperature after which a solution of sodium chloroacetate (2.82 g, 30 mmol) was added. The reaction mixture was stirred for a further 3 h at room temperature, then acidified with aqueous HCl (6 M, 30 mL). The resulting solution was refluxed for 16 h, then cooled to room temperature. The crude product was

extracted from the aqueous layer with ethyl acetate (3 × 50 mL), and the combined organic layer was dried with $MgSO_4$, filtered, and the solvent removed in vacuo. The products were obtained in 80–92 % yield.

### Preparation of 2-bromo-5-formylpyridine (8)

The compound was prepared following the literature procedure [27]. A suspension of 2-bromo-5-iodopyridine (3 g, 11 mmol) in dry $Et_2O$ (100 mL) was cooled to –78 °C prior to the addition of *n*-BuLi (2.2 M, 5.3 mL, 1.1 equiv). The reaction mixture was stirred for 1 h at –78 °C prior to the addition of dry DMF (1 mL). After stirring for 1 h, the reaction mixture was warmed to room temperature and quenched by the addition of dilute HCl (1 M, 20 mL). The organic layer was separated, and the aqueous layer was further extracted with $Et_2O$ (2 × 20 mL). The ether layer was dried with $MgSO_4$, filtered, and the solvent removed in vacuo. Column chromatography on silica using a gradient (7–60 % EtOAc, hexanes) yielded the pure product as a white solid (1.25 g, 64 % yield). $R_f$ (1:1 EtOAc/hexanes): 0.39. Mp 100–101 °C. (lit. [28] 100 °C). $^1$H NMR ($CDCl_3$): δ 7.69 (d, $J$ = 8 Hz, 1H), 8.01 (dd, $J$ = 8 and 2 Hz, 1H), 8.83 (d, $J$ = 2 Hz, 1H), 10.10 (s, 1H, CHO).

### General procedure for the Suzuki coupling of compound 8 to boronic acids 9a–d

To a solution of **8** (0.1 g, 0.54 mmol) in 1,4-dioxane (5 mL) was added the requisite boronic acid (1.1 equiv) and aqueous $K_3PO_4$ (0.23 g in 1 mL $H_2O$, 2 equiv). The reaction mixture was degassed for 1 min with argon before adding catalytic $Pd_2(dba)_3$ (10 mg, 2 mol %) and $PCy_3HBF_4$ (8 mg, 4 mol %). The reaction mixture was stirred at 100 °C for 16 h in a sealed tube. The reaction mixture was then cooled to room temperature, and the solvent was removed. The residue was partitioned between EtOAc (20 mL) and water (20 mL), and the aqueous phase was further extracted with EtOAc (2 × 20 mL). Column chromatography on silica (EtOAc/hexanes, 0–50 %) yielded the desired products as white or pale yellow solids (77–97 % yield).

### General procedure for the Knoevenagel condensation of aldehydes 10a–d with rhodanines 7a–f

To a solution of the aldehydes **10a–d** (typically 100 mg, 0.30 mmol) in glacial acetic acid (1–5 mL) and sodium acetate (0.11 g, 4 equiv) was added the desired rhodanine **7a–f** (2 equiv). The mixture was refluxed for 3 h, then cooled to room temperature. The solvent was removed in vacuo, and aqueous HCl (1 M, 50 mL) was added and the resulting mixture was refluxed for a further 1 h. The reaction mixture was then cooled to room temperature, and the precipitate was collected via vacuum filtration. Recrystallization from hot 1 M HCl, or column chromatography on silica, gave the pure products **11a–x** (65–98 % yields).

### Molecular docking

The X-ray crystal structures of Bcl-XL and Mcl-1 with the Bim-BH3 peptide were downloaded from the PDB database (PDB IDs 3DFL and 2PQK). Sequence aligments and percent identity were confirmed using the matchmaker scripts in UCSF Chimera version 1.3 [29]. The 3D structures of the inhibitors were generated using ChemBio 3D Ultra followed by MMFF energy minimization. AutoDock 4.0.1 and ADT were used for generating the docking models [21]. Grid maps covering the entire BH3 binding site of both proteins were used in AutoDock calculations using the standard grid spacing of 0.375 Å. The GA-LS algorithm was adopted using the default setting except for the maximum number of energy evaluations which was set to 1 000 000. For each docking job, 100 hybrid GA-LS runs were

 

employed. A total of 100 possible binding conformations were generated and grouped into clusters using a 1.0 Å root-mean-square tolerance. The docking models were analyzed using ADT.

### Biology: Fluorescence polarization assay

The Flu-Bak-BH3 peptide was purchased from Mimotopes (Clayton, Victoria, Australia). The peptide was prepared as 1 mM stock solution in DMSO, and stock solutions of the test compounds (4 mM in DMSO) were used for serial dilutions (250–0.65 μM final concentrations). The assay was carried out in a total volume of 100 μL/well containing 3 μg glutathione *S*-transferase (GST)-hBcl$_{XL}$ΔC19 or 3 μg glutathione *S*-transferase (GST)-hMcl-1ΔC20 and 60 nM labeled peptide in buffer (50 mM Tris, pH 8, 150 mM NaCl and 0.1 % bovine serum albumin). In each well was added 10 μL of the test compounds, and the reaction mixture was incubated at room temperature for 1 h. Each compound was tested in quadruplicate. The fluorescence polarization values were determined using Tecan GeniosPro plate reader using the excitation/emission wavelengths 485/535 nm. The data was analyzed using GraphPad Prism 5, and the results were fitted to a dose–response curve to obtain the $IC_{50}$ values and standard deviations. The $K_i$ values were calculated using an online calculator [20] using the following parameters: [L] = 0.06 μM for Flu-Bak, [Bcl-XL] = 1.1 μM, and [Mcl-1] = 1.6 μM. The dissociation constants employed were $K_d$ (Bcl-XL) = 0.14 μM and $K_d$ (Mcl-1) = 1.1 μM.

## SUPPLEMENTARY INFORMATION

Detailed compound characterization data is available online (doi:10.1351/PAC-CON-10-10-29). $^{1}$H and $^{13}$C NMR spectra are available from the authors upon request.

## ACKNOWLEDGMENTS

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