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Structural tailoring of a novel fluorescent IRE-1 RNase inhibitor to precisely control its activity

Andong Shao[†], Chang Won Kang[‡], Chih-Hang Anthony Tang[†], Christopher Cain[‡], Qin Xu[†], Claire Phoumyvong[†], Juan R. Del Valle[‡], and Chih-Chi Andrew Hu[†]

[†]The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104

[‡]Department of Chemistry, University of South Florida, Tampa, FL 33620

Abstract

Activation of the IRE-1/XBP-1 pathway has been linked to many human diseases. We report a novel fluorescent tricyclic chromenone inhibitor, **D-F07**, in which we incorporated a 9-methoxy group onto the chromenone core to enhance its potency and masked the aldehyde to achieve long-term efficacy. Protection of the aldehyde as a 1,3-dioxane acetal led to strong fluorescence emitted by the coumarin chromophore, enabling **D-F07** to be tracked inside the cell. We installed a photolabile structural cage on the hydroxy group of **D-F07** to generate **PC-D-F07**. Such a modification significantly stabilized the 1,3-dioxane acetal protecting group, allowing for specific stimulus-mediated control of inhibitory activity. Upon photo-activation, the re-exposed hydroxy group on **D-F07** triggered the aldehyde-protecting 1,3-dioxane acetal to slowly decompose, leading to the inhibition of the RNase activity of IRE-1. Our novel findings will also allow for spatiotemporal control of the inhibitory effect of other salicylaldehyde-based compounds currently in development.

Graphical Abstract:

Corresponding Authors: Chih-Chi Andrew Hu: chu@wistar.org, Juan R. Del Valle: delvalle@usf.edu.

Author Contributions

A.S., C.W.K., C.H.A.T., C.C., J.R.D. and C.C.A.H. designed research; A.S., C.W.K., C.H.A.T., C.C., Q.X., C.P., J.R.D. and C.C.A.H. performed research; A.S., C.W.K., C.H.A.T., C.C., J.R.D. and C.C.A.H. analyzed data; and A.S., C.W.K., J.R.D. and C.C.A.H. wrote the manuscript.

Notes

All authors have declared that no conflict of interest exists.

SUPPORTING INFORMATION

NMR spectra of all newly synthesized compounds and supplementary figures are available on-line free of charge at <http://pubs.acs.org>. Copies of ¹H and ¹³C NMR spectra; supplementary figures S1–S8; and molecular formula strings.

INTRODUCTION

Cellular stress phenotypes in cancer result from the increased rates of metabolism, mitosis, protein synthesis, and DNA damage associated with tumor progression. Cytoprotective signaling pathways activated in response to these phenotypes have emerged as important non-oncogenic targets for therapy.¹ The endoplasmic reticulum (ER) stress response is frequently hyperactivated in cancer due to an accumulation of unfolded proteins, hypoxic conditions, calcium imbalance, and other stimuli.^{2–5} Of note, the ER stress response can also be activated in response to the overexpression of oncogenes.^{6–7} The three branches of ER stress response are governed by the stress sensor proteins IRE-1, ATF6, and PERK.^{3, 8} IRE-1 is an ER-resident dual kinase/RNase that splices 26 nucleotides from the mRNA of the transcription factor, XBP-1. This spliced XBP-1 mRNA variant encodes the functional 54-kDa XBP-1s protein, which translocates into the nucleus and regulates the ER stress response genes.^{9–11} By genetic deletion of XBP-1s, we and others have shown that XBP-1s contributes to the progression of chronic lymphocytic leukemia (CLL) and triple-negative breast cancer.^{12–13} While most transcription factors are difficult to target with small molecules, the specific activation of XBP-1s via the RNase activity of IRE-1 provides an attractive opportunity to exploit the increased stress conditions associated with not only cancer but also many other diseases.^{14–15}

High-throughput screening of large chemical libraries has led to the discovery of various salicylaldehydes as potent *in vitro* inhibitors against the RNase activity of IRE-1.^{16–19} The aldehyde moiety of each of these inhibitors is believed to be critical for inhibition of RNase function, allowing the formation of an unusual but highly specific Schiff base with Lys907 in the RNase domain of IRE-1.^{18, 20} Although IRE-1 contains 25 lysine residues in its cytosolic domain, only covalent modification at Lys907 (and in some cases K599) is observed *in vivo* after treatment with salicylaldehyde-based inhibitors.¹⁸ Specific perturbation of the Lys907 ϵ -amino group pKa in the IRE-1 RNase domain results in enhanced Lys nucleophilicity, slower inhibitor off-rate, and desired phenotypic response.^{18, 20} Non-specific lysine modification by salicylaldehydes is generally short-lived (rapid

off-rate), resulting in minimal off-target effects. The first co-crystal structure of IRE-1 covalently bound to an ortho-hydroxy-aryl-aldehyde inhibitor validates this proposed mode of binding.²¹

We conducted the chemical synthesis of a library of salicylaldehyde analogues, and developed a family of potent tricyclic chromenone-based IRE-1 inhibitors via a Duff formylation that is attended by an unusual cyclization reaction.²² To improve the in vivo efficacy of these aldehyde inhibitors, we developed B-I09, in which the reactive aldehyde was protected as a 1,3-dioxane acetal.¹² B-I09 is effective in suppressing the growth of CLL and Myc-overexpressing Burkitt's lymphoma in vivo and in preventing the development of the graft-versus-host disease in mice.^{12, 23–25} Additionally, the ability of B cells to produce secretory IgM is potently inhibited by B-I09, leading to significantly decreased immunosuppressive functions of myeloid-derived suppressor cells and reactivation of anti-tumor CD8+ T cell functions in CLL and lung cancer mouse models.²⁶ Structural tailoring of IRE-1 inhibitors to investigate the influence of substituents on the drug stability and stimuli-specific release has not been explored.

Here, we report a unique prodrug strategy that could be used to precisely control the activity of IRE-1 inhibitors. We first developed a novel fluorescent IRE-1 inhibitor, **D-F07**, by lead optimization, in which the reactive aldehyde group was protected as a 1,3-dioxane acetal, resulting in strong emission of blue fluorescence from the coumarin chromophore. Such a protecting group could also be slowly hydrolyzed under physiological conditions to achieve long-term efficacy. We next installed a photo-labile structural cage at the C8 position of **D-F07** to achieve **PC-D-F07**. Such a chemical modification on the 8-hydroxy group could significantly stabilize the 1,3-dioxane acetal protecting group, thus allowing specific stimuli-mediated cleavage to re-expose the hydroxy group on **D-F07** to trigger the decomposition of the 1,3-dioxane acetal moiety. These strategies could be applied to other salicylaldehyde-based compounds to achieve spatiotemporal control of their biological activities.

RESULTS AND DISCUSSION

Tricyclic chromenone compounds with N-H and N-CH₃ are more potent IRE-1 inhibitors.

To develop inhibitors with improved potency to target the IRE-1/XBP-1 pathway, we previously synthesized and compared tricyclic chromenone-based IRE-1 RNase inhibitors with different amine substituents (R¹): *allyloxycarbonyl* (*alloc*) in **1**¹², *-H* in **2**¹², and *-CH₃* in **3**²² (Figure 1A). We showed that a biotin-tagged derivative of **1**, B-I06, could pull down endogenously expressed IRE-1 in B cells, while the reduced derivative B-I07 (aldehyde replaced by alcohol) failed to do so.¹² Here, we further confirmed the direct interaction of **2** and **3** with IRE-1 using surface plasmon resonance (SPR), and determined a K_d value of 295 nM for **2** and 138 nM for **3** (Figure 1B).

5TGM1 multiple myeloma cells were used to evaluate the effects of inhibitors because they constitutively activated the IRE-1/XBP-1 pathway for survival.²⁷ We treated 5TGM1 cells with 10 μM **1**, **2**, or **3** for 0, 3, 6, 12, or 24 h, and analyzed the levels of XBP-1s. While **1**, **2**, and **3** rapidly inhibited the expression of XBP-1s, **2** and **3** exhibited much better long-term inhibitory activities than **1** (Figure 1C).

Tricyclic chromenone compounds with the 1,3-dioxane protecting groups emit strong fluorescence.

We next explored the possibility of using different protecting groups (at R²) to mask the aldehyde of the tricyclic chromenone parent compounds. In the case of our previously reported inhibitor B-I09 (**4**; Figure 1D), this was achieved through introduction of a 1,3-dioxane acetal.¹² We synthesized compounds **5–9** in order to assess differences in XBP-1s suppression and duration of action attributable to protecting group stability. As in the case of **4**, installation of the 1,3-dioxane group resulted in strong fluorescence at around 452 nm for both **5** and **7**, with approximately 45- to 72-fold increase in intensity relative to their aldehyde counterparts, **2** and **3** (Figures 1E, 1F, and S1). As the aldehyde is strongly electron withdrawing, its conversion to the acetal dramatically alters the electronic character of the aromatic substituent at R², leading to the fluorescence recovery of the coumarin chromophore. When the aldehyde was converted to hydrazone derivatives, **6**, **8**, and **9**, weak or no fluorescence could be observed (Figure 1F). This is due to the similar electronic nature of hydrazine functionality. The fluorescence of derivatives from **2** and **3** in aqueous solution could also be visualized by fluorescence imaging under 365 nm illumination (Figure 1G).

Installation of the 1,3-dioxane protecting group allows for gradual release of active tricyclic chromenone compounds leading to enhanced long-term suppression of XBP-1s.

We treated 5TGM1 cells with **2** or **3** at 10 μ M over the course of 3 days. The expression levels of XBP-1s recovered significantly after treatment for 2 days (Figure 2A). However, when 5TGM1 cells were treated with **4** or **7** at 10 μ M, the levels of XBP-1s remained significantly suppressed after 3-day treatment (Figure 2A), suggesting that the slow hydrolysis of the 1,3-dioxane group allowed for increased cell uptake and gradual release of the active compounds to achieve long-term inhibition. To evaluate the cytotoxicity of these compounds, we treated 5TGM1 cells with indicated inhibitors at 20 μ M for a course of 4 days. Our data showed that **4** and **7** were more potent than their respective aldehyde counterparts, **2** and **3**, in inhibiting the growth of 5TGM1 cells (Figure 2B). We further treated 5TGM1 cells with **4** and **5** for 24 h, and found that **4** was more potent than **5** in suppressing the expression of XBP-1s at 5 μ M (Figure 2C). This is likely due to the enhanced stability of 2,2-dimethyl-1,3-dioxane acetals toward hydrolysis relative to their unsubstituted counterparts.²⁸ We evaluated the decomposition rate of **4** and **5** in PBS solution (containing 1% DMSO) by measuring fluorescence intensity. After incubation at 37 °C for 48 h, the fluorescence intensity of **4** declined to 5.1% (Figure 2D). Under the same conditions, the fluorescence intensity of **5** only declined to approximately 60% after 48 h (Figure 2E), supporting the established trend.

Tricyclic chromenone compounds with the *N*-acetylhydrazone protecting group rapidly inhibit the expression of XBP-1s.

We treated 5TGM1 cells with **6**, **4**, **7**, **8**, and **9** at the concentration of 2.5 μ M for 0, 1, 2, or 4 h, and observed that *N*-acetylhydrazone derivative **8** could rapidly inhibit XBP-1s (Figure 2F). We confirmed that **8** also rapidly suppressed the expression of XBP-1s in mouse B cells (Figure S2). We reasoned that the electron-withdrawing nature of the acetyl moiety in the *N*-

acetylhydrazone in **8** results in faster hydrolysis relative to **7**. HPLC degradation experiments confirmed that this was indeed the case (Figure 2G).

Installation of a methoxy group increases the inhibitory potency of tricyclic chromenone compounds.

In contrast to compound **3**, a tricyclic chromenone with an exposed aldehyde which began to lose its inhibitory effect within 4 h, installation of the *N*-acetylhydrazone protecting group in **8** resulted in prolonged inhibition of XBP-1s expression (Figure S3). To develop a potent inhibitor that could suppress the expression of XBP-1s for an extended period of time, we chose to incorporate a methoxy group onto the C9 position of the chromenone core because X-ray co-crystallization studies using a related inhibitor demonstrated that this substituent engages in a favorable hydrogen bond with IRE-1 in the RNase active site.²¹ The synthesis of *N*-acetylhydrazone derivative **15** from 4-methoxyresorcinol (**10**) was carried out as shown in Figure 3A. When we treated 5TGM1 cells with **8** and its methoxy analogue, **15**, at 2.5 μ M for 0, 1, 2, or 4 h, or at 10 μ M for a course of 3 days, **15** not only suppressed the expression of XBP-1s more rapidly than **8** (Figure S3) but also exhibited a long-term inhibitory effect (Figure S4). Nevertheless, the levels of XBP-1s still slightly recovered in cells treated with **15** for 3 days (Figure S4). Since **8** inhibited the expression of XBP-1s more rapidly than **7** (Figures 2F and S5), we reasoned that **7** could be more effective than **8** in suppressing the expression of XBP-1s for a longer term, and showed that it was indeed the case (Figure 3B). We further compared **7** with **14** (**D-F07**, the methoxy derivative of **7**), and found that **D-F07** was superior to **7** and **15** in suppressing the expression of XBP-1s for a longer term (Figure 3B). Intermediate **13** (Figure 3A), the methoxy analogue of **4**, was also more potent than **4** in suppressing the expression of XBP-1s (Figure 3B). However, **D-F07** exhibited enhanced potency in suppressing the expression of XBP-1s (Figure S6). To evaluate the cytotoxicity induced by these compounds, we treated 5TGM1 cells with **3** carrying the exposed aldehyde, **7** carrying the 1,3-dioxane protecting group, or **D-F07** carrying both the 1,3-dioxane protecting group and the C9 methoxy group, at 20 μ M for a course of 3 days. **D-F07** exerted higher cytotoxicity than **3** and **7** in 5TGM1 cells (Figure 3C). Previously, we showed that a human chronic lymphocytic leukemia cell line, WaC3, was sensitive to **4**.¹² We treated WaC3 cells with **7**, **8** as well as their respective methoxy derivatives, **D-F07** and **15**, at 20 μ M for a course of 3 days. Both **D-F07** and **15** were initially more potent than **7** and **8** in suppressing WaC3 cell growth (Figure 3D); however, acetylhydrazone **15** was incapable of continuously inhibiting the growth of WaC3 cells. **D-F07** carrying the 1,3-dioxane acetal and C9 methoxy group suppressed WaC3 cell growth potently.

Installation of a photo-labile cage on the C8 hydroxy of **D-F07** stabilizes the 1,3-dioxane acetal and allows for UV-triggered inhibition of IRE-1.

The fluorescent property of aldehyde-protected tricyclic chromenone compounds could be used to visualize the subcellular localization and evaluate intracellular pharmacokinetics of the inhibitors.^{29–30} We examined the fluorescence of methoxy-containing tricyclic chromenone derivatives, and confirmed that **D-F07** and **13**, both harboring the 1,3-dioxane protecting group, emitted bright fluorescence at 460 nm (Figures 3E and S7), while **15**, bearing the *N*-acetylhydrazone protecting group, showed weak fluorescence. Since **D-F07**

could emit strong blue fluorescence and had the most potent effect in inhibiting the expression of XBP-1s in whole cells, we further modified the hydroxy group of **D-F07** with 4-isopropyl-3-nitrobenzoate (Figure 4A) because this photo-labile moiety could quench the fluorescence from coumarin and could be efficiently cleaved by UV irradiation.^{31–32}

Alkylation of the 8-hydroxy group with this photo-labile cage indeed quenched the fluorescence of **D-F07**. The fluorescence of the photo-caged compound (**16** or **PC-D-F07**) could be ‘turned on’ by UV irradiation. Upon UV irradiation, the 4-isopropyl-3-nitrobenzoate moiety was cleaved, converting **PC-D-F07** into **D-F07** with an approximately 75-fold increase in fluorescence intensity (Figures 4B and S8). To visualize intracellular photoactivation of the inhibitor, we treated 5TGM1 cells with **PC-D-F07** at 10 μ M for 4 h and analyzed these cells by confocal microscopy (Figure 4C). A rapid increase in fluorescence intensity in the cytoplasm of **PC-D-F07**-treated 5TGM1 cells was detected after 10-s UV irradiation (Figure 4, C-D). We treated 5TGM1 cells with **D-F07** or **PC-D-F07** at 10 μ M, immediately UV-irradiated the cells for 0, 3, 5, or 10 min, and cultured cells for additional 4 h. We observed that **PC-D-F07** could only suppress the expression of XBP-1s after UV irradiation (Figure 4E), highlighting the importance of the hydroxy group of tricyclic chromenone compounds in suppressing the expression of XBP-1s.

Because **PC-D-F07** was incapable of inhibiting the expression of XBP-1s in the absence of UV irradiation, we hypothesized that the rate of 1,3-dioxane acetal hydrolysis is suppressed by alkylation of the C8 hydroxyl group. We measured the rate of 1,3-dioxane acetal hydrolysis for **D-F07** and **PC-D-F07** (in PBS and RPMI media at 37 °C) by analytical HPLC. **PC-D-F07** was completely stable in PBS for 48 h (Figure 4F) and in RPMI media for 96 h (Figure 4G). In contrast, the 1,3-dioxane acetal group in **D-F07** was approximately 70% hydrolyzed in the end of both experiments. We further treated 5TGM1 cells with **D-F07** or **PC-D-F07** at 10 μ M for 4 h, removed the compounds from culture media by washing cells with PBS for 3 times, resuspended cells in fresh RPMI media, UV-irradiated cells for 10 min, and incubated cells for additional 0, 4, or 8 h (Figure 4H). After removal of the inhibitor supply from the media, **D-F07** failed to suppress XBP-1s continuously. However, **PC-D-F07**, upon UV irradiation, was converted to **D-F07**, triggering hydrolysis of the 1,3-dioxane acetal group and revealing the aldehyde group to allow for IRE-1 inhibition and suppression of XBP-1s expression (Figure 4H). Together, these results indicate that the 1,3-dioxane acetal moiety is stabilized by *O*-alkylation of the phenol. This finding could be used to precisely control the inhibitory effect of these and related salicylaldehyde derivatives.

CONCLUSIONS

In summary, we have demonstrated a unique prodrug strategy that can be used to enhance the efficacy of salicylaldehyde-based inhibitors of IRE-1. This is the first report that the 1,3-dioxane acetal protecting group on a salicylaldehyde-based compound can be stabilized by installing a photo-labile group on its *ortho*-hydroxy position. **PC-D-F07** can be converted to **D-F07** at a precise time and location by UV irradiation, and **D-F07** can be tracked by fluorescence in vitro and in cells. Once liberated, the re-exposed hydroxy group on **D-F07** will trigger the aldehyde-protecting 1,3-dioxane acetal to slowly hydrolyze, allowing the inhibition of IRE-1 RNase activity to occur. Our strategy in generating highly stable tricyclic

chromenone IRE-1 inhibitors featuring stimulus-mediated release provides a promising platform for developing other potent salicylaldehyde-based inhibitors.

EXPERIMENTAL SECTION

General synthesis notes.

Unless stated otherwise, reactions were performed in flame-dried glassware under a positive pressure of argon or nitrogen gas using dry solvents. Commercial grade reagents and solvents were used without further purification except where noted. Toluene, Et₂O, DCM, DMF, and MeCN were used following passage through Pure Process Technologies solvent purification system. Other anhydrous solvents were purchased directly from Sigma-Aldrich or Acros Organic and used without further purification. Thin-layer chromatography (TLC) was performed using Merck 60 F254 silica gel pre-coated glass-backed plates (0.25 mm). Flash chromatography was performed using silica gel cartridges (40–65 µm particle size). Reaction progress was monitored by TLC analysis (single spot/two solvent systems) using a UV lamp, CAM (ceric ammonium molybdate), ninhydrin, or basic KMnO₄ stain(s) for detection purposes. NMR spectra were recorded on a 400 or 500 MHz spectrometer. Proton chemical shifts are reported as δ values relative to residual signals from deuterated solvents (D₂O, CDCl₃, CD₃OD, or DMSO-*d*₆). UV-vis and fluorescence spectra were carried out on a BioTek Synergy™ NEO2 instrument (96 well plate). Photo-cleavage of PC-D-F07 was achieved by irradiation using a portable Blak-Ray UV lamp (Model UVL-21, 4 W, 1 mW/cm²). All synthesized compounds were determined to be 95% pure by analytical RP-HPLC.

Allyl

7-(5,5-dimethyl-1,3-dioxan-2-yl)-8-hydroxy-3,4-dihydro-1 H-chromeno[3,4-c]pyridin-5(2H)-one (5). A mixture of **1**¹² (50 mg, 0.15 mmol), 2,2-dimethyl-1,3-propanediol (47 mg, 0.46 mmol) and pTsOH·H₂O (1.4 mg, 8.0 µmol) in benzene (5 mL) was heated at 85° C for 2 h. Triethylamine (3 drops) was added, the reaction was concentrated, and the crude material was diluted with EtOAc. The organic layer was washed with water and brine and dried over anhydrous MgSO₄. Purification by silica gel flash chromatography (30% EtOAc/hexanes) gave the intermediate acetal as a white solid (49 mg, 80%). ¹H NMR (500 MHz, CDCl₃) δ 9.01 (s, 1H), 7.39 (d, *J* = 8.9 Hz, 1H), 6.84 (d, *J* = 8.9 Hz, 1H), 6.20 (s, 1H), 5.93 (ddd, *J* = 16.4, 10.8, 5.4 Hz, 1H), 5.31 (dd, *J* = 17.2, 1.6 Hz, 1H), 5.22 (dd, *J* = 10.4, 1.5 Hz, 1H), 4.63 (dt, *J* = 5.7, 1.5 Hz, 2H), 4.42 (s, 2H), 3.80 (s, 4H), 3.76 (t, *J* = 5.8 Hz, 2H), 2.81 (t, *J* = 5.8 Hz, 2H), 1.31 (s, 3H), 0.83 (s, 3H); ¹³C NMR (126 MHz, CDCl₃) δ 159.3, 155.2, 150.7, 146.4, 132.7, 125.2, 117.9, 116.3, 114.4, 111.7, 109.4, 98.3, 77.9, 66.4, 41.8, 39.2, 30.4, 24.7, 23.1, 21.8; HRMS (ESI-TOF) *m/z* [M + H]⁺ calcd for C₂₂H₂₆NO₇ 416.1704, found 416.1706.

Tetrakis(triphenylphosphine)palladium(0) (6.0 mg, 5.2 µmol) was added to a mixture of the above acetal (43 mg, 0.10 mmol) and phenylsilane (56 mg, 0.52 mmol) in DCM (8 mL) and the reaction was stirred for 1 h at room temperature. The reaction mixture was concentrated and the residue purified by silica gel flash chromatography (0 to 10% MeOH/CHCl₃) to give **5** as a pale yellow solid (29 mg, 85%). ¹H NMR (500 MHz, CDCl₃) δ 7.41 (d, *J* = 8.8 Hz,

1H), 6.85 (d, J = 8.8 Hz, 1H), 6.23 (s, 1H), 3.82 (s, 6H), 3.17 (d, J = 5.7 Hz, 2H), 2.76 (dd, J = 5.1, 2.7 Hz, 2H), 2.09 (s, 1H), 1.33 (s, 3H), 0.84 (s, 3H); ^{13}C NMR (101 MHz, CDCl_3) δ 159.9, 158.9, 150.7, 146.5, 124.9, 118.8, 114.2, 112.3, 109.3, 98.3, 77.9, 43.2, 41.8, 30.3, 25.1, 23.0, 21.7; HRMS (ESI-TOF) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{18}\text{H}_{21}\text{NNaO}_5$ 354.1311, found 354.1325.

(E)-8-hydroxy-7-((piperidin-1-ylimino)methyl)-1,2,3,4-tetrahydro-5H-chromeno[3,4-c]pyridin-5-one (6).: A mixture of **1**¹² (50 mg, 0.15 mmol), 1-aminopiperidine (21 mg, 0.21 mmol) and anhydrous magnesium sulfate (60 mg, 0.50 mmol) in DCM (5 mL) was stirred for 3 h at room temperature. The reaction was diluted with DCM and water and the organic layer was washed with brine and dried over anhydrous MgSO_4 . Purification by silica gel flash chromatography (30% EtOAc/hexanes) gave the intermediate hydrazone as a pale yellow solid (50 mg, 80%). ^1H NMR (500 MHz, CDCl_3) δ 12.91 (s, 1H), 8.14 (s, 1H), 7.27 (d, J = 10.5 Hz, 1H), 6.81 (d, J = 8.8 Hz, 1H), 5.94 (ddt, J = 16.4, 10.9, 5.6 Hz, 1H), 5.31 (dd, J = 17.2, 1.8 Hz, 1H), 5.21 (d, J = 10.5 Hz, 1H), 4.63 (d, J = 5.7 Hz, 2H), 4.42 (s, 2H), 3.76 (t, J = 5.8 Hz, 2H), 3.18 (t, J = 5.7 Hz, 4H), 2.82 (s, 2H), 1.77 (t, J = 5.9 Hz, 4H), 1.64 – 1.44 (m, 2H); ^{13}C NMR (126 MHz, CDCl_3) δ 160.6, 159.5, 155.2, 150.3, 146.8, 132.7, 131.5, 123.3, 117.9, 115.7, 113.7, 110.9, 107.3, 66.4, 51.9, 41.8, 39.6, 39.2, 24.7, 23.7; HRMS (ESI-TOF) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{22}\text{H}_{26}\text{N}_3\text{O}_5$ 412.1867, found 412.1876.

The above hydrazone (47 mg, 0.11 mmol) was subjected to Alloc deprotection as described for the synthesis of **5**. The crude residue was purified by silica gel flash chromatography (0 to 10% MeOH/ CHCl_3) to give **6** as a pale yellow solid (37 mg, 79%). ^1H NMR (500 MHz, CDCl_3) δ 12.90 (s, 1H), 8.19 (s, 1H), 7.28 (d, J = 8.8 Hz, 1H), 6.83 (d, J = 8.8 Hz, 1H), 3.81 (s, 2H), 3.43 – 3.08 (m, 6H), 2.95 – 2.84 (m, 1H), 2.76 (d, J = 5.7 Hz, 2H), 1.82 – 1.71 (m, 4H), 1.67 – 1.45 (m, 2H); ^{13}C NMR (126 MHz, CDCl_3) δ 160.3, 160.1, 150.3, 146.9, 132.0, 123.1, 118.2, 113.5, 111.5, 107.3, 51.9, 43.2, 41.9, 25.1, 24.8, 23.7; HRMS (ESI-TOF) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{18}\text{H}_{21}\text{N}_3\text{NaO}_3$ 350.1475, found 350.1485.

7-(1,3-dioxan-2-yl)-8-hydroxy-3-methyl-1,2,3,4-tetrahydro-5H-chromeno[3,4-c]pyridin-5-one (7).: A mixture of **4** (100 mg, 330 μmol), 37% formaldehyde in H_2O (54 mg, 0.66 mmol) and Pd/C (10 wt.%, 80 mg) in 1:1 THF/dioxane (2 mL) was stirred for 6 h under H_2 atmosphere at room temperature. The reaction was filtered through celite, rinsed with MeOH and concentrated. Purification by silica gel flash chromatography (0 to 10% MeOH/ CHCl_3) gave **7** as a pale yellow solid (81 mg, 77%). ^1H NMR (400 MHz, CDCl_3) δ 8.83 (s, 1H), 7.41 (d, J = 8.8 Hz, 1H), 6.85 (d, J = 8.8 Hz, 1H), 6.33 (s, 1H), 4.31 (dd, J = 10.8, 5.1 Hz, 2H), 4.12 (td, J = 12.4, 2.4 Hz, 2H), 3.51 (d, J = 2.3 Hz, 2H), 3.01 – 2.92 (m, 2H), 2.87 – 2.79 (m, 2H), 2.58 (s, 3H), 2.29 (ddt, J = 13.9, 12.6, 5.1 Hz, 1H), 1.56 (ddt, J = 13.8, 2.6, 1.3 Hz, 1H); ^{13}C NMR (101 MHz, CDCl_3) δ 159.7, 159.2, 150.6, 145.8, 125.3, 116.5, 114.4, 111.8, 109.8, 98.1, 67.9, 51.9, 50.5, 45.2, 25.8, 25.4; HRMS (ESI-TOF) m/z $[2\text{M} + \text{H}]^+$ calcd for $\text{C}_{34}\text{H}_{39}\text{N}_2\text{O}_{10}$ 635.2599, found 635.2630.

(E)-N'-((8-hydroxy-3-methyl-5-oxo-1,3,4,5-tetrahydro-2H-chromeno[3,4-c]pyridin-7-yl)methylene)acetohydrazide (8).: A mixture of **3**²² (25 mg, 0.10 mmol), acetohydrazide (11 mg, 0.15 mmol) and acetic acid (1.2 mg, 19 μmol) in ethanol (5 mL) was heated at 95° C for 3 h. The reaction was cooled to room temperature and concentrated. Purification by silica

gel flash chromatography (0 to 10% MeOH/CHCl₃) gave **8** as a pale yellow solid (25 mg, 83%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.48 (s, 1H), 11.92 (s, 0.8H), 11.50 (s, 0.2H), 8.76 (s, 0.8H), 8.61 (s, 0.2H), 7.60 (d, *J* = 8.9 Hz, 1H), 6.91 (d, *J* = 8.8 Hz, 1H), 3.20 (s, 2H), 2.84 (t, *J* = 5.7 Hz, 2H), 2.62 (t, *J* = 5.6 Hz, 2H), 2.36 (s, 3H), 2.16 (s, 0.6H), 1.98 (s, 2.4H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.8, 160.5, 159.0, 151.2, 146.9, 141.9, 127.1, 117.6, 113.8, 111.6, 105.7, 52.3, 50.5, 45.7, 25.8, 21.7; HRMS (ESI-TOF) *m/z* [M + H]⁺ calcd for C₁₆H₁₈N₃O₄ 316.1292, found 316.1299.

(E)-8-hydroxy-3-methyl-7-((piperidin-1-ylimino)methyl)-1, 2,3,4-tetrahydro-5H-

chromeno[3,4-c]pyridin-5-one (9).: A mixture of **3**²² (25 mg, 0.10 mmol), 1-aminopiperidine (14 mg, 0.13 mmol) and anhydrous magnesium sulfate (60 mg, 0.50 mmol) in DCM (5 mL) was stirred for 30 min at room temperature. The reaction was diluted with DCM and water and the organic layer was washed with brine and dried over anhydrous MgSO₄. Purification by silica gel flash chromatography (0 to 10% MeOH/CHCl₃) gave **9** as a pale yellow solid (26 mg, 79%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.73 (s, 1H), 8.07 (s, 1H), 7.49 (d, *J* = 8.8 Hz, 1H), 6.85 (d, *J* = 8.8 Hz, 1H), 3.20 (s, 2H), 3.17 (t, *J* = 5.7 Hz, 4H), 2.85 (t, *J* = 5.8 Hz, 2H), 2.63 (t, *J* = 5.7 Hz, 2H), 2.36 (s, 3H), 1.69 (p, *J* = 5.6 Hz, 4H), 1.51 (td, *J* = 6.5, 3.5 Hz, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.8, 160.5, 159.0, 151.2, 146.9, 141.9, 127.1, 117.6, 113.8, 111.6, 105.7, 52.3, 50.5, 45.7, 25.8, 21.7; HRMS (ESI-TOF) *m/z* [M + H]⁺ calcd for C₁₉H₂₄N₃O₃ 342.1822, found 342.1813.

Allyl

(2-(7-hydroxy-6-methoxy-2-oxo-2H-chromen-4-yl)ethyl)carbamate (11).: A mixture of methyl 5-(((allyloxy)carbonyl)amino)-3-oxopentanoate (327 mg, 1.43 mmol) and 4-methoxybenzene-1,3-diol (200 mg, 1.43 mmol) in methanesulfonic acid (10 mL) was stirred for 1 h at room temperature. Ice was added to the reaction slowly to quench the reaction and diluted with EtOAc. After removing the aqueous layer, the organic layer was washed with sat. aq. NaHCO₃ and concentrated. The resulting solid was washed with EtOAc to give pure **11** as a yellow solid (382 mg, 84%). ¹H NMR (500 MHz, CD₃OD) δ 7.31 (s, 1H), 6.75 (s, 1H), 6.11 (s, 1H), 5.89 (ddt, *J* = 15.8, 10.6, 5.4 Hz, 1H), 5.26 (dd, *J* = 17.2, 1.7 Hz, 1H), 5.16 (dd, *J* = 10.6, 1.7 Hz, 1H), 4.51 (d, *J* = 5.4 Hz, 2H), 3.95 (s, 3H), 3.42 (t, *J* = 7.2 Hz, 2H), 2.97 (t, *J* = 7.1 Hz, 2H); ¹³C NMR (126 MHz, CD₃OD) δ 162.6, 157.3, 155.1, 151.4, 149.6, 145.6, 133.0, 116.1, 111.0, 110.3, 105.5, 102.8, 64.9, 55.6, 39.7, 32.3; HRMS (ESI-TOF) *m/z* [M + H]⁺ calcd for C₁₆H₁₈NO₆ 320.1129, found 320.1132.

Allyl

7-formyl-8-hydroxy-9-methoxy-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridine-3(4H)-carboxylate (12).: Acetic anhydride (1.12 g, 10.9 mmol) was added to a mixture of **11** (700 mg, 2.19 mmol) and pyridine (87 mg, 1.1 mmol) in acetonitrile (70 mL) and the reaction was stirred for 18 h at room temperature. The reaction was diluted with EtOAc, washed with brine and concentrated. The resulting crude material was dissolved in TFA (25 mL), treated with hexamethylenetetramine (768 mg, 5.48 mmol) and stirred for 18 h at 75° C. The reaction was concentrated, dissolved in THF (50 mL) and aq. 1M HCl (50 mL) and stirred for 2 h at 60° C. The reaction was diluted with EtOAc and the organic layer washed with brine. Purification by silica gel flash chromatography (0 to 10% MeOH/CHCl₃) gave **12** as a

yellow solid (383 mg, 49% over 2 steps). ^1H NMR (400 MHz, CDCl_3) δ 12.44 (s, 1H), 10.59 (s, 1H), 7.10 (s, 1H), 5.95 (ddt, $J = 16.4, 10.9, 5.6$ Hz, 1H), 5.33 (dd, $J = 17.2, 1.6$ Hz, 1H), 5.24 (dd, $J = 10.4, 1.5$ Hz, 1H), 4.64 (d, $J = 5.7$ Hz, 2H), 4.47 (s, 2H), 3.95 (s, 3H), 3.82 (t, $J = 5.7$ Hz, 2H), 2.86 (t, $J = 5.8$ Hz, 2H); ^{13}C NMR (101 MHz, CDCl_3) δ 193.5, 158.4, 155.6, 155.1, 148.2, 145.9, 145.5, 132.6, 118.1, 110.9, 110.1, 108.8, 66.5, 56.8, 41.9, 39.1, 25.0; HRMS (ESI-TOF) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{18}\text{H}_{18}\text{NO}_7$ 360.1078, found 360.1076.

7-(1,3-dioxan-2-yl)-8-hydroxy-9-methoxy-1,2,3,4-tetrahydr o-5H-chromeno[3,4-c]pyridin-5-one (13): A mixture of **12** (721 mg, 2.00 mmol), 1,3-propandiol (458 mg, 6.02 mmol) and pTsOH·H₂O (19 mg, 0.1 mmol) in toluene (70 mL) was refluxed with Dean-Stark apparatus at 150 °C for 4 h. Triethylamine (10 drops) was added and the reaction was concentrated. The crude material was then diluted with EtOAc. The organic layer was washed with water and brine and dried over anhydrous MgSO_4 . Purification by silica gel flash chromatography (0 to 5% MeOH/ CHCl_3) gave the intermediate acetal as a pale yellow solid (776 mg, 93%). ^1H NMR (400 MHz, CDCl_3) δ 8.86 (s, 1H), 6.77 (s, 1H), 6.24 (s, 1H), 5.89 (ddt, $J = 16.0, 10.9, 5.6$ Hz, 1H), 5.26 (dd, $J = 17.2, 1.6$ Hz, 1H), 5.17 (dd, $J = 10.4, 1.4$ Hz, 1H), 4.58 (d, $J = 5.7$ Hz, 2H), 4.36 (s, 2H), 4.25 (dd, $J = 10.8, 5.1$ Hz, 2H), 4.06 (td, $J = 12.4, 2.3$ Hz, 2H), 3.82 (s, 3H), 3.76 (t, $J = 5.8$ Hz, 2H), 2.75 (t, $J = 5.8$ Hz, 2H), 2.33 – 2.16 (m, 1H), 1.50 (d, $J = 14.4$ Hz, 1H); ^{13}C NMR (101 MHz, CDCl_3) δ 159.4, 155.2, 149.6, 146.1, 145.7, 144.7, 132.7, 117.8, 116.7, 110.9, 110.4, 105.6, 98.2, 67.9, 66.4, 56.6, 41.9, 39.2, 25.7, 24.8; HRMS (ESI-TOF) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{21}\text{H}_{24}\text{NO}_8$ 418.1496, found 418.1496.

The above hydrazone (26 mg, 62 μmol) was subjected to Alloc deprotection as described for the synthesis of **5**. The crude residue was purified by silica gel flash chromatography (0 to 10% MeOH/ CHCl_3) to give **13** as an orange solid (19 mg, 93%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.33 (s, 1H), 7.13 (s, 1H), 6.11 (s, 1H), 4.15 (dd, $J = 11.4, 4.7$ Hz, 2H), 3.93 (t, $J = 11.6$ Hz, 2H), 3.87 (s, 3H), 3.75 (s, 2H), 3.21 (s, 2H), 2.93 (s, 2H), 2.11 (tt, $J = 12.6, 7.5$ Hz, 1H), 1.43 (d, $J = 13.4$ Hz, 1H); ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 164.6, 153.3, 152.3, 150.0, 149.9, 123.6, 116.6, 116.1, 110.9, 101.6, 72.3, 63.1, 61.5, 48.0, 46.4, 30.4, 29.9; HRMS (ESI-TOF) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{20}\text{NO}_6$ 334.1285, found 334.1286.

7-(1,3-dioxan-2-yl)-8-hydroxy-9-methoxy-3-methyl-1,2,3,4- tetrahydro-5H-chromeno[3,4-c]pyridin-5-one (14; D-F07): A mixture of **13** (272 mg, 820 μmol), 37% formaldehyde in H₂O (133 mg, 660 μmol) and Pd/C (10 wt.%, 200 mg) in 1:1 THF/dioxane (30 mL) was stirred for 5 h under H₂ atmosphere at room temperature. The reaction was filtered through celite, rinsed with MeOH and concentrated. Purification by silica gel flash chromatography (0 to 7% MeOH/ CHCl_3) gave **14** as an orange solid (258 mg, 91%). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 9.43 (s, 1H), 7.11 (s, 1H), 6.11 (s, 1H), 4.15 (dd, $J = 11.5, 4.7$ Hz, 2H), 3.93 (t, $J = 11.8$ Hz, 2H), 3.85 (s, 3H), 3.20 (s, 2H), 2.88 (s, 1H), 2.64 (t, $J = 5.7$ Hz, 2H), 2.49 (s, 3H), 2.37 (s, 2H), 2.11 (qt, $J = 12.1, 4.8$ Hz, 1H), 1.43 (d, $J = 13.3$ Hz, 1H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 159.7, 148.6, 146.7, 145.3, 117.6, 111.9, 111.0, 106.3, 96.9, 67.5, 56.7, 52.5, 50.6, 45.6, 32.0, 26.0. 25.7; HRMS (ESI-TOF) m/z $[\text{M} + \text{K}]^+$ calcd for $\text{C}_{18}\text{H}_{21}\text{KNO}_6$ 386.1000, found 386.1005.

(E)-N'-((8-hydroxy-9-methoxy-3-methyl-5-oxo-1,3,4,5-tetrahydro-2H-chromeno[3,4-c]pyridin-7-yl)methylene)acetohydrazide (15).: 4M aq. HCl (4 mL) was added to a solution of **14** (40 mg, 0.12 mmol) in 1,4-dioxane (1 mL) and the reaction was stirred for 1h at room temperature. The reaction was neutralized with 4M aq. NaOH, poured to silica gel (3 g) and concentrated to silica gel powder. Purification by solid load silica gel flash chromatography (0 to 10% MeOH/CHCl₃) gave the intermediate aldehyde, which was used directly in the following step.

A mixture of the crude residue above, acetohydrazide (13 mg, 0.17 mmol) and acetic acid (1.4 mg, 23 μmol) in ethanol (10 mL) was heated at 95° C for 3 h. The reaction was cooled to room temperature and concentrated. Purification by silica gel flash chromatography (0 to 20% MeOH/CHCl₃) gave **15** as a orange solid (11 mg, 27% over 2 steps). ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.63 (s, 1H), 11.91 (s, 0.8H), 11.50 (s, 0.2H), 8.74 (s, 0.8H), 8.60 (s, 0.2H), 7.12 (s, 1H), 3.83 (s, 3H), 3.18 (s, 2H), 2.86 (t, *J* = 5.8 Hz, 2H), 2.61 (t, *J* = 5.7 Hz, 2H), 2.34 (s, 3H), 2.14 (s, 0.6H), 1.96 (s, 2.4H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.8, 159.2, 147.0, 145.4, 145.3, 142.2, 139.7, 118.0, 117.8, 107.8, 106.0, 56.7, 52.5, 50.5, 45.7, 26.1, 21.7; HRMS (ESI-TOF) *m/z* [M + H]⁺ calcd for C₁₇H₂₀N₃O₅ 346.1398, found 346.1394.

2-(trimethylsilyl)ethyl

4-(1-((7-(1,3-dioxan-2-yl)-9-methoxy-3-methyl-5-oxo-1,3,4,5-tetrahydro-2H-chromeno[3,4-c]pyridin-8-yl)oxy)ethyl)-3-nitrobenzoate (16; PC-D-F07).: A mixture of **14** (100 mg, 290 μmol), 2-(trimethylsilyl)ethyl 4-(1-bromoethyl)-3-nitrobenzoate (162 mg, 430 μmol) and *N,N*-diisopropylethylamine (74 mg, 0.58 mmol) in acetonitrile (5 mL) was stirred for 24 h at 65° C. The reaction and diluted with EtOAc, washed with water and brine and dried over anhydrous MgSO₄. Purification by silica gel flash chromatography (0 to 5% MeOH/CHCl₃) gave **16** as an orange solid (24 mg, 13%). ¹H NMR (400 MHz, CDCl₃) δ 8.48 (s, 1H), 8.22 (s, 2H), 6.79 (s, 1H), 6.27 (s, 1H), 6.11 (q, *J* = 6.3 Hz, 1H), 4.46 – 4.36 (m, 2H), 4.30 – 4.16 (m, 2H), 4.04 – 3.91 (m, 2H), 3.59 (s, 3H), 3.37 (s, 2H), 2.78 (t, *J* = 5.8 Hz, 2H), 2.67 (t, *J* = 5.4 Hz, 2H), 2.45 (s, 3H), 2.36 – 2.19 (m, 1H), 1.71 (d, *J* = 6.3 Hz, 3H), 1.41 (d, *J* = 13.4 Hz, 1H), 1.18 – 1.03 (m, 2H), 0.05 (s, 9H); ¹³C NMR (101 MHz, CDCl₃) δ 164.6, 159.7, 148.8, 147.8, 147.5, 145.1, 144.6, 143.9, 133.6, 130.6, 129.1, 124.8, 120.3, 119.5, 115.0, 106.1, 96.5, 67.8, 67.7, 64.2, 56.0, 52.4, 50.6, 45.5, 29.7, 26.0, 25.7, 23.1, 17.5, (–)1.5; HRMS (ESI-TOF) *m/z* [M + H]⁺ calcd for C₃₂H₄₁N₂O₁₀Si 641.2516, found 641.2538.

Antibodies and reagents.

Antibodies against XBP-1s (Cell Signaling Technology) and p97 (Fitzgerald) were obtained commercially. LPS was purchased from Sigma-Aldrich.

Cell culture.

5TGM1 mouse multiple myeloma cells, primary mouse B cells, and WaC3 human CLL cells were cultured at 37 °C in a 5% CO₂ incubator using RPMI 1640 media (Gibco) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin G sodium, 2 mM L-

glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 µg/mL streptomycin sulfate and 0.1 mM β-mercaptoethanol (β-ME).

Protein isolation and immunoblotting.

Cells were lysed using RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, and 1 mM EDTA) supplemented with protease inhibitors. Protein concentrations were measured by BCA assays. Samples were boiled in the SDS-PAGE sample buffer with β-ME, and analyzed by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes, blocked in 5% nonfat milk (w/v in PBS), and immunoblotted with primary antibodies and appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. The p97 serves as a loading control. Immunoblots were developed using Western Lighting Chemiluminescence Reagent.

Surface plasmon resonance.

Surface plasmon resonance measurement was performed using a Biacore T200 system (GE Healthcare). A total of 9700 resonance units of human IRE-1 (59.2 kD polyhistidine-tagged puritin-hIRE-1 fusion protein¹²) was immobilized through amine coupling (EDC/NHS) in the flow well of a CM5 sensor chip at a speed of 5 µL/min, and a control well was similarly treated with buffer only (PBS, pH 7.4, containing 2% DMSO). Inhibitors of various concentrations were injected at a speed of 40 µL/min in PBS, pH 7.4, containing 2% DMSO. Chip surface was regenerated by injecting 10 µL solution of 50 mM aq. NaOH at a speed of 5 µL/min. Resonance differences between the wells containing proteins and the control wells were plotted against inhibitor concentrations. Determination of K_d was performed through asymptotic 1 fitting of the curve to steady-state one site binding model (OriginPro 8.5).

Cell proliferation assays.

5TGM1 and WaC3 cells were suspended in phenol red-free complete RPMI media, seeded in 96-well cell culture plates, and treated with IRE-1 inhibitors for indicated times. Cells were spun down before proliferation was assessed by XTT assays following the manufacturer's instructions (Roche). Briefly, 1 µL electron-coupling reagent, 50 µL XTT labeling reagent, and 100 µL phenol red-free complete RPMI media were combined and added to each well of the 96-well plates. Cells were incubated in a 5% CO₂ incubator for additional 4 h to allow for the yellow tetrazolium salt XTT to be cleaved by mitochondrial dehydrogenases in metabolically active cells to form the orange formazan dye, which can be detected at 492 nm using a BioTek Synergy™ NEO2 Multimode Reader.

Compound degradation HPLC assay.

A 20 mM DMSO stock solution of the analyte compound (**7**, **8**, **D-F07**, or **PC-D-F07**) was diluted to 400 µM with PBS, pH 7.4, or to 800 µM with complete RPMI media. After incubation at 37 °C, aliquots at various time intervals were injected onto an Agilent Infinity II 1260 analytical HPLC instrument and the peaks integrated (UV monitored at 220 nm, 30 min linear gradient 5–95% MeCN in H₂O). Intact compound relative to t = 0 was plotted as a function of time.

Confocal microscopy.

5TGM1 cells were cultured in the 12-well plate for 12 h before treatment. Live cells were treated with DMSO or 10 μ M **PC-D-F07** for 4 h, washed with PBS for 3 times, fixed in 4% paraformaldehyde solution at room temperature for 15 min, washed with PBS again, resuspended with 100 μ L PBS, seeded onto a clean coverslip, and air-dried. Cells were rehydrated with PBS, mounted on a glass slide, and observed using a Leica TCS SP5 II confocal microscope. Photo-activation of **PC-D-F07** was conducted using the DAPI filtered diode laser (50 mW). **D-F07**, resulted from **PC-D-F07**, was excited at 488 nm, observed at the emission wavelength from 498 to 605 nm, and expressed as green.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

XBP-1s	X-box binding protein 1 spliced form
IRE-1	inositol-requiring enzyme 1

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Figure 1. Development of potent tricyclic chromenone inhibitors of IRE-1 by installation of different amine substituents and protective moieties; IRE-1 inhibitors with 1,3-dioxane acetal protective groups emit strong fluorescence.

(A) Chemical structures of **1**, **2**, and **3** with different amine substituent groups (R^1). (B) IRE-1 binding affinities of **2** and **3** as determined by surface plasmon resonance. (C) 5TGM1 cells were treated with **1**, **2**, and **3** at 10 μM for 0, 3, 6, 12, or 24 h, lysed, and analyzed for indicated proteins by immunoblots. (D) Chemical structures of derivatives of **2** and **3** carrying different protective groups (R^2). (E) Fluorescence intensities of **2** and **3** derivatives with different protective groups in aqueous solution (10 μM , DMSO/PBS mixture (v/v = 1:99), $E_x = 360 \text{ nm}$). (F) The enlarged fluorescence spectra of **2**, **3**, **6**, **8**, and **9** in aqueous solution (10 μM , DMSO/PBS mixture (v/v = 1:99), $E_x = 360 \text{ nm}$). (G) Fluorescence images of **2** and **3** derivatives with different protective groups in aqueous solution (10 μM , DMSO/PBS mixture (v/v = 1:99)) under illumination at 365 nm.

Figure 2. Tricyclic chromenone inhibitors with an N-acetohydrazone protective group can rapidly inhibit the expression of XBP-1s in cancerous B cells.

(A) 5TGM1 cells were treated with **2**, **4**, **3**, or **7** at 10 μM for a course of 3 days, lysed, and analyzed for indicated proteins by immunoblots. (B) 5TGM1 cells were treated with DMSO or with 20 μM **2**, **4**, **3**, or **7** for 4 days and subjected to XTT assays. Percentages of growth were determined by comparing inhibitor-treated groups with DMSO-treated groups. Data from four independent groups receiving the same treatment were plotted as means $\pm$ SD. Results are representative of three independent experiments. (C) 5TGM1 cells were treated with **4** or **5** using indicated concentrations for 24 h, lysed, and analyzed for indicated proteins by immunoblots. (D-E) Fluorescence stability of **4** (D) or **5** (E) in aqueous solution at 37 $^{\circ}\text{C}$ from 0 to 48 h (10 μM , DMSO/PBS mixture (v/v = 1:99), $E_{\lambda} = 360$ nm). The values of I/I_0 were plotted as a function of time and shown in the inset. I_0 was the initial fluorescence intensity of **4** (D) or **5** (E) at 0 h. Results (means $\pm$ SD) are representative of three independent experiments. (F) 5TGM1 cells were treated with **6**, **4**, **7**, **8**, or **9** at 2.5 μM for 1, 2, or 4 h, lysed, and analyzed for indicated proteins by immunoblots. (G) The decomposition rates of **7** and **8** in RPMI media at 37 $^{\circ}\text{C}$ were plotted as a function of time. Compounds in medium aliquots ($n = 3$) were analyzed by HPLC and the peaks integrated. Results (means $\pm$ SD) are representative of three independent experiments.

Figure 3. Installation of a methoxy group to tricyclic chromenone inhibitors of IRE-1 enhances their activities in suppressing the expression of XBP-1s.

(A) Chemical synthesis of IRE-1 inhibitors carrying a methoxy group. (B) 5TGM1 cells were treated with indicated inhibitors at 10 μ M for a course of 3 days, lysed, and analyzed for indicated proteins by immunoblots. (C) 5TGM1 cells were treated with DMSO or with 20 μ M **3**, **7**, or **D-F07** for 3 days and subjected to XTT assays. Percentages of growth were determined by comparing inhibitor-treated groups with DMSO-treated groups. Data from four independent groups receiving the same treatment were plotted as means $\pm$ SD. Results are representative of three independent experiments. (D) WaC3 cells were treated with DMSO or with 20 μ M **7**, **8**, **15**, or **D-F07** for 3 days and subjected to XTT assays. Percentages of growth were determined by comparing inhibitor-treated groups with DMSO-treated groups. Data from four independent groups receiving the same treatment were plotted as means $\pm$ SD. Results are representative of three independent experiments. (E) Fluorescence intensities of **13**, **15**, and **D-F07** in aqueous solution (10 μ M, DMSO/PBS mixture (v/v = 1:99), E_x = 360 nm).

Figure 4. Installation of a photo-labile cage on the hydroxy group stabilizes the 1,3-dioxane acetal of PC-D-F07 and allows for UV irradiation-mediated generation of D-F07.

(A) The chemical structure of **PC-D-F07**. (B) The fluorescence of **PC-D-F07** (10 µM in DMSO/PBS solution (v/v = 1:99), $E_x = 360$ nm) could be ‘turned on’ by increasing exposure time to UV irradiation (4W, 1 mW/cm²). The inset showed gradually enhanced blue fluorescence resulted from liberated **D-F07**. (C) 5TGM1 cells were treated with DMSO or **PC-D-F07** (10 µM) for 4 h, washed with PBS for 3 times, fixed and analyzed by confocal microscopy. The fluorescence spectrum scan was recorded in the range of 498–650 nm. Scale bar = 10 µm. (D) The fluorescence, within the linear range, in the cytoplasm of 95 DMSO-treated (control) and 185 **PC-D-F07**-treated 5TGM1 cells was used to calculate for the mean fluorescence intensity. The fluorescence intensity at 10 s (I) was compared with the background fluorescence at 0 s (I_0 , DAPI filtered). Data (I/I_0) were plotted as means $\pm$ SD. (E) 5TGM1 cells were treated with DMSO, **D-F07**, or **PC-D-F07** at 10 µM, immediately subjected to UV irradiation (4W, 1 mW/cm²) for 0, 3, 5, or 10 min, cultured for

additional 4 h, lysed, and analyzed for indicated proteins by immunoblots. (**F** and **G**) The decomposition rates of **D-F07** and **PC-D-F07** were plotted as a function of time in PBS (**F**) or RPMI media (**G**) at 37 °C. Compounds in PBS and medium aliquots (n = 3) were analyzed by HPLC and the peaks integrated. Results (means ± SD) are representative of three independent experiments. (**H**) 5TGM1 cells were treated with **D-F07** or **PC-D-F07** at 10 µM for 4 h, washed for 3 times with cold PBS, resuspended in fresh RPMI media, subjected to UV irradiation (4W, 1 mW/cm²) for 0 or 10 min, cultured for additional 0, 4, and 8 h, lysed, and analyzed for indicated proteins by immunoblots.