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Photodegradation and inhibition of drug-resistant influenza virus neuraminidase using anthraquinone–sialic acid hybrids†

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The anthraquinone–sialic acid hybrids designed effectively degraded not only non-drug-resistant neuraminidase but also drug-resistant neuraminidase, which is an important target of anti-influenza therapy. Degradation was achieved using long-wavelength UV radiation in the absence of any additives and under neutral conditions. Moreover, the hybrids efficiently inhibited neuraminidase activities upon photo-irradiation.

Influenza, caused by the influenza virus, is a serious disease, and influenza pandemics continue to be a major medical threat.[1] In anti-influenza therapies, influenza virus neuraminidase (NA) is one of the most important targets. The NA cleaves the linkage between progeny virus from the surface sialo-receptor of host cells, which is essential for replication of infective virus.[2] Hence, NA has been the subject of attention as a drug target.[3] One effective approach for inhibition of NA activity is to employ small molecules, which have a high affinity for NA, such as oseltamivir[4] and zanamivir.[5] However, the emergence of drug-resistant influenza viruses has caused problems for effective medical treatment.[6] Therefore, new types of drugs for treatment of drug-resistant influenza viruses are needed. A recent study reported that certain anthraquinone derivatives could degrade proteins upon photo-irradiation under neutral conditions and in the absence of any additives.[7] Here, the fundamental result of this study was applied to design anthraquinone–sialic acid hybrids that could effectively degrade NA and inhibit its enzymatic activity under photo-irradiation. In addition, the hybrids effectively inhibited drug (oseltamivir)-resistant NA as well as non-drug (oseltamivir)-resistant NA upon photodegradation.

A previous study showed that anthraquinone derivatives degraded proteins upon photo-irradiation in the absence of any additives and under neutral conditions.[7] Sialic acid is a native ligand for NA, and is essential for NA recognition. Thus, a hybrid molecule consisting of anthraquinone and sialic acid would be expected to effectively degrade not only a non-drug-resistant NA but also a drug-resistant NA, because even drug-resistant NA must recognize the sialo-receptor (sialic acid) of host cells for replication. This inspired the design of several anthraquinone–sialic acid hybrids, **1–3**, which consist of an anthraquinone **4** attached to sialic acid at different positions (Fig. 1).

After preparation of anthraquinone–sialic acid hybrids **1–3** (see ESI,† Schemes S1–S3), photo-induced degradation assays of non-drug-resistant NA (influenza A virus (A/California/04/2009) H1N1 substrain)[8] were conducted using **1–3** along with **4** under UV light irradiation (365 nm, 100 W); reaction progress was monitored by SDS-PAGE and immunoblotting. The results are summarized in Fig. 2. Comparisons of lane 1 with lane 2 in Fig. 2(a) and lane 1 in Fig. 2(a) with lanes 1 in Fig. 2(b)–(e) showed that neither photo-irradiation of NA in the absence of **1–4** nor treatment of NA with **1–4** in the absence of photo-irradiation resulted in a change in the SDS-PAGE profile. In contrast, the SDS-PAGE band corresponding to NA in lanes 2 in Fig. 2(b)–(e) disappeared after exposure to each compound under photo-irradiation, indicating that degradation of NA occurred. Furthermore, for hybrids **2** and **3**, the disappearance of the SDS-PAGE band was observed at lower concentrations

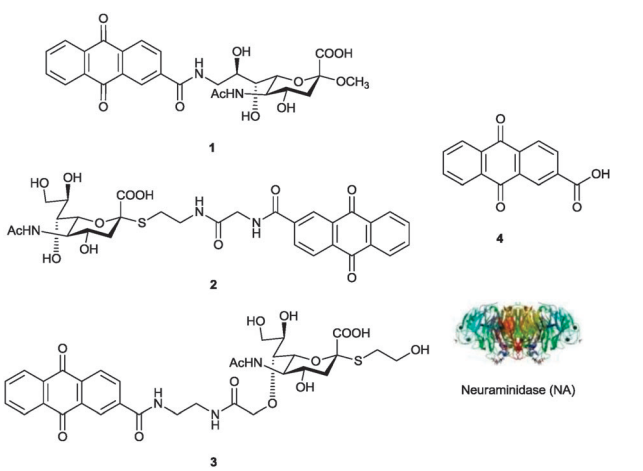

Fig. 1 Chemical structures of anthraquinone–sialic acid hybrids **1–3**, anthraquinone derivative **4**, and model structure of neuraminidase (NA).

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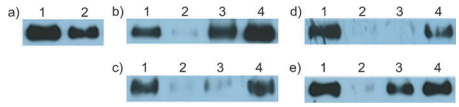

Fig. 2 Photodegradation of non-drug-resistant NA [influenza A virus (A/California/04/2009) H1N1 substrain] using **1–4** under long-wavelength UV irradiation. The enzyme (125 mU) was incubated with each compound in Tris buffer (pH 7.5, 50 mM) at 25 °C for 60 min under irradiation with a UV lamp (365 nm, 100 W) placed 10 cm from the sample. The products were analyzed by SDS-PAGE and immunoblotting with monoclonal antibody 2F10E12G1. Gels (a–e) represent (a) enzyme without (lane 1) and with photo-irradiation (lane 2) in the absence of a compound, (b–e) enzyme with **1**, **2**, **3**, and **4**, respectively. In (b–e), lane 1, enzyme + each compound (100 μM) without photo-irradiation; lanes 2–4, enzyme + each compound (concentrations 100, 10, and 1 μM, respectively) upon photo-irradiation.

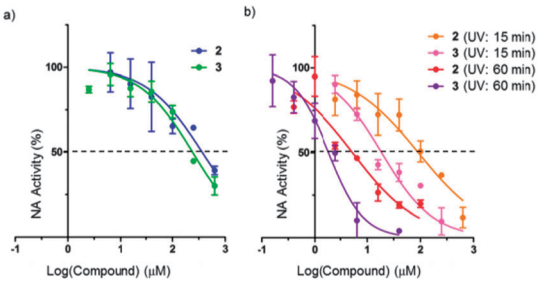

Fig. 3 Relation between concentration of **2** or **3** and non-drug-resistant NA [influenza A virus (A/California/04/2009) H1N1 substrain] activity (a) without photo-irradiation and (b) with photo-irradiation (UV lamp, 365 nm, 100 W, 10 cm, 15 or 60 min). The curve represents the concentration–inhibition curve for the estimated IC_{50} value. Assays were performed in Tris buffer (pH 7.5, 50 mM) at 25 °C. Nonlinear regression analysis with Prism® version 5 (Graphpad Software, Inc.) was used for curve fitting of the substrate cleavage reaction.

(Fig. 2(c) and (d)). These results demonstrated that anthraquinone derivatives are capable of degrading the target enzyme NA upon irradiation with long-wavelength UV light. In addition, certain anthraquinone–sialic acid hybrids effectively photodegraded NA upon photo-irradiation.

To investigate the mechanism behind the photodegradation of NA, electron paramagnetic resonance (EPR) studies were conducted.[9] 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was used as a spin-trapping agent for the detection of superoxide anions ($O_2^{\bullet -}$) or hydroxyl radicals ($^{\bullet}OH$) (see ESI,† Fig. S1). Results showed that photo-irradiation of **1–4** in the presence of DMPO gave products with EPR spectra characteristic of the DMPO–hydroxy radical spin adduct DMPO–$^{\bullet}OH$. The results also confirmed that no peaks corresponding to DMPO–$^{\bullet}OH$ were detected, either upon treatment of DMPO with each compound without photo-irradiation or upon photo-irradiation of DMPO in the absence of each compound. Thus, the $^{\bullet}OH$, produced by reaction of the photo-excited anthraquinone moieties and O_2, was the major contributor to photodegradation of NA by these anthraquinone derivatives.[10]

To confirm that **1–4** bind to non-drug-resistant NA and inhibit its activity, an enzyme inhibition assay using 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid as a substrate[11] hydrolyzed by NA was conducted (Fig. 3).[12] As a result, although **1** and **4** did not inhibit enzymatic activity of NA (IC_{50}: >500 μM), **2** and **3** showed moderate inhibitory activity against NA (IC_{50}: 364 and 240 μM, respectively), indicating that **2** and **3** bind to the active site of NA. The difference in the inhibitory effects of **2** and **3** on NA in the presence or absence of photo-irradiation was also examined. The IC_{50} values were 91.8 and 18.1 μM for **2** and **3**, respectively, upon 15 min photo-irradiation. The enzyme inhibitory activities of **2** and **3** increased 4.0- and 13-fold, respectively, with photo-irradiation. In addition, when the photo-irradiation time was increased to 60 min, the IC_{50} values were 5.18 and 1.87 μM for **2** and **3**, respectively. The enzyme inhibitory activities of **2** and **3** increased 70- and 128-fold, respectively, upon photo-irradiation. These results demonstrate that **2** and **3** inhibit the enzymatic activity of non-drug-resistant NA much more efficiently under photo-irradiation. In addition, the inhibitory activity can be controlled by adjusting both the compound's concentration and the photo-irradiation time. Next, the photodegradation activity of **2** and **3** against drug (oseltamivir)-resistant NA (influenza A virus (A/California/04/2009) H1N1 (H274Y) substrain) was investigated.[8]

Reaction progress was monitored by SDS-PAGE and results are summarized in Fig. 4. Comparisons of lane 1 with lane 2 in Fig. 4(a) and lane 1 in Fig. 4(a) with lanes 1 in Fig. 5(b) and (c) showed that neither photo-irradiation of drug (oseltamivir)-resistant NA in the absence of the hybrid nor treatment of drug (oseltamivir)-resistant NA with **2** or **3** in the absence of photo-irradiation resulted in a change in the SDS-PAGE profile. In contrast, lanes 2–4 in Fig. 4(b) and (c) show the disappearance of or reduction in the SDS-PAGE band corresponding to drug (oseltamivir)-resistant NA after exposure to each compound under photo-irradiation, which indicates that degradation of drug (oseltamivir)-resistant NA occurred. These results showed that anthraquinone–sialic acid hybrids **2** and **3** were capable of degrading not only a non-drug-resistant NA but also a drug-resistant NA upon irradiation with long-wavelength UV light.

To confirm that **2** and **3** inhibited drug (oseltamivir)-resistant NA upon photo-irradiation, an enzyme inhibition assay was performed (Fig. 5). Results showed that **2** and **3** possessed moderate inhibitory activity against the drug (oseltamivir)-resistant NA without photo-irradiation (IC_{50}: 394 and 148 μM, respectively). In contrast, IC_{50} values were 82.3 and 8.77 μM for **2** and **3**, respectively, with 15 min photo-irradiation. Furthermore, IC_{50} values were 6.58 and 2.09 μM for **2** and **3**, respectively, upon 60 min photo-irradiation. The enzyme inhibitory activities of **2** and **3** increased 60- and 71-fold, respectively, upon photo-irradiation. In contrast, for oseltamivir (carboxylic acid form), IC_{50} values were 0.003 and 0.454 μM against non-drug (oseltamivir)-resistant NA

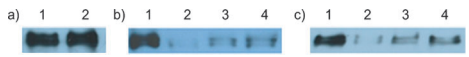

Fig. 4 Photodegradation of drug-resistant NA [influenza A virus (A/California/04/2009) H1N1 (H274Y) substrain] using **2** and **3** under long-wavelength UV irradiation. The enzyme (125 mU) was incubated with each compound in Tris buffer (pH 7.5, 50 mM) at 25 °C for 60 min under irradiation with a UV lamp (365 nm, 100 W) placed 10 cm from the sample. Products were analyzed by SDS-PAGE and immunoblotting with monoclonal antibody 2F10E12G1. Gels (a–c) represent (a) enzyme without photo-irradiation (lane 1) and with photo-irradiation (lane 2) in the absence of a compound; (b) and (c) enzyme with **2** and **3**, respectively. In (b) and (c), lane 1, enzyme + each compound (100 μM) without photo-irradiation; lanes 2–4, enzyme + each compound (concentrations 100, 10, and 1 μM, respectively) upon photo-irradiation.

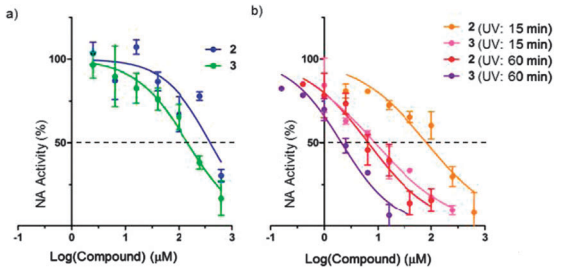

Fig. 5 Relation between concentration of **2** or **3** and drug-resistant NA [influenza A virus (A/California/04/2009) H1N1 (H274Y) substrain] activity (a) without photo-irradiation and (b) with photo-irradiation (UV lamp, 365 nm, 100 W, 10 cm, 15 or 60 min). The concentration–inhibition curve represents the estimated IC_{50} value. Assays were performed in Tris buffer (pH 7.5, 50 mM) at 25 °C. Nonlinear regression analysis with Prism® version 5 (Graphpad Software, Inc.) was used for curve fitting of the substrate cleavage reaction.

and drug (oseltamivir)-resistant NA, respectively (Fig. 6a). The inhibitory activity for drug (oseltamivir)-resistant NA decreased 151-fold compared to that for non-drug (oseltamivir)-resistant NA. Furthermore, the IC_{50} values for 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (DANA),[2a,13] which is the lead drug of oseltamivir and zanamivir, were 6.45 and 7.69 μM against non-drug (oseltamivir)-resistant NA and drug (oseltamivir)-resistant NA, respectively (Fig. 6b). The inhibitory activity for drug (oseltamivir)-resistant NA was similar to that for non-drug (oseltamivir)-resistant NA. The results revealed that, although the inhibition activities of **2** and **3** against drug (oseltamivir)-resistant NA were slightly weaker than that of oseltamivir, **2** and **3** inhibited the activity of drug (oseltamivir)-resistant NA much more efficiently under photo-irradiation. In addition, **2** and **3** inhibited not only non-drug-resistant NA but also a drug-resistant NA at the same level. Furthermore, the inhibitory activities of **3** against non-drug (oseltamivir)-resistant NA and drug (oseltamivir)-resistant NA upon photo-irradiation were greater than those of the lead drug of oseltamivir, DANA.

In conclusion, designed anthraquinone–sialic acid hybrids degraded not only non-drug-resistant NA but also a drug-resistant NA upon irradiation with long-wavelength UV light in the absence of any additives and under neutral conditions. Thus, a new and innovative method for effective inhibition of not only non-drug-resistant NA but also a drug-resistant NA was developed using photo-irradiation and the hybrids. The present photodegradation method using a hybrid molecule consisting of a protein photodegradation agent and a native ligand for the specific enzyme allows development of new strategies for inhibiting drug-resistant enzymes with a wide range of applications. In addition, the results presented here contribute to the molecular design of novel artificial protein photodegrading agents and agents for controlling the functions of proteins involved in many diseases or infections. Additional studies with respect to the target-selectivity are currently underway in our laboratory.

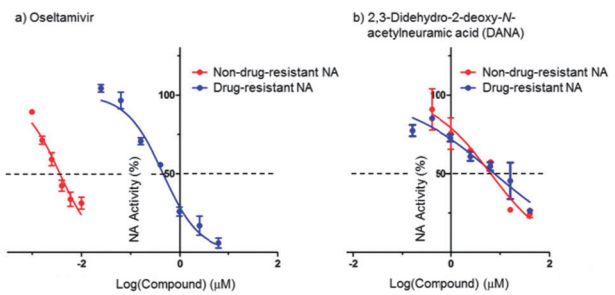

Fig. 6 Relation between concentration of oseltamivir or DANA and non-drug-resistant NA [influenza A virus (A/California/04/2009) H1N1 substrain] or drug-resistant NA [influenza A virus (A/California/04/2009) H1N1 (H274Y) substrain] activity. (a) Oseltamivir and (b) DANA. The concentration–inhibition curve represents the estimated IC_{50} value. Assays were performed in Tris buffer (pH 7.5, 50 mM) at 25 °C. Nonlinear regression analysis with Prism® version 5 (Graphpad Software, Inc.) was used for curve fitting of the substrate cleavage reaction.

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