2314 J. Med. Chem. 2010, 53, 2314–2318
DOI: 10.1021/jm901469p

Journal of Medicinal Chemistry
Brief Article

# Structural Insights into the Design of Small Molecule Inhibitors That Selectively Antagonize Mcl-1

Paul H. Bernardo,*,† Thirunavukkarasu Sivaraman,‡ Kah-Fei Wan,§ Jin Xu,† Janarthanan Krishnamoorthy,‡ Chun Meng Song,∥ Liming Tian,† Jasmine S. F. Chin,† Diane S. W. Lim,† Henry Y. K. Mok,‡ Victor C. Yu,⊥ Joo Chuan Tong,∥,# and Christina L. L. Chai*,†

†Institute of Chemical and Engineering Sciences, Agency for Science Technology and Research (A*STAR), 1 Pesek Road, Jurong Island, Singapore 627833, ‡Departmental of Biological Sciences, National University of Singapore, Singapore 117543, §Experimental Therapeutics Centre, Agency for Science Technology and Research (A*STAR), 31 Biopolis Way, Singapore 138669, ∥Institute for Infocomm Research, Agency for Science Technology and Research (A*STAR), 1 Fusionopolis Way, No. 21-01 Connexis, Singapore 138632, ⊥Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore 117543, and #Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, 8 Medical Drive, Singapore 117597

Received July 12, 2009

The screening of a small focused library of rhodanine derivatives as inhibitors of Bcl-2 proteins led to the discovery of two structurally related compounds with different binding profiles against the Bcl-XL and the Mcl-1 proteins. Subsequent NMR studies with mutant proteins and in silico docking studies provide a possible rationale for the observed specificity.

## Introduction

In recent years, the Bcl-2 family of prosurvival proteins has gained considerable interest as viable drug targets for cancer chemotherapy.[1,2] It is thus not surprising that intense efforts have focused on the development of Bcl-2 inhibitors for cancer chemotherapy.[1,3,4] One of the most promising inhibitors to date is **1** (ABT-737) (Figure 1) and its orally active congener **2** (ABT-263).[5,6] These compounds were developed by Abbot Laboratories, and **2** is currently in phase I clinical trials. Interestingly, **1** and **2** are potent inhibitors of multiple members of the prosurvival proteins of the Bcl-2 family, but they do not inhibit Mcl-1.[6] It has also been observed that cancer cells that are resistant to the cytotoxic activity of **1** become resensitized toward **1** when the Mcl-1 protein is neutralized.[7−9] This could mean that a selective inhibitor of Mcl-1, in conjunction with **1**, would constitute a powerful anticancer therapy against a broad spectrum of cancers.[10]

A recent publication reported that the small molecule **3** (obatoclax, GX15-070) can antagonize Mcl-1, and hence, it could be useful for circumventing the resistance conferred by Mcl-1 against cell death.[11] However, **3** is an example of a pan-Bcl-2 inhibitor with moderate to weak activities, and a number of these inhibitors are already known.[1,3] It remains unanswered whether a selective inhibitor of Mcl-1 can be developed.

In this report, we describe our discovery of an inhibitor that selectively antagonizes Mcl-1 and provide some fundamental insights into the future design and development of Bcl-XL and Mcl-1 inhibitors.

## Results and Discussion

In the course of our studies, we screened a small focused synthetic compound library of 34 compounds specially designed to incorporate the structural elements of **4** (BH3I-1)[12] which is known to bind to the BH3 binding site of Bcl-2 proteins, as well as aryl and pyridyl fragments which are structural elements present in the natural product sanguinarine (**5**).[13,14] The $IC_{50}$ values of these compounds were determined using fluorescent polarization assay (FPA[a]) based on a competition assay against fluorescein labeled Bak-BH3 peptide (Flu-Bak), then converted to $K_i$ values using a method reported in the literature.[15,16] Intriguingly, two constitutional isomers, **6** and **7**, were found to possess very different specificities for Bcl-XL and Mcl-1 protein. The $K_i$ values for **6** against Bcl-XL and Mcl-1 protein are 3.7 and 6.9 $\mu$M, respectively, while isomer **7** is not active at the concentration range studied (> 100 $\mu$M) for Bcl-XL but has a $K_i$ of 8 $\mu$M against the Mcl-1 protein. Where activity is observed, the $K_i$ values are smaller (i.e., better inhibitors) than that of **4** ($K_i$ for Bcl-XL is 10 $\mu$M; $K_i$ for Mcl-1 is 44 $\mu$M). However, as FPA assays only provide a rough gauge of the inhibitory activities of compounds based on competitive experiments, a more accurate measurement of the direct binding of compounds to the target proteins was determined via isothermal titration calorimetry (ITC) methods. Thus, the binding affinities ($K_d$) measured using ITC show a trend that closely mirrors the results obtained by FPA. FPA and ITC show that **4** binds to Bcl-XL as previously reported but in contrast does not bind well to Mcl-1 as earlier reported.[3] The FPA and ITC results also show that **6** binds to Bcl-XL and Mcl-1 at low micromolar concentrations ($K_d$ of 3.4 and 0.25 $\mu$M, respectively). Furthermore, the ITC results verify that **7** binds exclusively to Mcl-1 ($K_d$ of 10 $\mu$M). The $K_i$ values calculated from the FPA results and the $K_d$ values from ITC measurements are summarized in Table 1.

*To whom correspondence should be addressed. For P.H.B.: phone, +65 6796 3913; fax, +65 6316 6184; e-mail, paul_bernardo@ices.a-star.edu.sg. For C.L.L.C.: phone, +65 6796 3902; fax, +65 6316 6184; e-mail, christina_chai@ices.a-star.edu.sg.

[a] Abbreviations: ADT, AutoDockTools; CCSP, combined chemical shift perturbation; FPA, fluorescence polarization assay; HSQC, heteronuclear single quantum correlation; ITC, isothermal titration calorimetry; $K_i$, inhibition constant; $K_d$, dissociation constant.

 Published on Web 02/16/2010 

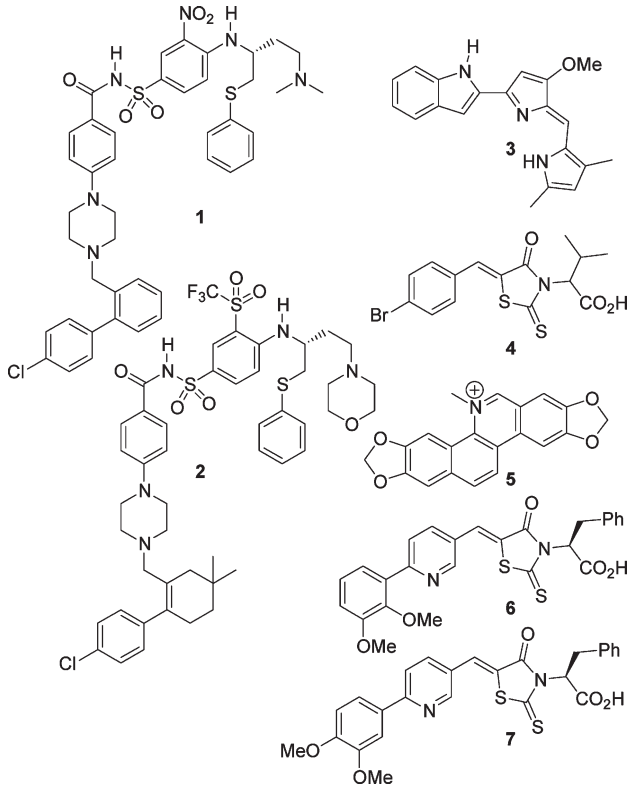

**Figure 1**

**Table 1.** $K_i$ Values from FPA and $K_d$ Values from ITC

| | Bcl-XL ($\mu$M) | | Mcl-1 ($\mu$M) | |
|---|---|---|---|---|
| compd | $K_i$ | $K_d$ | $K_i$ | $K_d$ |
| **4** | $10 \pm 1$ | $8.7 \pm 1.8$ | $44 \pm 4$ | $84 \pm 8$ |
| **6** | $3.7 \pm 0.3$ | $3.4 \pm 0.3$ | $6.9 \pm 0.4$ | $0.25 \pm 0.09$ |
| **7** | $>100$ | $>750$ | $8 \pm 1$ | $10 \pm 2$ |

$^{15}$N HSQC NMR titration studies were used to determine the possible binding site of each compound to Bcl-XL and Mcl-1. The various ligand-induced combined chemical shift perturbation (CCSP) signals were studied in detail, and the specific regions where the CCSP occurs were noted. For instance, in Bcl-XL−**6**, 40% of the residues that were perturbed above the threshold value (0.1 ppm) were located in the BH3 binding groove (Figure 2). These chemical shifts may be largely due to direct ligand−protein interactions and indirect ligand-induced conformational changes in the protein. The data showed that most of the residues perturbed by **6** were in the classical BH3-binding pocket of Bcl-XL. For Mcl-1, the complexes of Mcl-1−**6** and Mcl-1−**7** show that more than 50% of the residues perturbed above the threshold value were located in the BH3 binding groove of the protein. However, the CCSP for the Mcl-1 complexes are not identical. Compound **6** induced more CCSP signals than **7**, which may be due to a tighter fit within the BH3 binding site. The differences between the actual residues perturbed by **6** and **7** in the BH3 binding groove suggest that each compound has a different mode of interaction within the binding site. This suggests that the isomers may be bound in different orientations within the BH3 binding site.

The in silico docking results using Autodock 4.0.1 in conjunction with AutoDock Tools (ADT) are shown in Figure 3. The protein structures were obtained from the Protein Data Bank (Bcl-XL, PDB code 1LXL; Mcl-1 PDB code 1WSX).[17,18] The docking results for **6** on Bcl-XL show 98% of the possible conformations as a single cluster. Similar results were obtained for Mcl-1−**6** and Mcl-1−**7**.

Subsequent $^1$H−$^{15}$N HSQC experiments using mutants of Bcl-XL and Mcl-1 with **4**, **6**, and **7** provide further confirmation of the predicted docking results. The mutations were based on residues that were predicted by the docking model to be essential for binding activity. For Bcl-XL, the essential residues included amino acids within the BH3 binding groove (F105A, L108A, L130A, R139A, and Y173F) and two residues from the BH1 domain (E129A and A142G). The qualitative results are summarized in Table 2. Thus, for the binding of **6** to Bcl- XL, residues F105, L108, and A142 have a moderate effect on ligand binding while residue L130 is essential for binding. The residues E129 and R139 have little effect on binding, while Y173 has no effect on the binding of **6** to Bcl-XL. With respect to **4**, residues L108, L130, and R139 are essential for binding while residues F105 and A142 have a moderate effect on binding. The residue Y173 has no effect on the binding of **4** to Bcl-XL.

With respect to Mcl-1, all the residues chosen were from the BH3 binding groove (H205A, A208G, M212A, V230A, K236A, T247A, F251A). The qualitative results from the mutation studies are shown in Table 3. The binding of **6** to Mcl-1 is moderately affected by residues A208, K236, and T247 and greatly affected by residues M212 and F251. The residues H205 and V230 have only a weak effect on the binding of **6** to Bcl-XL. The binding of **7** is moderately affected by residues H205, A208, M212, V230, K236 and strongly affected by F251.

The full rigid models incorporating the docking results and mutation studies are shown in Figure 4. For the mutation results, mutations with little effect on CCSP are colored yellow (+++++), mutations with moderate effects are colored orange (+++), and mutations that greatly attenuate CCSP are colored red (++).

The studies above suggest a possible rationale for the specificity observed for the constitutional isomers **6** and **7**. The dimethoxy substituents in the 2- and 3-positions of the aromatic ring of **6** still fit within the binding pocket of Bcl-XL. However, the model suggests that a substituent at the 4-position of **7** is likely to be sterically repulsed by the Y195 residue. The rhodanine portion of **6** is also located in a large hydrophobic pocket and provides a scaffold for the appropriate positioning of the substituents of the rhodanine ring for critical interactions with the protein. The BH3 binding site for the Mcl-1 protein is wider than that for Bcl-XL, so it is not surprising that **6** and **7** bind to Mcl-1. The predicted models suggest that **6** and **7** occupy almost the same region on the BH3 binding site, although **6** appears to span across helices 2 and 3 while **7** is confined to the groove between these helices. What is surprising is the suggestion from the docking and NMR studies that **6** and **7** are bound to the BH3 binding site in orientations opposite each other. NMR chemical perturbation studies with mutant proteins do not show similar levels of interaction. In Mcl-1−**6**, the mutation M212A (in red) results in a decrease in CCSP. This may be due to the smaller alanine residue having a weaker lipophilic interaction with the ligand. This mutation leads to a weaker CCSP signal for Mcl-1−**7**. Mutation of the A208 residue also weakens the hydrophobic interaction between ligands **6** and **7** and the Mcl-1 protein. In addition, **6** is less affected than **7** by the H205A mutation, as the docking model shows that it is outside the effective volume of **6**. These studies suggest that the presence of a methoxy group at the ortho or para position of the aryl ring of these

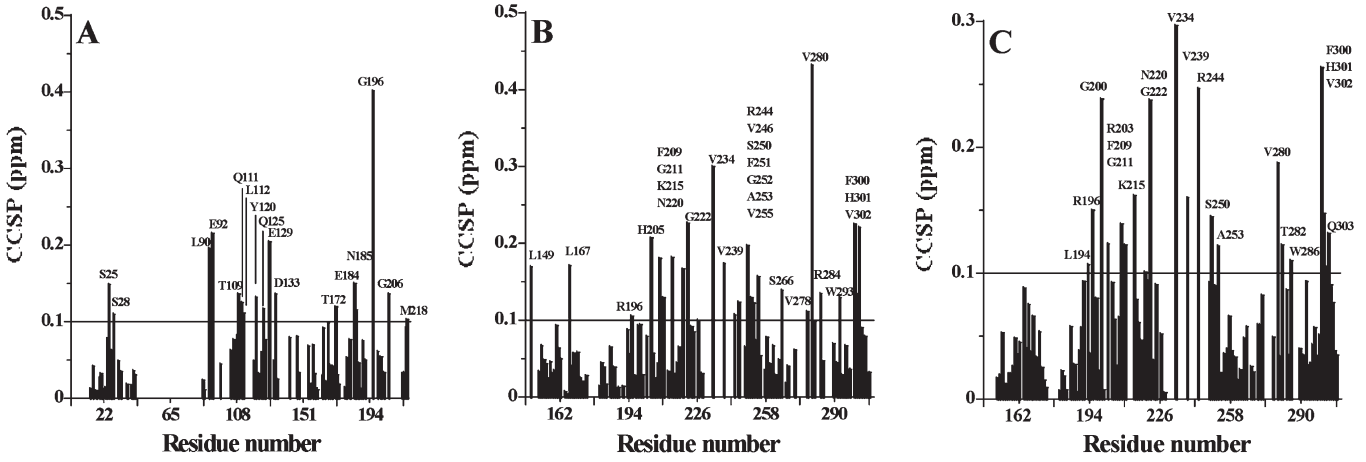


**Figure 2.** Amide proton and nitrogen CCSP of Bcl-XL or Mcl-1 derived from the $^{1}H-^{15}N$ HSQC spectra after titration with (A) **6** to Bcl-XL, (B) **6** to Mcl-1, and (C) **7** to Mcl-1 plotted against the residual number of the corresponding protein. The molar ratios of ligands to proteins were 3:1 and 1:1 in the titrations of **6** to Bcl-XL and of **6** or **7** to Mcl-1. Residues that are perturbed by more than an arbitrary threshold value of 0.1 ppm are labeled.

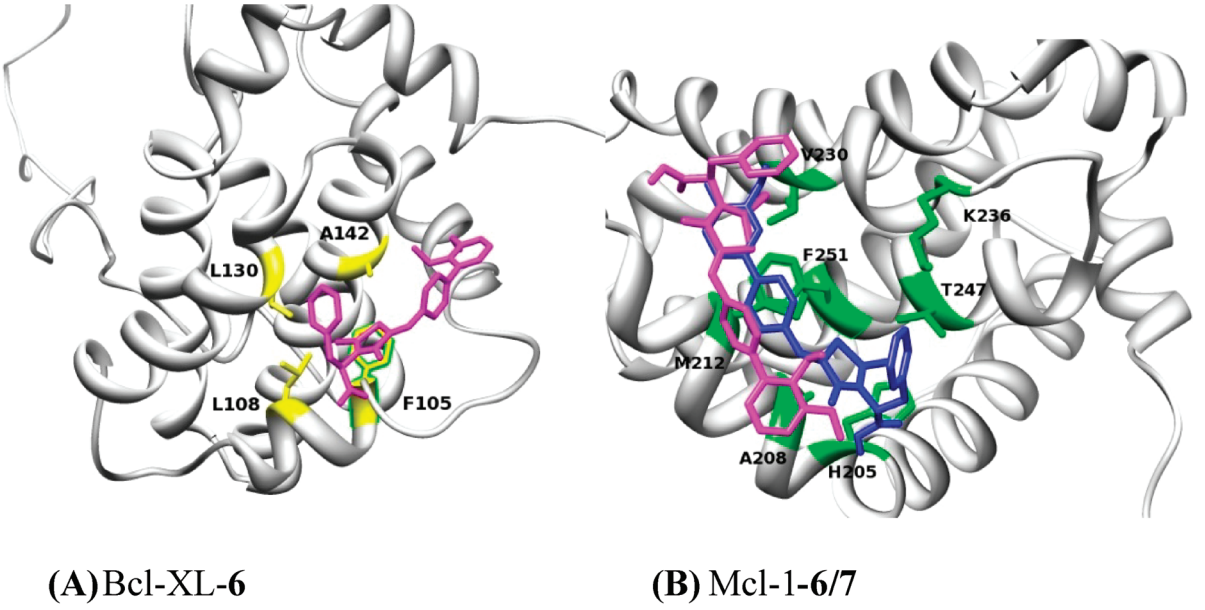


**Figure 3.** Docking models of Bcl-XL-**6** (A) and Mcl-1-**6**/**7** complexes (B) are shown. Residues that are essential for the binding of **6** in Bcl-XL are in yellow (F105, L108, L130, and A142). Residues that are essential for binding of **6**/**7** in Mcl-1 are in green (H205, A208, M212, V230, K236, T247, and F251).

**Table 2.** Degree of Overall Chemical Shift Perturbation Caused by **4** and **6** on the Backbone NHs of Various Bcl-XL Mutants As Examined by $^{15}N$ HSQC Spectra[a]

| Bcl-XL mutant | structural context | **4** | **6** |
|---|---|---|---|
| F105A | BH3 groove | +++ | +++ |
| L108A | BH3 groove | ++ | +++ |
| E129A | BH1 | +++++ | ++++ |
| L130A | BH3 groove | ++ | ++ |
| R139A | BH3 groove | ++ | ++++ |
| A142G | BH1 | +++ | +++ |
| Y173F | BH groove | +++++ | +++++ |

[a] The plus sign (+) indicates the relative degree of overall chemical shift perturbation observed during NMR titration. For reference, the wild-type CCSP is "+++++" by default.

**Table 3.** Degree of Overall Chemical Shift Perturbation Caused by **6** and **7** on the Backbone NHs of Various Mcl-1 Mutants As Examined by $^{15}N$ HSQC Spectra[a]

| Mcl-1 mutant | structural context | **6** | **7** |
|---|---|---|---|
| H205A | BH3 groove | ++++ | +++ |
| A208G | BH3 groove | +++ | +++ |
| M212A | BH3 groove | ++ | +++ |
| V230A | BH3 groove | ++++ | +++ |
| K236A | BH3 groove | +++ | +++ |
| T247A | BH3 groove | +++ | ++++ |
| F251A | BH3 groove | ++ | ++ |

[a] The plus sign (+) indicates the relative degree of overall chemical shift perturbation observed during NMR titration. For reference, the wild-type CSP is "+++++" by default.

compounds can determine the orientation of the compound at the binding site. Compound **6** has the aryl ring lying above A208 with the methoxy groups oriented inside the BH3 binding groove; however, the presence of the T207 residue effectively blocks the para position of this aryl ring. Thus, a para-substituted compound like **7** would be sterically

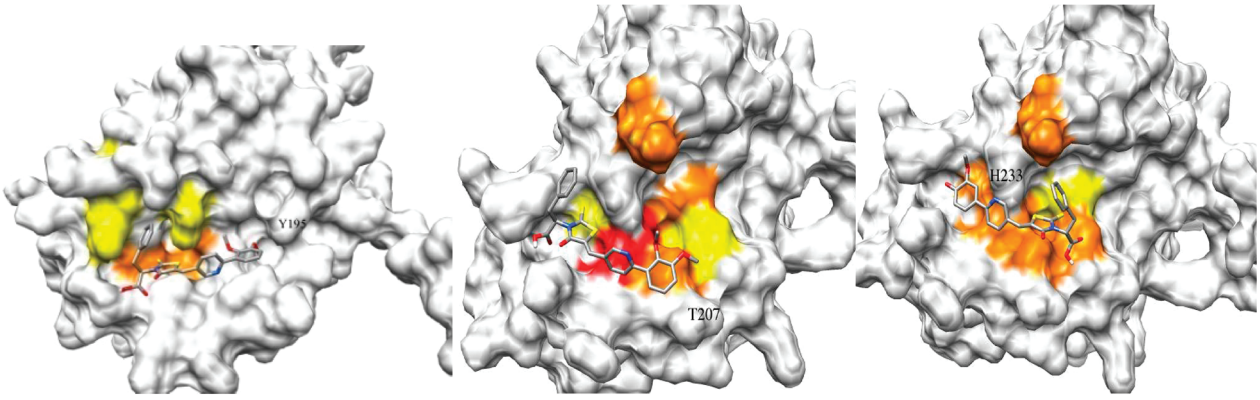


**Figure 4.** Docking model of Bcl-XL with (A) **6** and docking model of Mcl-1 with (B) **6** and (C) **7**. Residues from mutation studies are colored according to the degree of the corresponding chemical shift perturbation observed: yellow (+++++, ++++) = strong shift; orange (+++) = moderate shift; red (++) = weak shift. The labeled residues indicate areas of potential steric clashes that prevent the isomers from occupying the same pocket.

repulsed. Similarly, the aryl ring of **7** lies within a small pocket at the BH3 binding site. The aryl ring is in proximity to a bulky H233 residue which may prevent an ortho-substituted compound such as **6** from binding at this position. The structural features in the antagonists that subtly control the orientation of binding could perhaps be exploited in the design of new and more potent inhibitors of Mcl-1. For example, each orientation may provide new possible sites for interactions with appropriately placed functionalities in the ligands.

An important opportunity that has arisen from the findings reported here is the scope for rational and selective drug design for inhibitors of Bcl-XL and Mcl-1. To the best of our knowledge, this is the first report where molecular determinants governing the specificity of ligand binding to Bcl-XL and Mcl-1 have been compared and delineated. This opens up new challenges and provides new directions in selective drug design for the Bcl-2 family of proteins.

## Experimental Section

The procedures for the synthesis of **6** and **7** are described in detail in the Supporting Information. Purities of compounds (> 95%) are determined via elemental analysis.

**6.** Yellow solid, 54% yield. Mp 160−162 °C. $^1$H NMR ($CDCl_3$): $\delta$ 3.64 (d, $J$ = 7 Hz, 2H), 3.70 (s, 3H, $OCH_3$), 3.92 (s, 3H, $OCH_3$), 6.00 (br s, 1H, NCH), 7.03 (d, $J$ = 8 Hz, 1H), 7.20 (m, 6H), 7.40 (d, $J$ = 8 Hz, 1H), 7.74 (s, 1H), 7.81 (d, $J$ = 8 Hz, 1H), 8.04 (d, $J$ = 8 Hz, 1H), 8.88 (s, 1H). $^{13}$C NMR ($CDCl_3$): $\delta$ 33.9, 56.0, 58.2, 61.2, 113.9, 122.6, 124.2, 124.6, 125.6, 127.1, 127.8, 128.5, 128.9, 129.2, 132.1, 135.9, 136.7, 147.4, 151.4, 153.1, 156.5, 166.9, 170.9, 191.6. FTIR (KBr, cm$^{-1}$) 3410 (br), 2920, 1723, 1609, 1263, 1174, 1031, 831, 739, 670. HRMS (ESI$^-$): calcd 505.0897 for $C_{26}H_{21}N_2O_5S_2$ [M − H]$^-$. Found: 505.0873. Anal. ($C_{26}H_{22}N_2O_5S_2$) C, H, N.

**7.** Obtained as the HCl salt, orange solid, 86% yield. Mp 202−203 °C. $^1$H NMR (DMSO-$d_6$): $\delta$ 3.53 (d, $J$ = 4 Hz, 2H, $PhCH_2$), 3.83 (s, 3H, $CH_3$), 3.86 (s, 3H, $CH_3$), 5.86 (br s, 1H, NCH), 7.09 (d, $J$ = 8 Hz, 1H), 7.15−7.23 (m, 5H), 7.77 (m, 2H), 7.86 (s, 1H), 7.98 (dd, $J$ = 2 and 9 Hz, 1H), 8.13 (d, $J$ = 9 Hz, 1H), 8.90 (d, $J$ = 2 Hz, 1H). $^{13}$C NMR ($CDCl_3$ + $CD_3OD$): $\delta$ 33.6, 55.8, 58.2, 109.8, 111.0, 120.0, 120.1, 123.0, 126.8, 127.0, 128.3, 128.95, 129.0, 130.5, 136.0, 137.0, 149.2, 150.8, 151.8, 157.8, 166.9, 169.5, 191.8. FTIR (KBr, cm$^{-1}$) 3476 (br), 2907, 2360, 1719, 1584, 1263, 1229, 1023, 836, 738, 698. HRMS (ESI$^-$): calcd 505.0897 for $C_{26}H_{21}N_2O_5S_2$ [M − H]$^-$. Found: 505.0873. Anal. ($C_{26}H_{23}ClN_2O_5S_2$) C, H, N.

**Acknowledgment.** We thank the Agency for Science, Technology and Research (A*STAR), Singapore, for the funding for this project. This work was also supported by Grant BMRC 04/1/21/19/320 (to H.Y.K.M.) from the Biomedical Research Council of Singapore.

**Supporting Information Available:** Full experimental details and $^1$H and $^{13}$C NMR spectra and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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