Spongiacidin C, a pyrrole alkaloid from the marine sponge *Stylissa massa*, functions as a USP7 inhibitor

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ABSTRACT

USP7, a deubiquitylating enzyme hydrolyzing the isopeptide bond at the C-terminus of ubiquitin, is an emerging cancer target. We isolated spongiacidin C from the marine sponge *Stylissa massa* as the first USP7 inhibitor from a natural source. This compound inhibited USP7 most strongly with an IC_{50} of 3.8 μM among several USP family members tested.

The ubiquitin-proteasome system regulates various cellular events including cell-cycle control, transcription, and signal transduction through protein degradation by the 26S proteasome.[1,2] In the ubiquitin system, client proteins are polyubiquitylated by the sequential actions of three enzymes: ubiquitin-activating enzyme (E1); ubiquitin-conjugating enzyme (E2); and ubiquitin-protein ligase (E3).[1] This protein ubiquitylation is reversed by deubiquitylating enzymes (DUBs) that cleave the isopeptide bond at the C-terminus of ubiquitin,[3-5] leading to deconjugation of the ubiquitin chain in client proteins prior to the degradation. Thus, the proteasome-mediated degradation is controlled by the coordinated actions of the ubiquitin system and DUBs. Two proteasome inhibitors, VELCADE® (bortezomib)[6] and Kyprolis™ (carfilzomib),[7] were approved for the treatment of multiple myeloma by the FDA in 2003 and 2012, respectively, and the components of the ubiquitin-proteasome system have become attractive targets for the development of anticancer agents.[8,9] Among inhibitors of the enzymes in the ubiquitin system, E3 inhibitors are expected to be more specific and less toxic drugs than proteasome inhibitors because the respective E3s recognize specific target proteins. In addition, inhibitors of specific DUBs are also expected to be specific drugs because the respective DUBs show specific intracellular localization, biological roles, and substrate specificities.[3-5,10] The human genome encodes at least 98 DUBs, and altered DUB functions are related to several diseases including cancer. Among them, USP7 is an emerging oncology target because it stabilizes Hdm2 (human Mdm2), a major E3 for the tumor suppressor protein p53.[11,12] Hdm2 is auto-ubiquitylated, resulting in its own degradation by the proteasome. USP7 is capable of binding to and deubiquitylating auto-ubiquitylated Hdm2, which leads to stabilization of Hdm2 and consequent degradation

of p53. Hence, a USP7 inhibitor stabilizes p53 in cells through degradation of Hdm2, which subsequently results in the suppression of cancer. Several USP7 inhibitors have been developed by screening libraries of synthetic small molecules and by chemical modifications of lead compounds.[13-17] As an alternative approach, we screened our natural product library composed of marine invertebrate extracts, which are expected to contain structurally diverse and biologically significant metabolites. Here we report the identification of spongiacidin C (**1**) from the marine sponge *Stylissa massa* as the first USP7 inhibitor isolated from a natural source.

The screening of EtOAc- and water-soluble fractions derived from EtOH-extracts of 700 marine invertebrates was performed in 96-well plates using recombinant full-length USP7[18] with ubiquitin-Rh110 (LifeSensors, Inc.) as a quenched, fluorescent substrate. After USP7 (0.9 nM) in 80 μL of 0.2 M Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$, 0.05% CHAPS, and 2 mM 2-mercaptoethanol was incubated with a test sample (5 μg/mL) at 25˚C for 30 min, 0.5 μM ubiquitin-Rh110 in 20 μL of the buffer was added to the wells, and the mixture was further incubated at 25 ˚C. Then, the fluorescence intensity due to rhodamine, produced by USP7-catalyzed cleavage of the amide bond between the C-terminal glycine of ubiquitin and rhodamine in ubiquitin-Rh110, was measured within the linear range of the assay on a fluorometric plate reader with excitation and emission wavelengths of 490 nm and 530 nm, respectively.

One of the hits of this screen was the extract from the marine sponge *Stylissa massa*,[19] which was collected at a depth of 10 m in North Sulawesi, Indonesia, in December 2006 and soaked in EtOH immediately. The extract was concentrated and the residual aqueous layer

was partitioned with EtOAc and then *n*-BuOH. Purification from the EtOAc-soluble fraction (3.3 g) by SiO_2 column chromatography (MeOH/$CHCl_3$) and ODS column chromatography (MeOH/H_2O) followed by ODS HPLC (MeOH/H_2O) afforded spongiacidin C[20] (**1**, 0.67 mg) and dibromophakellin[21] (**2**, 3.6 mg). In addition, purification from the *n*-BuOH-soluble fraction (12.4 g) by ODS column chromatography (MeOH/H_2O) and SiO_2 column chromatography (MeOH/$CHCl_3$) followed by ODS HPLC (MeOH/H_2O and CH_3CN/H_2O) afforded debromohymenialdisine[22] (**3**, 116.6 mg), hymenialdisine[23] (**4**, 8.7 mg), manzacidin A[24] (**5**, 6.1 mg), manzacidin B[24] (**6**, 3.1 mg), manzacidin C[24] (**7**, 2.2 mg), and *N*-methylmanzacidin C[25] (**8**, 3.3 mg) (Fig. 1). The compounds were identified based on NMR and MS spectral data.

Among the eight compounds, **1** was the strongest inhibitor (IC_{50}, 3.8 μM), followed by **3** and **4** (congeners of **1**) (20% inhibition at 20 μM in both cases), but the others had minimal inhibitory activity. Although the structures of **1**, **3**, and **4** are similar, **1** exhibited a stronger inhibitory effect against USP7 than **3** and **4**, suggesting that the presence of a hydantoin ring in **1** was necessary for the USP7 inhibition instead of an aminoimidazolinone ring in **3** and **4**. Next we examined the inhibitory activity of **1** against USP7 and additional USP family members including the core catalytic domains of USP2, USP8, and USP21,[17,18] and the core catalytic domain of SENP1 (SUMO-specific peptidase 1),[26] with their respective substrates, i.e., ubiquitin-EK_L (Ub-CHOP2)[17] for USP7, diubiquitin IQF K4804 (LifeSensors, Inc.) for USP2, ubiquitin-Rh110 for USP8 and USP21, and SUMO3-EK_L (SUMO-CHOP2)[17] for SENP1, according to the methods described previously.[15-18,27] As shown in Table 1, **1** inhibited USP7 strongly (IC_{50}, 3.8 μM) and USP21 moderately (IC_{50}, 16.6 μM), but was

inactive against USP2, USP8, or SENP1 over the dose range tested. However, it should be noted that when **1** was tested at concentrations up to 50 μM in follow-up cellular studies, there was no evidence of growth inhibition against the p53 wild type, USP7 expressing human colorectal cancer cell line HCT-116.[28]

In conclusion, we found that spongiacidin C (**1**), isolated from the marine sponge *Stylissa massa*, is a relatively selective inhibitor of USP7. Due to a lack of cytotoxic activity, additional cellular evaluations of USP7 pharmacodynamic markers such as Hdm2 and p53 were not performed. A recent publication demonstrated that a ubiquitin variant, engineered to bind tightly to USP7, inhibits USP7 selectively (K_D, 56 nM).[29] Additionally, several USP7 inhibitors have been reported, including HBX 41,108 (IC_{50}, 0.42 μM),[13] HBX 19,818 (IC_{50}, 28.1 μM) and HBX 28,258 (IC_{50}, 22.6 μM),[14] P5091 (IC_{50}, 4.2 μM),[15,16] P22077 (IC_{50}, 7.8 μM),[16,17,27] and compound 14 (IC_{50}, 0.42 μM),[16] all of which were obtained from libraries of synthetic chemicals or by subsequent synthetic modifications. Several natural products with a broad DUB inhibitory profile have been described,[30] but to the best of our knowledge, **1** is the first USP7 inhibitor isolated from a natural source and exhibited a potency similar to those of some of the previously described USP7 inhibitors derived from synthetic sources. We are currently studying alternative USP7 inhibitory natural product extracts with the goal of adding to this list in the near future.

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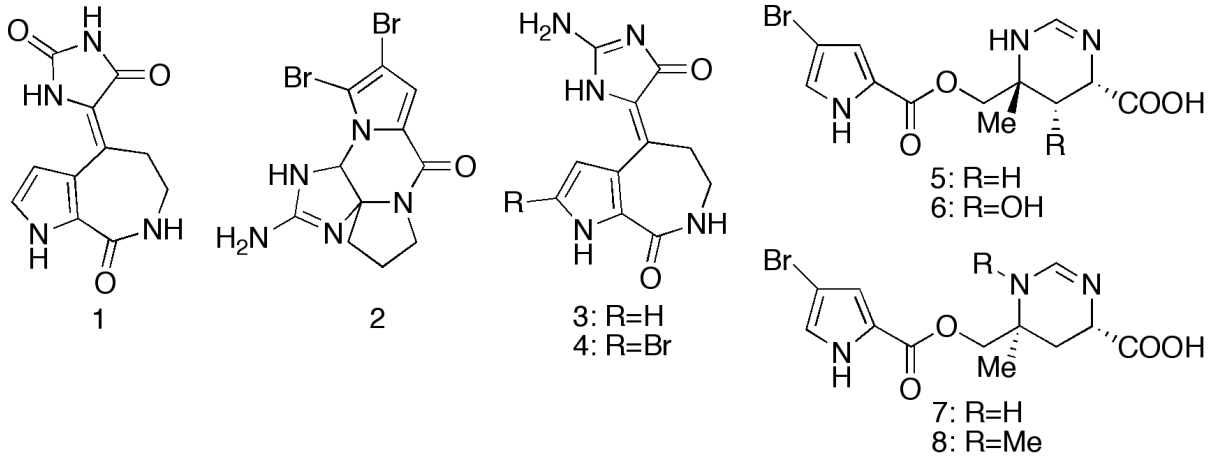

Figure 1. Structures of **1**-**8**.

Table 1. Inhibitory activities of

Enzyme	IC_{50} (μM)[a]
USP7	3.8 ± 1.8[b]
USP2c[c]	>31.6
USP8c[c]	>31.6
USP21c[c]	16.6 ± 2.8[b]
SENP1c[c]	>31.6

[a]The mean of three independent experiments.

[b]SD

[c]Expressed as the core catalytic domain.