

Valosin-containing Protein is a Target of 5'-I Fuligocandin B and Enhances TRAIL Resistance in Cancer Cells

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Dedicated to Professor Stuart L. Schreiber on the occasion of his 60th birthday

Fuligocandin B (**2**) is a novel natural product that can overcome TRAIL resistance. We synthesized enantiomerically pure fuligocandin B (**2**) and its derivative 5'-I fuligocandin B (**4**), and found that the latter had an improved biological activity against the human gastric cancer cell line, AGS. We attached a biotin linker and photoactivatable aryl diazine group to 5'-I fuligocandin B (**4**), and employed a pull-down assay to identify valosin-containing protein (VCP/p97), an AAA ATPase, as a 5'-I fuligocandin B (**4**) target protein. Knock-down of VCP by siRNA

enhanced sensitivity to TRAIL in AGS cells. In addition, **4** enhanced CHOP and DR5 protein expression, and overall intracellular levels of ubiquitinated protein. These data suggest that endoplasmic reticulum stress caused through VCP inhibition by **4** increases CHOP-mediated DR5 up-regulation, which enhances TRAIL-induced cell death in AGS cells. To the best of our knowledge, this is the first example to show a relationship between VCP and TRAIL-resistance-overcoming activity in cancer cells.

1. Introduction

Fuligocandin A (**1**) and B (**2**) are novel natural cycloanthranilyl-proline derivatives that were initially isolated from the slime mold, *Fuligo candida*, in 2004 by our group (Figure 1).^[1] We previously reported the ability of **2** to overcome tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) resistance in adult T-cell leukemia/lymphoma (ATLL)^[2] and human gastric adenocarcinoma (AGS) cells.^[3] TRAIL can induce apoptosis in various cancer cells, but not in most normal cells, and thus represents a promising target for cancer therapy.^[4] Apoptosis can be triggered through the interaction between TRAIL and its receptors, death receptor 4 and 5 (DR4 and DR5, respectively). However, many cancer cells are resistant to TRAIL-induced apoptosis. Therefore, overcoming TRAIL resist-

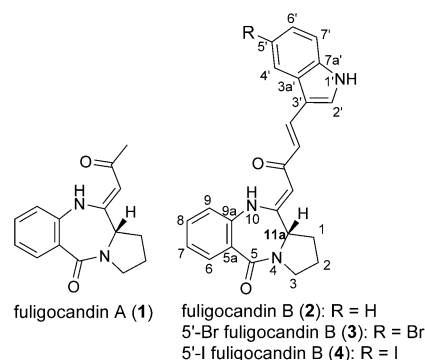


Figure 1. The structure of fuligocandin A (**1**), fuligocandin B (**2**), 5'-Br fuligocandin B (**3**), and 5'-I fuligocandin B (**4**).

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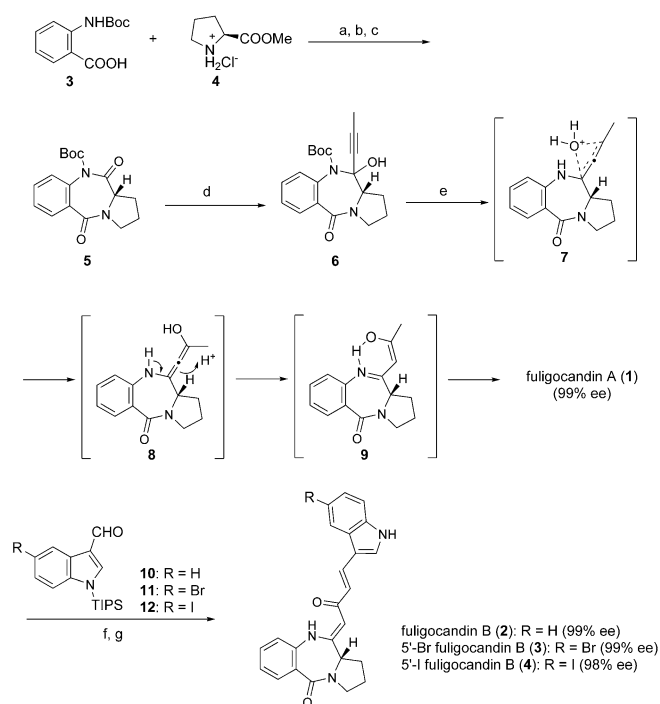
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ance by treatment with small molecules would be a useful approach for cancer treatment.^[5] Although several small-molecule TRAIL inducers have been reported, including luteolin,^[6a] flavopiridol,^[6b] azadirone,^[6c] and 2-methoxyestradiol,^[6d] there remains a strong impetus to find more efficient small molecules that can sensitize cancer cells to TRAIL. To identify natural products that make cancer cells sensitive to TRAIL, we have isolated potential natural products and evaluated their TRAIL-resistance-overcoming activity.^[7]

In this study, we describe the synthesis of derivatives of **2** and their activities. To identify a target protein of 5'-I fuligocandin B (**4**), which is a more active form of **2**, a biotin derivative of **4** was synthesized for a pull-down assay. The valosin-containing protein (VCP/p97), an AAA (ATPases associated with diverse cellular activities) ATPase family protein, was identified as a molecular target of **4**. A plausible mechanism of action of **4** is also presented.

2. Results and Discussion

An optically pure fuligocandin A (**1**) was synthesized by More et al. in 2009, using the aza-Wittig method.^[8] A practical racemic first total synthesis of fuligocandin B (**2**) was achieved by Bergman and co-workers^[9] in 2010, using Eschenmoser episulfide contraction as the key step. We also achieved a total synthesis of fuligocandin A (**1**) and B (**2**) with moderate enantiomeric excess (**1**; 70% *ee*, **2**; 61% *ee*).^[3] The first asymmetric total synthesis of **2** was reported in 2011,^[10] at which time the synthesis of interesting vinylogous amides was also reported. Our synthesis utilizes the Meyer–Schuster rearrangement as a key step, and fuligocandin B (**2**) can be synthesized in seven steps. We were able to apply our synthetic route to begin to explore the structure–activity relationships (SARs) in **2**. Following our established protocol, we undertook a coupling reaction of *N*-Boc-anthranilic acid, **3**, and *L*-proline methyl ester (**4**). After hydrolysis of the methyl ester with LiOH, intramolecular cyclization produced *N*-Boc-cycloanthranilylproline (**5**).^[3] To obtain optically pure fuligocandins, we altered our protocol. We were able to eliminate the racemization caused by propynyllithium by using propynylmagnesium bromide (Scheme 1). The Meyer–Schuster rearrangement of **6** under acidic conditions proceeded at -20°C to generate fuligocandin A (**1**) in 86% yield, with 99% *ee*, as a *Z* isomer.^[11] The rearrangement proceeded through the corresponding hydroxyallene **8**, fol-



Scheme 1. Synthesis of fuligocandin B derivatives. Reagents and conditions: a) *L*-proline methyl ester hydrochloride (**4**) (1.5 equiv), EDC-HCl (1.2 equiv), HOBt (1.3 equiv), TEA (5.0 equiv), CH_2Cl_2 , RT, 24 h, 77%; b) LiOH-H₂O (2.0 equiv), THF/MeOH/H₂O, RT, 17 h, 95%; c) PyBOP (1.3 equiv), DIEA (4.0 equiv), CH_2Cl_2 , RT, 72 h, 90%; d) propynyl magnesium bromide (1.5 equiv), THF, -78°C , 1 h, 90%; e) TFA/ CH_2Cl_2 = 1:1, -20°C , 8 h, 86%, 99% *ee*; f) **10**–**12** (10 equiv), diisopropylamine (10 equiv), *n*BuLi (10 equiv), THF, -78 to 0°C , 1.5 h; g) THF/1 *N* HCl, 24 h, 24% (**2**), 63% (**3**), 36% (**4**) (2 steps).

lowed by protonation to give **9**. Fuligocandin B (**2**) was synthesized through an aldol condensation with indole aldehyde **10** and **1** in 24% yield, with 99% *ee*.^[12] 5'-Br fuligocandin B (**3**) (91% *ee*) and 5'-I fuligocandin B (**4**) (95% *ee*) were also synthesized.

Next, we assessed the TRAIL-resistance-overcoming activity of the synthesized fuligocandin B derivatives (**2**–**4**), and found that three compounds were active (Figure 2). It should be emphasized that fuligocandin A (**1**) was not active (data not shown). To compare of the activities of **2**–**4**, we assessed cell

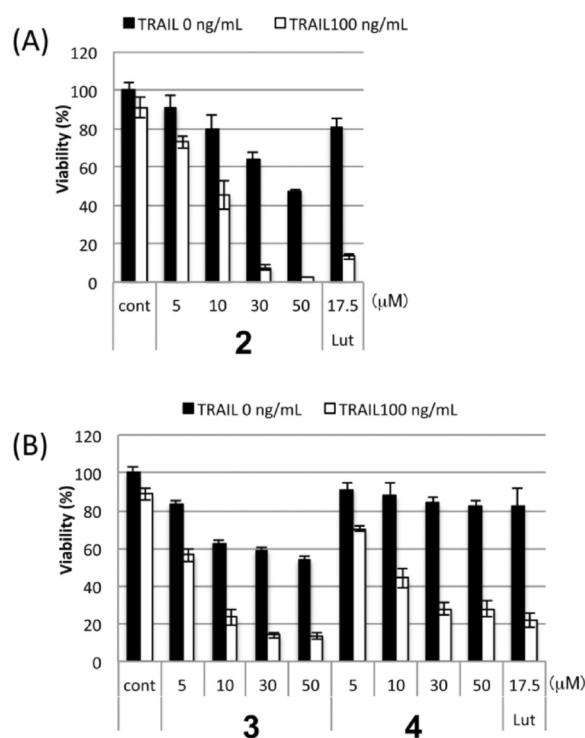


Figure 2. TRAIL-resistance-overcoming activity of fuligocandin B (**2**), 5'-Br fuligocandin B (**3**), and 5'-I fuligocandin B (**4**) against AGS cells. Lut: luteolin (positive control).

viability by using each compound at $10\ \mu\text{M}$. The larger the difference in cell viability between that measured in the absence and that in the presence of TRAIL, the more active the compound is. 5'-I fuligocandin B (**4**) ($10\ \mu\text{M}$) produced a 43.4% reduction in cell viability in the presence of TRAIL, whereas fuligocandin B (**2**) and 5'-Br fuligocandin B (**3**) produced 35.0 and 39.4% reductions, respectively. We, therefore, selected **4** as the most active in this assay to be used for further analysis. To address the molecular mechanisms underlying the mode of action of **4**, we isolated the target proteins of **4** by using a pull-down method.^[13] For a pull-down experiment, compound **4** needs to be modified to allow its immobilization on a matrix. We chose avidin-immobilized beads as a matrix and a biotin tag to modify **4**.

As a first step, a biotin probe **13** (Figure 3), without a photoactivatable group, was synthesized (see the Supporting Information). However, pull-down experiments were unsuccessful and no specific protein was detected. Therefore, a biotin probe

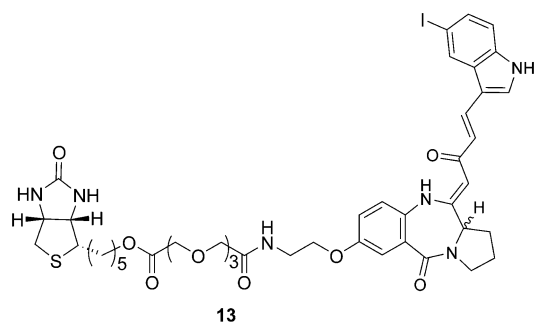


Figure 3. Biotin probe 13.

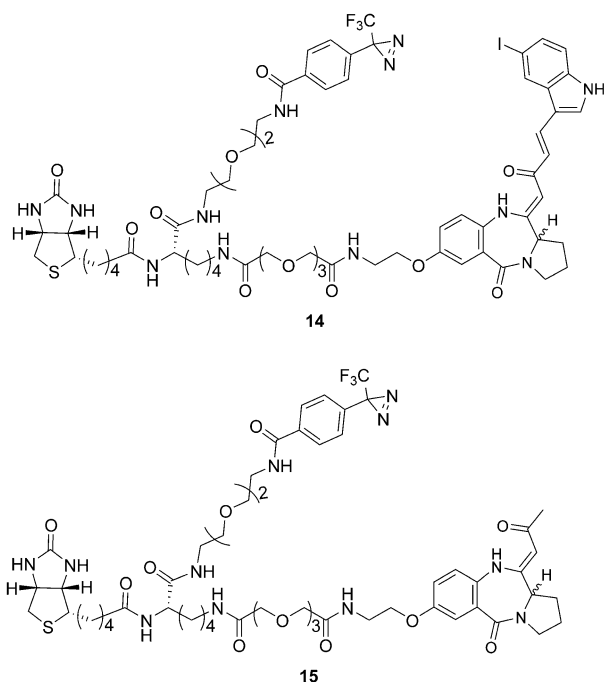
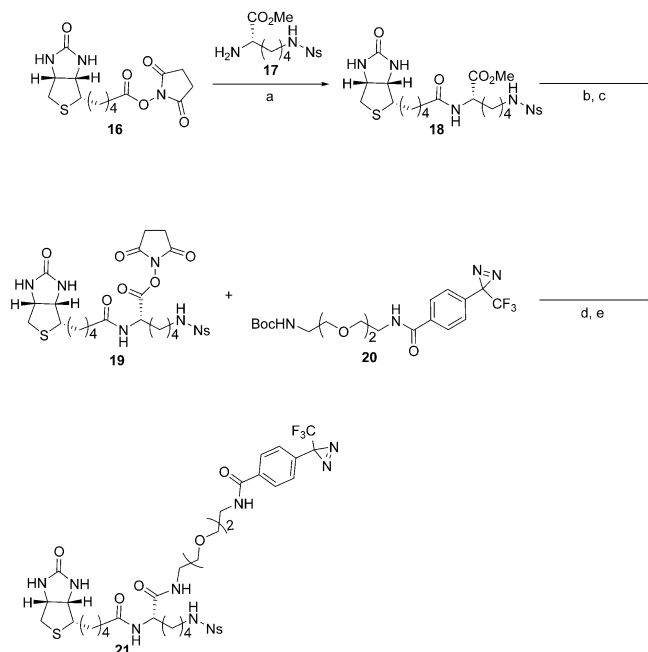


Figure 4. Biotin probe 14 and negative control probe 15.

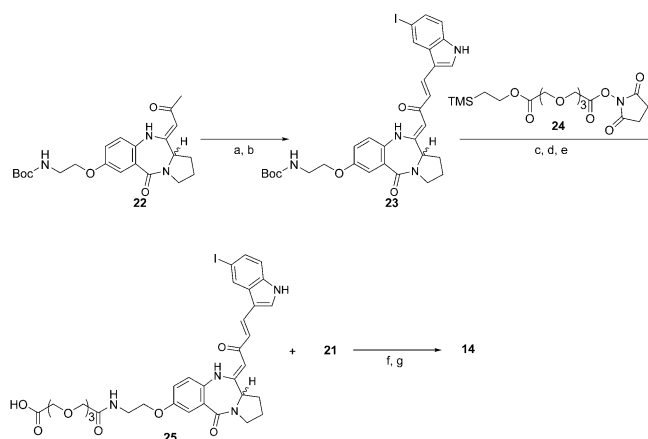
with 3-trifluoromethyl-3-aryl diazirine^[14] as a photoactivatable unit (14) was designed, as shown in Figure 4. As the indole unit is most important for activity,^[15] a point distant from the indole unit was selected for linker attachment. However, because there was some evidence that bulkiness around the 7-position decreased activity, the linker was made as small as possible. L-Lysine was chosen as the branch point to attach the photoaffinity unit. To improve hydrophilicity, a polyethylene glycol unit was introduced. As 1 did not have TRAIL-resistance-overcoming activity, a negative control probe 15 was also designed.^[16]

We planned a convergent synthesis by coupling a biotin unit 21 and fuligocandin B unit 25. For the synthesis of biotin unit 21, a diazirine unit was introduced at the latter step. Starting from the biotinylated compound 16, an amide formation with a L-lysine derivative 17 produced compound 18 (Scheme 2). After making a succinimidyl ester 19, a coupling reaction with a diazirine unit generated the biotinylated com-



Scheme 2. Reagents and conditions: a) 17 (1.2 equiv), TEA (2.0 equiv), DMF, 1.5 h, quant.; b) LiOH (2.0 equiv), THF/MeOH/H₂O, 3 h; c) NHS (1.3 equiv), EDC (1.3 equiv), DMF, 2 h; d) TFA/CH₂Cl₂, -10 °C, 30 min; e) TEA (10.0 equiv), DMF, 35 min, 89% (2 steps).

pound 21. The 5'-I fuligocandin B unit was synthesized as shown in Scheme 3. An aldol reaction between fuligocandin A derivative 22 and indole aldehyde 12 gave compound 23. Amide formation with linker unit 24, followed by hydrolysis, generated compound 25. Synthesis of 14 was achieved through a coupling reaction with 21. By using the same approach, a version of fuligocandin A 15 was synthesized as a negative control (see the Supporting Information). Probe 14



Scheme 3. Reagents and conditions: a) 12 (5.0 equiv), diisopropylamine (10.2 equiv), *n*BuLi (10.0 equiv), -78 °C → 0 °C, 3 h; b) TBAF (5.0 equiv), THF/1N aq. HCl, 15 h, 36% (2 steps from 22); c) TFA/CH₂Cl₂ = 1:1, -20 °C, 1 h; d) 24 (1.2 equiv), TEA/CH₂Cl₂ = 1:1, overnight, 59% (2 steps from 23); e) LiOH (2.0 equiv), THF/MeOH/H₂O, 26 h, 44%; f) PhSH (1.5 equiv), K₂CO₃ (2.0 equiv), DMF, 80 °C, 5 min; g) PyBOP (3.0 equiv), DIEA (4.0 equiv), DMF, 30 min, 29% (purified by HPLC).

retained TRAIL-resistance-overcoming activity against AGS cells (see the Supporting Information).

Pull-down experiments were performed by using the newly synthesized probes. The AGS cells treated with ligands **14** or **15** were UV irradiated (365 nm for 20 min on ice) and lysed, and the bound proteins were collected on avidin-immobilized beads. After SDS-PAGE (Figure 5A), the proteins pulled down

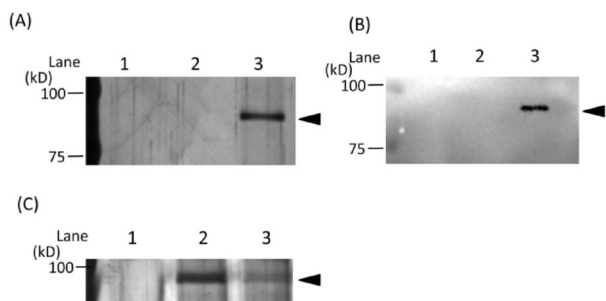


Figure 5. Detection of proteins bound by probes **14** and **15**. AGS cells were incubated with or without compounds at 37 °C for 24 h. Cells were UV irradiated, Nuetravidin beads were added, and the lysate was incubated at 4 °C for 1 h. The beads were washed, re-suspended in SDS sample buffer, heated and subjected to SDS-PAGE. The gel was visualized by silver staining. A) Lane 1: AGS lysate from DMSO treated cells (control); Lane 2: AGS lysate from cells incubated with probe **15** (negative control); Lane 3: AGS lysate from cells incubated with **14**. B) Western blot analysis. Protein bound specifically to probe **14** was reactive to anti-VCP antibody. C) A competitive experiment. Lane 1: AGS lysate from cells incubated with probe **15** (5 μM) (negative control); Lane 2: AGS lysate from cells incubated with **14** (5 μM); Lane 3: AGS lysate from cells incubated with **14** (5 μM) and **4** (25 μM).

by **14** were excised and digested with trypsin. Peptide fragments were analyzed by using LC-MS/MS and several proteins were identified. The dominant 89 kDa protein band appearing on the gel was determined to be VCP,^[17] which was confirmed by western blot analysis (Figure 5B). When the cells were treated with **14** (5 μM) and 5'-I fuligocandin B (**4**) (25 μM), the VCP band was dramatically decreased (Figure 5C). VCP is a member of the AAA ATPase family of chaperone-like proteins that control important cellular processes, such as ubiquitin-dependent protein degradation, through proteasomes, endoplasmic reticulum (ER)-associated degradation, the cell cycle, and autophagy. A number of VCP inhibitors have been reported including quinazolines, such as ML240, which have been shown to be ATP-competitive inhibitors,^[18] and CB-5083, which recently entered Phase I cancer clinical trials.^[19a,b] In addition, NMS-873^[20] and SMDC818909^[21] have been reported to be allosteric inhibitors of VCP, and the natural products withaferin A^[22] and xanthohumol^[23] were also identified as potent VCP inhibitors. Increased levels of VCP in cancer cells have been reported to be associated with poor clinical outcomes.^[24] Also, recent studies have indicated that cancer cells are sensitive to VCP inhibition.^[25] However, the involvement of VCP in TRAIL resistance in cancer cells has not been reported previously.^[26]

We examined the effect of the siRNA-mediated knock-down of VCP expression in AGS cells (Figure 6). Interestingly, VCP knock-down increased TRAIL-mediated cell death by 32.3%, in-

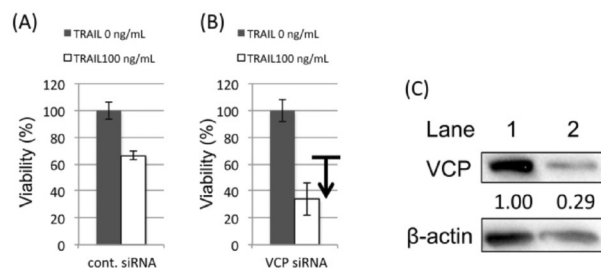


Figure 6. Effect of knock-down of VCP expression on TRAIL resistance of AGS cells. A) AGS cells treated with control siRNA. AGS cells survived treatment with TRAIL (100 ng mL⁻¹), with 66.2% viability. B) TRAIL resistance was weakened in AGS cells treated with VCP siRNA. Cell viability was 33.9%. C) Western blot analysis of VCP expression in AGS cells. Lane 1: control siRNA; Lane 2: VCP siRNA.

dicating that reduced VCP expression weakened the TRAIL resistance of AGS cells.

Based on our findings, we speculated that 5'-I fuligocandin B (**4**) inhibited VCP activity. If this is the case, the levels of cellular ubiquitinated proteins should be increased in 5'-I fuligocandin B (**4**)-treated cells. Indeed, after treatment with **4**, many ubiquitinated proteins were detected (see Figure 1 in the Supporting Information). It is known that ER stress is caused by high levels of ubiquitinated protein; therefore, this result indicated that inhibition of VCP by **4** may be linked to ER stress.

There are several small molecule TRAIL sensitizers that target ER stress induction,^[27] and it is known that expression of the DR5 regulator, CHOP, is increased during ER stress.^[28] Consistent with these findings, we found that the CHOP and DR5 protein levels increased markedly in response to 5'-I fuligocandin B (**4**) treatment, in a dose-dependent manner (Figure 7). These data suggest that CHOP upregulation following ER stress could be one of the mechanisms by which 5'-I fuligocandin B (**4**) enhances TRAIL-induced cell death in AGS cells.

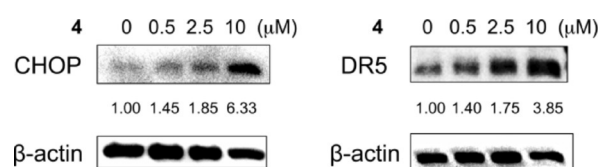


Figure 7. Effect of 5'-I fuligocandin B (**4**) on CHOP and DR5 protein expression in AGS cells. Cells were treated with increasing concentrations of 5'-I fuligocandin B (**4**) and analyzed by western blotting.

There are many reports that dissociation constant (K_d) values were determined from the circular dichroism (CD) spectrum after the binding of the ligand to a protein.^[29a,b] The binding of 5'-I fuligocandin B (**4**) to glutathione *S*-transferase (GST) fused VCP (GST-VCP) was measured by using CD (Figure 8). The CD spectra of GST-VCP and GST were analyzed between 205 and 250 nm in the presence of different concentrations of **4**, and the changes in magnitude at a wavelength of 212.5 nm, reflecting the α -helix structure, were plotted. The CD value changed in the presence of **4**, indicating that **4** induces a con-

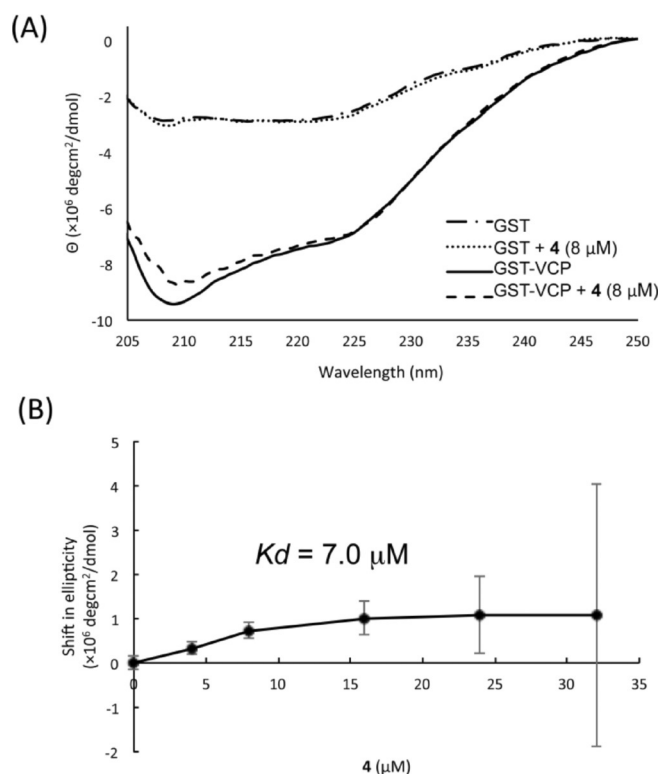


Figure 8. Effects of **4** on CD spectra of proteins. A) CD spectra of GST-VCP and GST. B) Concentration-dependent effects of 5'-I fuligocandin B (**4**) on the magnitude of GST-VCP response at 212.5 nm. CD measurements were performed as described in the Supporting Information. Values are the mean \pm SD. The K_d value of 5'-I fuligocandin B (**4**) was determined from the double-reciprocal plots.

formational change in GST-VCP upon binding. On the other hand, there was no significant change for GST. The K_d value for **4** and GST-VCP was 7.0 μM , according to the double-reciprocal plots.

3. Conclusions

We report the synthesis of photoreactive biotin probe **14**, which is a useful tool for the identification of 5'-I fuligocandin B (**4**) target proteins. We identified VCP as a target protein of **4**, and found that VCP was directly involved in AGS TRAIL resistance. Compound **4** also enhanced the level of cellular protein ubiquitination as well as CHOP and DR5 protein expression in AGS cells. As VCP plays an important role in the degradation of ubiquitinated proteins, the inhibition of VCP by **4**, and concomitant ER stress, may be important mechanisms underlying TRAIL-resistance-overcoming activity (Figure 9). To the best of our knowledge, this is the first experimental data linking VCP and TRAIL-resistance-overcoming activity in cancer cells. We believe that this finding is useful and gives a new perspective to developing small molecules that have TRAIL-resistance-overcoming activity.

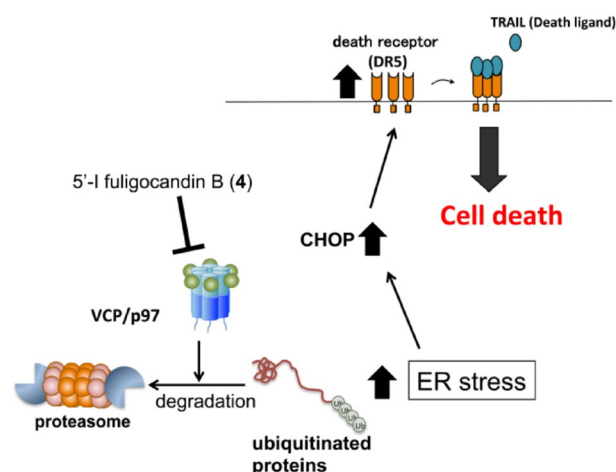


Figure 9. A model for TRAIL-resistance-overcoming activity of 5'-I fuligocandin B (**4**) in AGS cells.

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Keywords: endoplasmic reticulum stress • natural products • target identification • TRAIL resistance • valosin-containing protein

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- [15] Fuligocandin A (**1**), which does not have an indole unit, did not show the TRAIL-resistance-overcoming activity.
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