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Photodegradation of lipopolysaccharides and the inhibition of macrophage activation by anthraquinone–boronic acid hybrids†

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Target-selective photodegradation of 3-deoxy-D-*manno*-2-octulopyranosonic acid (KDO) was achieved without additives and under neutral conditions using a designed anthraquinone–boronic acid hybrid and long wavelength UV light irradiation. The hybrid can photodegrade lipopolysaccharides (LPS) and inhibit macrophage activation induced by LPS.

Carbohydrates in living cells play important roles in many biological events, including bacterial recognition and infection.[1] Consequently, the development of innovative methods for selectively controlling the specific function of certain carbohydrates has attracted much attention in chemistry, biology, and medicine. Lipopolysaccharides (LPS) represent the main outer membrane component of Gram-negative bacteria such as *E. coli* (Fig. 1), and play a key role during severe Gram-negative infection, sepsis, and sepsis shock.[2] However, there are few effective therapeutic agents for LPS-induced sepsis shock that do not cause serious side effects. There is therefore an urgent need for the development of novel therapeutic agents which can selectively bind to LPS and neutralize bacterial endotoxins. Herein we describe the molecular design, chemical synthesis, and biological evaluation of novel light-activatable small molecules which can selectively photodegrade LPS and inhibit macrophage activation, without the need for additives and under neutral conditions. To the best of our knowledge, this is the first demonstrated example of the target-selective degradation of glycolipid LPS by light switching under neutral conditions.

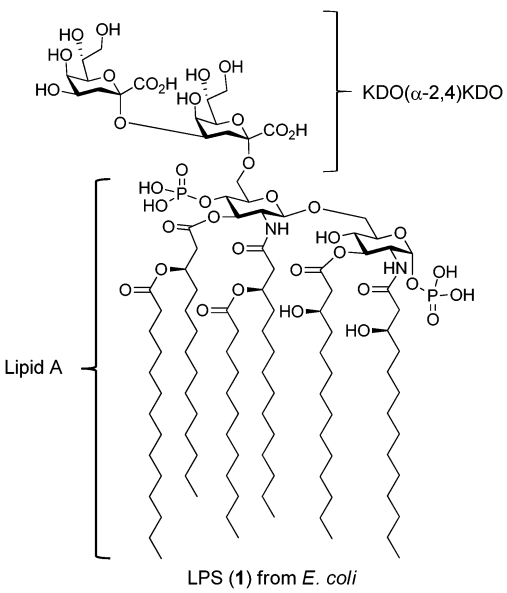

Fig. 1 Chemical structure of lipopolysaccharides (LPS) (**1**) from *E. coli*.

In our previous studies, designed anthraquinone (AQ)–lectin hybrids with high affinities for specific oligosaccharides were found to selectively degrade target oligosaccharides upon irradiation with long wavelength UV light and without the need for further additives.[3,4] In addition, designed AQ–[5] or fullerene–boronic acid hybrids[6] were found to selectively bind to and effectively degrade the target D-galactofuranosides (Gal*f*s) under neutral conditions not only upon UV irradiation, but also upon visible light irradiation. Furthermore, upon photo-irradiation, AQ–boronic acid hybrids showed bactericidal activity against *Mycobacterium bovis* BCG, which contains Gal*f*s in the cell wall. On the basis of our preliminary findings,[7] we set out to design and synthesize target-selective photodegrading agents for LPS.

We first targeted the structure of the KDO (3-deoxy-D-*manno*-2-octulopyranosonic acid) residue that is covalently linked to lipid A, a membrane anchoring element and the toxic principal of LPS. KDO is a unique and common structural component of LPS and is not found in mammals. Furthermore, KDO possesses an acyclic 1,2-diol on the exocyclic side chain. Arylboronic acid was chosen as the recognition moiety. It is already known that phenylboronic acid can bind to 1,2- or 1,3-diols through reversible boronate formation under physiological conditions.[8] Thus, we expected that a hybrid molecule consisting of a boronic acid, which would preferentially bind to an acyclic diol on KDO, and a photo-degrading AQ derivative, could be used for target-selective photodegradation of LPS.

To investigate our hypothesis, we first designed AQ–boronic acid hybrids **2** and **3**, which have different length spacers between the AQ moiety and the boronic acid moiety (Fig. 2). After chemical synthesis of the hybrids **2** and **3** (see Scheme S1 in the ESI†), the binding affinities of the hybrids to different glycosides, including KDOα–OMe (**4**), Neu5Acα–OMe (**5**), Galβ–OMe (**6**), Glcα–OMe (**7**) and Manα–OMe (**8**), were examined using a quantitative three-component alizarin red S (ARS) assay[9] under

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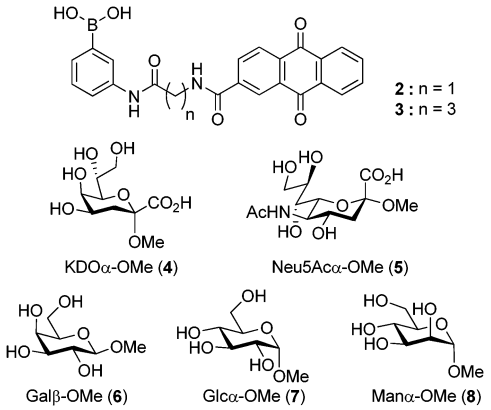

Fig. 2 Chemical structures of AQ–boronic acid hybrids and several glycosides.

neutral (buffered pH 7.4) aqueous conditions. The results are summarized in Table 1. Hybrid molecules **2** and **3** bound with good affinity to **4** and **5**, which possess an acyclic 1,2-diol on the exocyclic side chain, and not to 1,2- or 1,3 diols on other glycosides. In addition, **2**, which possesses a short spacer, exhibited very similar K_a values towards **4** and **5**. On the other hand, the K_a value obtained for hybrid **3**, which possesses a long spacer, for binding to **4**, was almost three times greater than that of **3** binding to **5**. These results suggest that our designed hybrid molecule **3** selectively binds to the target glycoside **4** rather than to other glycosides. Although the reason for this specificity remains unclear, we believe that not only the interaction between the boronic acid in **3** and an acyclic 1,2-diol in KDO, but also other interactions, such as CH–π interaction between the AQ moiety in **3** and the hydrogen atoms on a pyran ring of KDO, are important factors for the binding.

Next, we examined the photoinduced glycoside-degradation abilities of **2** and **3** (1.0 mM) against glycosides **4–8** (1.0 mM) in 10% DMF/0.1 M phosphate buffer (pH 7.4) at 25 °C for 2 h, using a long-wavelength UV lamp (365 nm, 100 W) placed 10 cm from the sample. The progress of the photodegradation reaction was monitored by HPLC/UV (215 nm or 254 nm) analysis after appropriate modifications of the resulting products (see ESI†). The percent degradation was calculated based on the peak area corresponding to each modified glycoside; the results are shown in Fig. 3. When neither hybrid was added (control), more than 95% of each modified glycoside was detected by HPLC analysis. When hybrid **2** or **3** was exposed to glycosides **6–8**, less than 10% degradation took place, owing to low affinity

Table 1 Association constants (K_a) for the hybrid molecules **2** and **3** with several glycosides

Entry	Glycoside	K_a[a] [M^{-1}] Hybrid **2**	Hybrid **3**
1	KDOα–OMe (**4**)	54	99
2	Neu5Acα–OMe (**5**)	48	34
3	Galβ–OMe (**6**)	10	9
4	Glcα–OMe (**7**)	12	12
5	Manα–OMe (**8**)	11	9

[a] The K_a values were determined by an ARS assay in 10% DMF/0.1 M phosphate buffer (pH 7.4) and are the average of at least two reproducible measurements; see ESI.

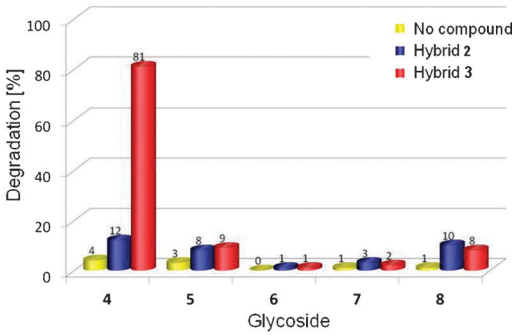

Fig. 3 Each glycoside (1.0 mM) was incubated with AQ–boronic acid hybrid **2** or **3** (1.0 mM) in 10% DMF/0.1 M phosphate buffer (100 μL, pH 7.4) at 25 °C for 2 h under irradiation with a UV lamp (365 nm, 100 W) placed 10 cm from the sample. The reaction mixture was analysed by HPLC (Mightysil RP-18 GP 5 μm, 4.6 × 150 mm); 40 °C; detection by UV (210 nm or 254 nm) after appropriate modifications of the resulting products.

of the hybrids for the glycosides. In addition, the degradation activities of **2** for **4** and **5**, which have moderate affinities for **2**, were also found to be low. In sharp contrast, when **3** was exposed to **4** under photo-irradiation, significant degradation took place. Moreover, the degradation of **4** by **3** was found to be much more effective than that of **5** by **3**. These results suggest that the binding affinity of the hybrid **3** to glycoside, and the distance between the protons in the glycoside and the photo-activated AQ moiety are influential factors in the target-selective photodegradation of glycosides by **3**.

Next, we examined the photo-induced degradation of LPS (**1**) (5.0 μg mL^{-1}, 2.15 μM, 1.0 equiv.) by hybrid **2** or **3** at concentrations of 215 (100 equiv.), 64.5 (30 equiv.), 21.5 (10 equiv.) and 6.45 μM (3 equiv.) in 10% DMF/0.1 M phosphate buffer (pH 7.4). The progress of the photodegradation reaction was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results are shown in Fig. 4. When **2** was exposed to LPS (**1**) and photo-irradiated, no change in the SDS-PAGE profile was observed (Fig. 4a). On the other hand, when LPS (**1**) was treated with **3** at a concentration of 215 μM with photo-irradiation, the SDS-PAGE band corresponding to LPS (**1**) disappeared (lane 4 in Fig. 4b). These results indicate that our designed hybrid **3** photodegraded not only KDO but also lipid A in the target LPS (**1**) upon irradiation with UV light in the absence of any

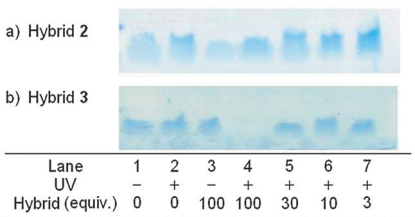

Fig. 4 Photodegradation of LPS (**1**) by hybrids **2** and **3**. **1** (5.0 μg mL^{-1}, 2.15 μM, 1.0 equiv.) was incubated with (a) **2** or (b) **3** in 10% DMF/0.1 M phosphate buffer (pH 7.4) at 25 °C for 2 h under irradiation with a UV lamp (365 nm, 100 W) placed 10 cm from the sample. The reaction mixture was analysed using tricine–SDS-PAGE. Lane 1, **1** alone; lane 2, **1** following UV irradiation; lane 3, **1** + **2** or **3** without UV irradiation; lanes 4–7, **1** + **2** or **3** (equiv. to **1**; 100, 30, 10 and 3 equiv., respectively) following UV irradiation.

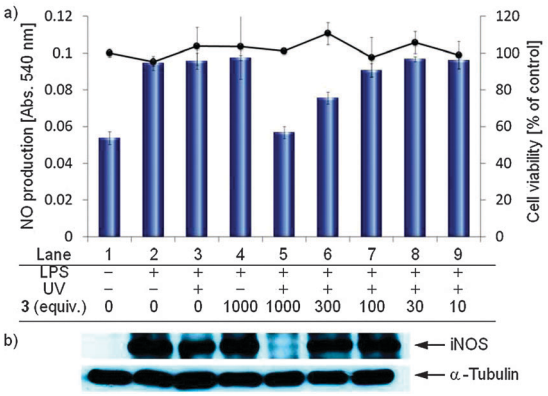

Fig. 5 LPS-induced NO production and iNOS expression in RAW 264.7 cells. LPS (**1**) (10 μg mL^{-1}, 4.30 μM, 1.0 equiv.) was pre-incubated with **3** in 10% DMF/H_2O at 25 °C for 2 h with or without photo-irradiation using a UV lamp (365 nm, 100 W) placed 10 cm from the sample. The resulting mixture was incubated with RAW 264.7 cells, which were seeded into 96-well plates, at 37 °C for 24 h. NO production was measured by the Griess assay (column). Cell viability was measured by the MTT assay (circle). Lane 1, control (without treatment); lane 2, **1** without photo-irradiation; lane 3, **1** with photo-irradiation; lane 4, **1** + **3** (1000 equiv.) without photo-irradiation; lanes 5–9, **1** + **3** (equiv. to **1**; 1000, 300, 100, 30 and 10 equiv., respectively) with photo-irradiation. (b) iNOS expression was assessed by western blotting. Lane 1, control (without treatment); lane 2, **1** without photo-irradiation; lane 3, **1** with photo-irradiation; lane 4, **1** + **3** (1000 equiv.) without photo-irradiation; lanes 5–7, **1** + **3** (equiv. to **1**; 1000, 300, and 100 equiv., respectively) with photo-irradiation.

additives at neutral pH. However, an excess amount of **3** to **1** was required for photodegradation due to the low concentration of **1** used in the reaction.

Next, we conducted EPR studies[10] using **3** and DMPO with or without UV irradiation in order to confirm the generation of ROS, a reactive species for oligosaccharide photodegradation.[3–7] Photo-irradiation of **3** in the presence of DMPO gave the DMPO-hydroxy radical spin adduct DMPO–•OH. Furthermore, no peaks corresponding to DMPO–•OH were detected either when **3** was photo-irradiated or when photo-irradiation was conducted in the absence of **3** (see Fig. S1 in the ESI†).

Finally, the macrophage activation ability of photodegraded LPS by AQ–boronic acid hybrid **3** was evaluated based on LPS-induced nitric oxide (NO) production in macrophage RAW 264.7 cells using the Griess assay.[11] In addition, cell viability was determined by the MTT assay. These results are summarized in Fig. 5a. When the cells were treated with only LPS (0.1 μg mL^{-1}), NO production was significantly induced (lanes 1–3 in Fig. 5a). In addition, it was found that pre-incubation of LPS with **3** without photo-irradiation resulted in neither cytotoxicity nor inhibition of NO production (lane 4 in Fig. 5a). In contrast, pre-incubation of LPS with **3** upon photo-irradiation inhibited NO production in a concentration-dependent manner (lanes 5–9 in Fig. 5a). Next, to reveal whether these changes in NO production were caused by changes in iNOS expression, we assessed iNOS expression in RAW 264.7 cells by western blotting (Fig. 5b). It was confirmed that pre-incubation of LPS with **3** upon photo-irradiation did not induce iNOS expression in the cells (lane 5 in Fig. 5b). These results clearly indicate that photodegradation of LPS by AQ–boronic acid hybrid **3** upon photo-irradiation occurred effectively and inhibited NO production and iNOS expression by LPS-induced macrophage activation.

In conclusion, we have developed a new chemical agent, an AQ–boronic acid hybrid, which can selectively bind to and photo-degrade target glycoside KDO upon irradiation with UV light, without the need for additives and under neutral conditions. In addition, the hybrid molecule can photodegrade target glycolipid LPS. Furthermore, it was revealed that photo-degradation of LPS by the hybrid inhibits NO production and iNOS expression by LPS-induced macrophage activation. The results presented here will contribute to the molecular design of novel artificial carbohydrate photodegradation agents, which should find wide application in chemistry, biology, and medicine. The development of more specific and tighter binding hybrid molecules for LPS is now under investigation in our laboratory.

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