## The natural product hybrid of Syringolin A and Glidobactin A synergizes proteasome inhibition potency with subsite selectivity<sup>†</sup>‡

Jérôme Clerc,<sup>a</sup> Nan Li,<sup>b</sup> Daniel Krahn,<sup>a</sup> Michael Groll,<sup>c</sup> André S. Bachmann,<sup>d</sup> Bogdan I. Florea,<sup>b</sup> Herman S. Overkleeft<sup>\*b</sup> and Markus Kaiser<sup>\*a</sup>

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The preparation of a Syringolin A/Glidobactin A hybrid (SylA–GlbA) consisting of a SylA macrocycle connected to the GlbA side chain and its potent proteasome targeting of all three proteasomal subsites is reported. The influence of the syrbactin macrocycle moiety on subsite selectivity is demonstrated.

Proteasomal protein degradation plays a critical role in innumerous biological processes such as cell division, immune responses, apoptosis and gene expression.<sup>1</sup> Proteolysis takes place in the 20S core particle (CP), a 670 kDa cylinder built up from four stacked heptameric rings, in which only the  $\beta$ 1,  $\beta$ 2 and  $\beta$ 5 subunits harbor distinct proteolytic activities with different substrate specificities.<sup>2</sup>

Besides the constitutive 20S CP, alternative 20S proteasome species are expressed in a tissue-specific manner. In the immunoproteasome, the constitutive  $\beta 1$ ,  $\beta 2$  and  $\beta 5$  subunits are replaced by the  $\beta 1i$ ,  $\beta 2i$  and  $\beta 5i$  subunits while the thymoproteasome encompasses the proteolytically active  $\beta 1i$ ,  $\beta 2i$  and  $\beta 5t$  subunits.<sup>3,4</sup> These subunits display alternative cleavage activities, thereby modulating the overall trimming of proteins during degradation.

Active-site directed proteasome inhibitors are valuable anticancer agents as well as versatile research tools.<sup>5</sup> Consequently, various rationally-designed small molecules and natural products have been explored in recent years.<sup>6</sup> These inhibitors usually target the proteasomal proteolytic subsites with different potencies. To date, the physiological consequences of such subsite specific inhibition are not well understood although distinct roles of individual proteasomal subunits for certain biological processes have already been observed.<sup>7</sup> This pinpoints that proteasome inhibitors with different subsite potencies could induce diverse pharmacological effects and consequently demands for a deeper understanding of the structural determinants governing subsite selectivity of proteasome inhibitor classes.

We recently elucidated the syrbactin natural products syringolin A (SylA), syringolin B (SylB) and glidobactin A (GlbA) as mechanism-based irreversible proteasome inhibitors (for the chemical structures of the syrbactins used during this study, see ESI<sup>‡</sup>).<sup>8</sup> These natural products exert distinct proteasomal subsite selectivities in biochemical activity assays as well as in structural studies and selectively target the proteasome in complex proteomes.<sup>9</sup> SylA targets all three proteolytic activities, albeit with different potencies. SylB and GlbA on the other hand selectively inhibit  $\beta 2$  and  $\beta 5$  even at high concentrations, inviting the hypothesis that the macrocyclic residue critically influences subsite selectivity while the N-terminal lipophilic tail strongly enhances inhibition potency. This hypothesis is supported by the finding that model compound SylA-LIP, consisting of a SylA core structure attached to a lipophilic side chain, inhibits all three proteolytic sites, thereby displaying the most potent inhibition of all syrbactins reported so far.<sup>10</sup> While SylA, GlbA and SylA-LIP showed promising anticancer activities in cell-based assays, the order of activity was somewhat surprising, with GlbA being more potent than SylA–LIP.<sup>11</sup>

To gain further insights into syrbactins' structure-activity relationships, we decided to put to the test two additional derivatives, being SylA–GlbA as a 'true' hybrid of Syringolin A and Glidobactin A, together with sat-SylA as a negative control. The synthesis of SylA–GlbA started with a Horner–Wadsworth–Emmons (HWE) olefination of octanal (1) using (*E*)-triethyl-4-phosphonocrotonate and LDA (Scheme 1). The resulting ethyl ester **2** was hydrolyzed with LiOH to yield the free acid **3**. Peptide coupling of Boc–Thr(O*t*Bu)–OH to the previously reported SylA macrocycle **4**<sup>12</sup> led to intermediate **5** which upon deprotection and PyBOP-mediated coupling of **3** yielded the desired SylA–GlbA hybrid (**6**). The synthesis of sat-SylA is reported in the ESI.‡

With these additional derivatives in hand, their inhibition potency and subsite selectivity was established using competitive activity-based protein profiling (ABPP) in cell lysates and living cells in comparison with known syrbactins.<sup>13</sup> To this end, HEK cell lysates were preincubated with varying concentrations of the syrbactins and profiled with the proteasomal activity based probe (ABP) MV151 (Fig. 1, for chemical structures of the ABPs, see ESI‡).<sup>14</sup> The results from these experiments were generally in good agreement with the results from the previous biochemical inhibition studies with

<sup>&</sup>lt;sup>a</sup> Zentrum für Medizinische Biotechnologie,

Fakultät für Biologie und Geographie, Universität Duisburg-Essen, 45117 Essen, Germany. E-mail: markus.kaiser@uni-due.de; Fax: +49 201 1833135; Tel: +49 201 1832949

 <sup>&</sup>lt;sup>b</sup> Leiden Institute of Chemistry and Netherlands Proteomics Centre, Gorlaeus Laboratories, Einsteinweg 55, 2333 CC Leiden, The Netherlands. E-mail: h.s.overkleeft@chem.leidenuniv.nl; Fax: +31 71 5274307; Tel: +31 71 5274342

<sup>&</sup>lt;sup>c</sup> Center for Integrated Protein Science at the Department Chemie, Technische Universität München, Lichtenbergstr. 4, 85747 Garching, Germany

<sup>&</sup>lt;sup>d</sup> Cancer Research Center of Hawaii, University of Hawaii at Manao, 1236 Lauhala Street, Honolulu, Hawaii 96813, USA

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Scheme 1 Structure and synthesis of the SylA–GlbA hybrid. (A) Chemical structures of SylA and GlbA and their mergence to the hybrid compound SylA–GlbA. (B) Chemical synthesis of the SylA–GlbA hybrid. (a) (*E*)-triethyl-4-phosphono crotonate (1.5 eq.), LDA (2 eq.), THF, -78 °C – rt, 2.5 h, 67%. (b) LiOH (3 eq.), MeOH/H<sub>2</sub>O (1 : 1), 50 °C, 1 h, 82%. (c) Boc–Thr(OtBu)–OH (1.2 eq.), PyBOP (1.5 eq.), HOAt (1.5 eq.), DIPEA (2 eq.), DMF, 0 °C – rt, 1 h, 62%. (d) (i) 20% TFA in DCM, rt, 30 min; (ii) **3** (1.2 eq.), PyBOP (1.5 eq.), HOAt (1.5 eq.), DIPEA (4 eq.), rt, o/n, 25%.

purified 20S CP, with only slight deviations in the activity pattern. All syrbactins except the non-reactive negative control sat-SylA bind the  $\beta$ 2 and  $\beta$ 5 subsites, although with different affinities. Furthermore, all syrbactins inhibited the B5 subunit more potently than  $\beta$ 2. A direct comparison of the different syrbactins revealed GlbA as the most potent inhibitor, followed by the only slightly less potent SylA-GlbA hybrid. Surprisingly and in contrast to what was found in the biochemical assay, SylA-LIP showed less affinity than GlbA, and we argue this discrepancy is the result of unspecific binding of the highly hydrophobic SylA-LIP to other proteins in the cell lysate. This finding might explain the unexpected activity pattern of GlbA and SylA-LIP in the previous cancer cell assays.<sup>11</sup> SylA and in particular SylB appeared much less active than the syrbactins with the N-terminal lipophilic chain. Importantly, while the syrbactins with a SylA macrocycle and in particular SylA-GlbA also bind, at high inhibitor concentrations, to the ß1 subunits, GlbA did not



Fig. 1 Competitive ABPP of HEK cell lysates using 1  $\mu$ M MV151 as ABP and varying concentrations of syrbactins. MV151-targeted proteasomal subsites (for assignment, see ref. 14) are marked by arrows on the left side of the gel. 20  $\mu$ g protein/lane was loaded. M = 25 kDa marker lane.

inhibit this subsite. These findings support our hypothesis that the type of macrocycle moiety critically influences  $\beta 1$  subsite selectivity.

Encouraged by these results, we continued our investigations by competitive ABPP of thymus lysates which besides the constitutive 20S CP also contain the immuno- and thymoproteasome (Fig. 2).<sup>15</sup> For the immunoproteasome, a similar trend for subunit selectivity and inhibition potency was observed with all syrbactins. While the negative control sat-SylA was completely inactive, the other derivatives preferentially bound to the  $\beta$ 5i subunit, followed by the  $\beta$ 2i subunit. Direct comparison of syrbactins revealed GlbA and SylA-GlbA as the most potent compounds, followed by SylA-LIP and SylA and lastly SylB. Interestingly, SylA-GlbA also bound to the  $\beta$ 1i subunit while GlbA showed (if any) only very weak inhibition. Thus, the immunoproteasome displays a similar inhibition pattern as the constitutive proteasome. Moreover, despite  $\beta$ 5t visualization by the MVB003 probe is generally only weak<sup>15</sup> (β5t being expressed in parts of the thymus only,<sup>4</sup> whereas we applied extracts from the organ as a whole), we observed that GlbA concentrations of 10 µM completely abolished ß5t labelling, while no such activity could be seen for any SylA-derived syrbactin. This different activity profile is in accordance with literature predictions<sup>4,15</sup> that ß5t displays a rather unique cleavage specificity, preferring hydrophilic residues such as the hydroxylysine and threonine moieties of GlbA in their active site pockets. Finally, as GlbA shows promising anti-cancer activities in cell-based and mouse



Fig. 2 Competitive ABPP of thymus lysates of 3-month old mice using 1  $\mu$ M MVB003 as ABP and varying concentrations of syrbactins. MVB003-targeted proteasomal subsites are marked by arrows (for assignment, see ref. 15) on the left side of the gel. 20  $\mu$ g protein/lane was loaded. M = 25 kDa marker lane.

models,<sup>11,16</sup> we compared its cellular inhibition properties with the hybrid SylA–GlbA (ESI‡). Both compounds exhibited potent activities, displaying concentration-dependent inhibition of the different proteasome subunits in a similar order as observed during the studies with lysates at biologically relevant concentrations. The observed potent inhibition of the proteasome by GlbA also in living cells is in agreement with its anticancer properties. Moreover, the SylA–GlbA hybrid shows roughly equipotent cellular inhibition properties for  $\beta 5/\beta 5i$  and  $\beta 2/\beta 2i$ . The additional  $\beta 1/\beta 1i$  inhibition of SylA–GlbA *vs*. GlbA therefore might turn SylA–GlbA into an interesting probe for investigating the function of this subsite.

In summary, we have demonstrated with the SylA–GlbA hybrid that the SylA or GlbA macrocycle moiety has critical influence on the  $\beta$ 1 subsite selectivity. Moreover, hydrophilic functionalities, such as in GlbA, appear to favour binding to  $\beta$ 5t in comparison with  $\beta$ 5/ $\beta$ 5i and this finding may serve as a guideline for the development of inhibitors/ABPs specific for this yet poorly understood thymoproteasome-specific subunit. Finally, SylA–GlbA demonstrates potent proteasome inhibition properties in lysates as well as in living cells, suggesting its use as a small molecule to probe proteasome function and as a promising anticancer agent.

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