ChemComm

RSC Publishing

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2013, **49**, 6027

Received 22nd April 2013,
Accepted 15th May 2013

DOI: 10.1039/c3cc42957a

www.rsc.org/chemcomm

Target-selective photo-degradation of verotoxin-1 and reduction of its cytotoxicity to Vero cells using porphyrin–globotriose hybrids†

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Designed and synthesized porphyrin–globotriose hybrids effectively degraded verotoxin-1, which causes severe bloody diarrhoea and fetal hemolytic uremic syndrome (HUS). Degradation was achieved using long-wavelength UV or visible light irradiation in the absence of any additives and under neutral conditions. Moreover, the hybrids neutralized the cytotoxicity of verotoxin upon photo-irradiation.

Verotoxin is an exotoxin produced by entero-hemorrhagic *Escherichia coli* (EHEC). There have been many outbreaks of EHEC over the past several decades.[1] Verotoxin causes severe bloody diarrhea and fetal hemolytic uremic syndrome (HUS).[2] In this context, the development of specific inhibitors for verotoxin would be useful for clinical use and would provide an efficient countermeasure against outbreaks of EHEC. Verotoxin is classified into two subgroups, verotoxin-1 (VT-1) and verotoxin-2 (VT-2).[3] The toxin consists of a pentameric B subunit, which is responsible for the recognition of cell surface oligosaccharide units, and an A subunit, which damages ribosomal RNA once internalized by the cell.[4] Cell surface recognition is a necessary step in allowing the toxin to be internalized and fulfill its enzymatic function. In mammalian cells, globotriaosyl ceramide, a glycolipid which contains globotriose (Gb3; Galα(1–4)-Galβ1–4)-Glc, Fig. 1), is the recognition element responsible for the binding of the B subunit.[5] The conventional approach for neutralizing the cytotoxicity of verotoxin is to synthesize analogues which possess several or a large number of Gb3 moieties to permit multivalent interaction with verotoxin. Recently, Gb3-conjugated molecules[6] and Gb3-grafted polymers[7] were reported as inhibitors of the B subunit of verotoxin. Unfortunately, no effective clinical agents which can selectively bind to verotoxin and neutralize its cytotoxicity have been developed to date, in part because conventional inhibitors generally bind to verotoxin reversibly, so the protein regains its activity when the inhibitors diffuse away. An alternative strategy is to utilize a photosensitizer to degrade proteins when triggered by specific photo-irradiation conditions. Using this approach, even if the inhibitor diffuses away, the target protein is degraded and remains inactive, resulting in irreversible inhibition of protein function and an apparent increase in potency. In our previous study, porphyrin derivative **3** was found to degrade proteins by emitting reactive oxygen species under photo-irradiation conditions.[8] Based on these findings, we hypothesized that a protein photo-degrading agent possessing a Gb3 structure as a recognition moiety for the B subunit of verotoxin would selectively degrade verotoxin under photo-irradiation conditions and could be used to neutralize the cytotoxicity of verotoxin. In this communication, we present the molecular design, chemical synthesis and biological evaluation of novel and artificial light activatable porphyrin–Gb3 hybrid molecules that can effectively degrade verotoxin and neutralize its cytotoxicity under photo-irradiation.

To investigate our hypothesis, we utilized porphyrin derivative **3** as a protein photo-degrading agent and designed porphyrin–Gb3 hybrids **4–8** (Fig. 1). After chemical synthesis of the designed hybrids **4–8** with an appropriate spacer between **1** and **3** (see ESI,† Scheme S1 and Fig. S1), we first examined the photo-induced protein degradation activities of porphyrin derivative **3** and porphyrin–Gb3 hybrid **4** as representative samples using VT-1 under aerobic conditions. The progress of the reaction was monitored by SDS-PAGE.[9] The results are shown in Fig. 2. It is clear (Fig. 2(a)) that porphyrin derivative **3** had no degradation activity towards VT-1 whether the mixture of VT-1 and **3** was irradiated with a long-wavelength UV lamp (365 nm, 100 W) or not. In Fig. 2(b) using **4**, comparison of lanes 3 and 4 with lane 2 shows that neither photo-irradiation of VT-1 in the absence of **4** or treatment of VT-1 with **4** in the absence of photo-irradiation resulted in a change in the SDS-PAGE profile. In contrast, lane 5 shows the disappearance of the bands corresponding to both the A and B subunits of VT-1, indicating that porphyrin–Gb3 hybrid **4** caused degradation of VT-1 upon photo-irradiation. These results clearly demonstrated that interaction between **4**, which possesses a Gb3 moiety, and VT-1 at the B subunit is important to effectively degrade VT-1. In addition, to confirm the protein selectivity of **4** in photo-degradation, a mixture of VT-1, bovine serum albumin (BSA) and hen egg lysozyme (Lyso) was subjected to the photo-degradation conditions.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3cc42957a

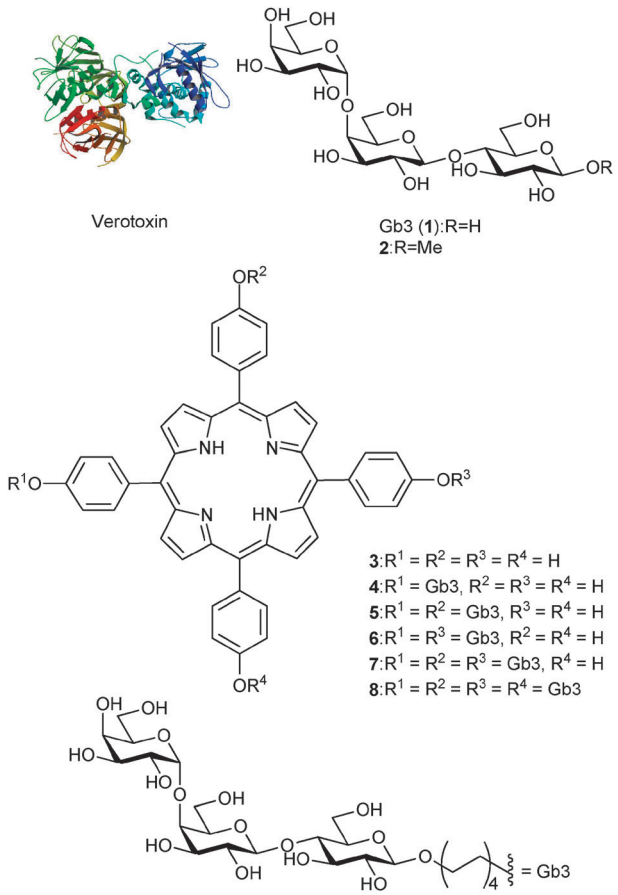

Fig. 1 Model structure of verotoxin (PDB ID: 1DM0) and chemical structures of globotriose (**1**), methyl globotrioside **2**, porphyrin derivative **3** and designed porphyrin–globotriose hybrids **4–8**.

Neither BSA nor Lyso were degraded even in the presence of **4** upon photo-irradiation at the concentration range examined (Fig. 2(c)). In addition, whether visible light (diffuse sunlight, 100 W) was used or UV, the photo-degrading ability of **4** was quite similar (Fig. 2(d)). This outcome indicated that designed hybrid **4** could be activated even using visible light, which is more harmless and permeable than UV.

In addition, the photo-degradation efficiency was found to be dependent on photo-irradiation time. Importantly, lanes 5 and 6 in Fig. 2(b)–(d) indicate that the VT-1 B subunit was degraded prior to the VT-1A subunit. These results suggested that reactive oxygen species, which have very short lifetimes, caused severe degradation of the VT-1 B subunit due to the high affinity between **4** and the VT-1 B subunit. Our previous study revealed that hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) are key players in the photo-degradation of proteins using porphyrins.[8] No other SDS-PAGE bands corresponding to degraded peptide fragments or the aggregated product were observed after photo-irradiation with **4**. This suggests that the degradation reaction occurred non-site-specifically and that VT-1 was degraded into small peptide fragments that were too small to observe by SDS-PAGE analysis.[10,11]

Next, to compare the efficacy of photo-degradation of designed hybrids **4–8**, highly sensitive immunoblot analysis using the anti-VT-1 B subunit antibody was performed using a visible light lamp. The results are summarized in Fig. 3. Comparisons of lanes 2 and 3

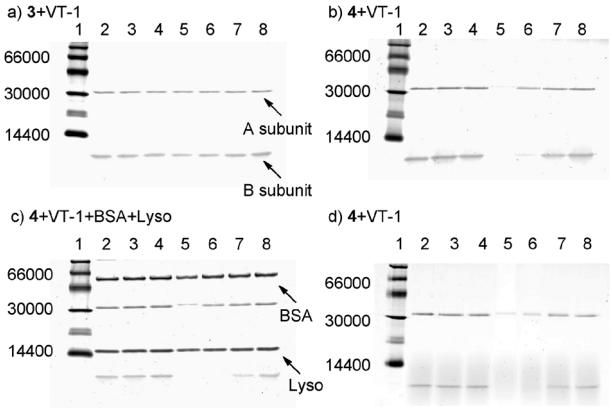

Fig. 2 Photo-degradation of VT-1 using **3** and **4** under long-wavelength UV or visible light irradiation. Each protein (0.5 μM) was incubated with **3** or **4** in 0.1 M Tris–HCl buffer (pH 7.9, 0.1 M NaCl) containing 3% DMF at 25 °C for 2 h under irradiation with a UV lamp (365 nm, 100 W) or a visible light lamp (diffuse sunlight, 100 W) placed 10 cm from the sample. The products were analysed by tricine–SDS-PAGE. The gels were stained with Sypro Ruby protein gel stain. Gels (a–d) represent (a) **3** + VT-1 with UV, (b) **4** + VT-1 with UV, (c) **4** + VT-1 + BSA + Lyso with UV, (d) **4** + VT-1 with visible light. Lanes 1, size marker; lanes 2, proteins alone; lanes 3, proteins upon photo-irradiation; lanes 4, proteins + compound (15 μM) without photo-irradiation; lanes 5–8, proteins + compound (at concentrations of 15, 5, 1.5 and 0.5 μM, respectively) upon photo-irradiation.

with lanes 1 in Fig. 3(a)–(e) showed that neither photo-irradiation of VT-1 in the absence of each compound or treatment of VT-1 with each compound in the absence of photo-irradiation affected VT-1. Furthermore, no significant degradation of VT-1 B subunits was observed when VT-1 was treated with **6** or **7** under photo-irradiation conditions (Fig. 3(c) and (d)). In contrast, lanes 4 and 5 in Fig. 3(a), (b) and (e) show the disappearance of or reduction in the SDS-PAGE band corresponding to the VT-1 B subunit after exposure to **4**, **5** or **8** under photo-irradiation, which indicated that degradation of the VT-1 B subunit had occurred. In addition, for hybrid **5**, the disappearance of the SDS-PAGE band was observed at lower

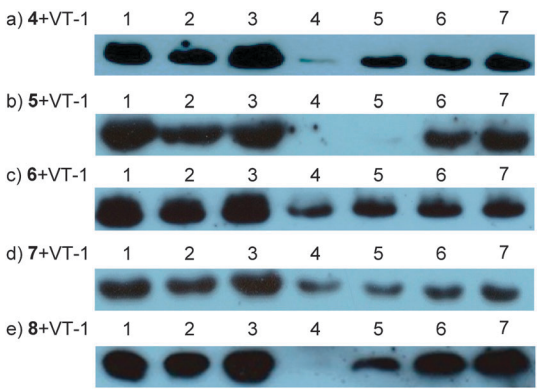

Fig. 3 Photo-degradation of VT-1 B subunits using **4–8** under visible light irradiation. VT-1 (70 nM) was incubated with **4–8** in PBS (pH 7.4) containing 10% DMF at 25 °C for 2 h under irradiation with a visible light lamp (diffuse sunlight, 100 W) placed 45 cm from the sample. The products were analysed by tricine–SDS-PAGE and immunoblotting with monoclonal antibody. Gels (a–e) represent the results using **4**, **5**, **6**, **7**, and **8**, respectively. Lanes 1, protein alone; lanes 2, protein upon photo-irradiation; lanes 3, protein + compound (70 nM) without photo-irradiation; lanes 4–7, protein + compound (at concentrations of 70, 21, 7, and 2.1 nM, respectively) upon photo-irradiation.

Table 1 EC_{100} of **2–8** to VT-1 with or without 30 min of photo-irradiation using a visible light lamp (diffuse sunlight, 100 W)

Compound	2	3	4	5	6	7	8
VIS(−) (μM)	>100	>20	1.9	1.6	3.4	22	19
VIS(+) (μM)	>100	>20	0.32	0.19	0.93	1.1	0.62

concentrations (Fig. 3(b)). These results indicated that hybrid **5** exhibited the most potent photo-degrading activity towards VT-1 among the tested hybrids **4–8**. The photo-degrading activities of **4** and **8** were similar, whereas hybrids **6** and **7** showed the weakest activities among the hybrids **4–8**. These results indicated that the photo-degrading activity of the hybrids did not simply increase as the number of Gb3 units increased; rather, two GB3 units were most effective, and their optimal position was when positioned *cis* to each other.

To confirm that hybrids **4–8** reduce the cytotoxicity of VT-1 to Vero cells, cell proliferation after treatment of VT-1 with hybrids **4–8**, and with **2** and **3**, was measured by staining with crystal violet. The effective concentrations (EC_{100}: a minimum concentration to show 100% inhibition of the cytotoxicity of VT-1 to Vero cells) of **2–8** are summarized in Table 1. Gb3 trisaccharide **2** and porphyrin derivative **3**, which does not possesses a Gb3 moiety, did not reduce the cytotoxicity of VT-1 even at concentrations of 100 and 20 μM (the maximum concentration due to the low solubility of **3**), respectively. On the other hand, the EC_{100} values of hybrids **4–8** were 1.9, 1.6, 3.4, 22, and 19 μM, respectively, without visible light irradiation. These results suggested that the hydrophobic porphyrin scaffold **3** is not sufficient to inhibit the cytotoxicity of VT-1. Moreover, Gb3 trisaccharide **2** did not significantly reduce cytotoxicity due to its low affinity[5e,12] for the VT-1 B subunit. In sharp contrast, the porphyrin–globotriose hybrids **4–8**, which possess the Gb3 moiety, potently reduced the activity compared to **2** and **3**. This may be due to hydrophobic interactions between the porphyrin scaffold and the target protein and another interaction between the Gb3 scaffold and the target protein synergistically enhancing the binding ability of hybrids **4–8** to VT-1. In addition, hybrid **5** exhibited the most potent neutralizing activity among the hybrids **4–8**. Moreover, as expected, treatment of VT-1 with **4–8** under photo-irradiation using visible light resulted in much more efficient reduction of VT-1 cytotoxicity compared to that in the absence of photo-irradiation. The EC_{100} values of hybrids **4–8** were 0.32, 0.19, 0.93, 1.1, and 0.62 μM, respectively, under photo-irradiation conditions. These results indicated that photo-degradation of VT-1 using hybrids **4–8** efficiently enhanced reduction of VT-1 cytotoxicity to Vero cells. In addition, hybrid **5**, which showed the most potent photo-degradation activity, exhibited the most potent reducing activity against the cytotoxicity of VT-1 under photo-irradiation conditions. The order of reducing activities of hybrids **4–8** under photo-irradiation conditions roughly followed that of photo-degrading activities of hybrids **4–8** towards VT-1.

In conclusion, the present work demonstrates not only the molecular design and chemical synthesis of novel porphyrin–Gb3 hybrids, but also their target-selective protein-degradation profiles and potency for reducing the cytotoxicity of VT-1 to Vero cells under photo-irradiation conditions. The described chemical synthesis and biological evaluation provide significant information about the molecular design of novel and artificial protein photo-degrading agents for controlling the functions of proteins involved in diseases.

This research was supported in part by the High-Tech Research Center Project for Private Universities: Matching Fund Subsidy, 2006–2010, Scientific Research (B) (No. 20310140 and 23310153) and Scientific Research on Innovative Areas ''Chemical Biology of Natural Products'' from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) and Grant-in-Aid for JSPS fellows (22-5423).

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