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Activity-Based Proteomics

Receptor-Mediated Targeting of Cathepsins in Professional Antigen Presenting Cells**

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Activity-based profiling probes (ABPs) have received considerable attention in recent years as tools for the detection of enzyme activities both in cell extracts and in living cells.[1] An eminent example of an ABP is the biotinylated peptide epoxysuccinate DCG-04 (**1**; Figure 1).[2] Peptide epoxysuccinates covalently and irreversibly inhibit cysteine proteases of the papain family, and attachment of an affinity or identification handle enables visualization and/or enrichment of the thus modified proteases for ensuing studies in a proteomics setting.[3] In this way, and depending on the research setting, the action of known cysteine proteases may be monitored in the appropriate physiological conditions, and new proteolytic activities may be unearthed. The cathepsins, of which the majority are cysteine proteases, reside mostly in the endosomal and lysosomal compartments.[4] Here they partake in the processing and degradation of proteins brought to these compartments by endocytosis and phagocytosis events, but also by specific targeting of newly synthesized proteins through the secretory pathway. Cathepsin activity is however not restricted to the endo/lysosomal pathway. For instance, cathepsin L is excreted to the extracellular space by some tissues and cathepsin activities are found in other subcellular compartments as well. Measuring cathepsin activity in cell lysates therefore may not accurately reflect the relevant activity being studied in a given subcellular compartment, even more so since the optimum pH value for a given cathepsin (encountered upon gradual acidification in the course of endosome maturation) may differ from that of the next cathepsin.

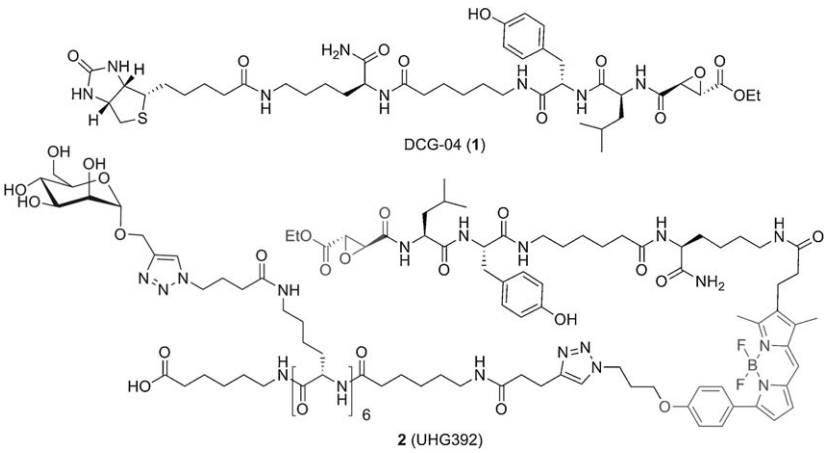

Figure 1. Structures of epoxysuccinate DCG-04 (**1**) and the envisioned mannosylated fluorescent epoxysuccinate construct **2**.

Information on lysosomal and endosomal cysteine protease activity is of particular interest in the context of processing of antigenic peptides and their presentation on both MHC (major histocompatibility complex) class II molecules and MHC class I molecules[5] (the latter in a process referred to as cross-presentation[6]). For this purpose, cathepsin-directed ABPs that specifically target the endosomal/lysosomal compartments would be of particular interest. Some years ago Ploegh and co-workers reported a strategy in which streptavidin-coated latex beads were loaded with the biotinylated ABP, DCG-04.[7] The resulting constructs appeared suitable for phagocytosis by macrophages after which the encountered cysteine proteases were trapped for post-lysis analysis and identification by strip domain structure polyacrylamide gel electrophoresis (SDS-PAGE). We report herein the design of fluorescent epoxysuccinate construct **2** (Figure 1) equipped with a mannose cluster,[8] with which a broad panel of cysteine proteases are selectively targeted in the endosomal pathway in both dendritic cells and macro-

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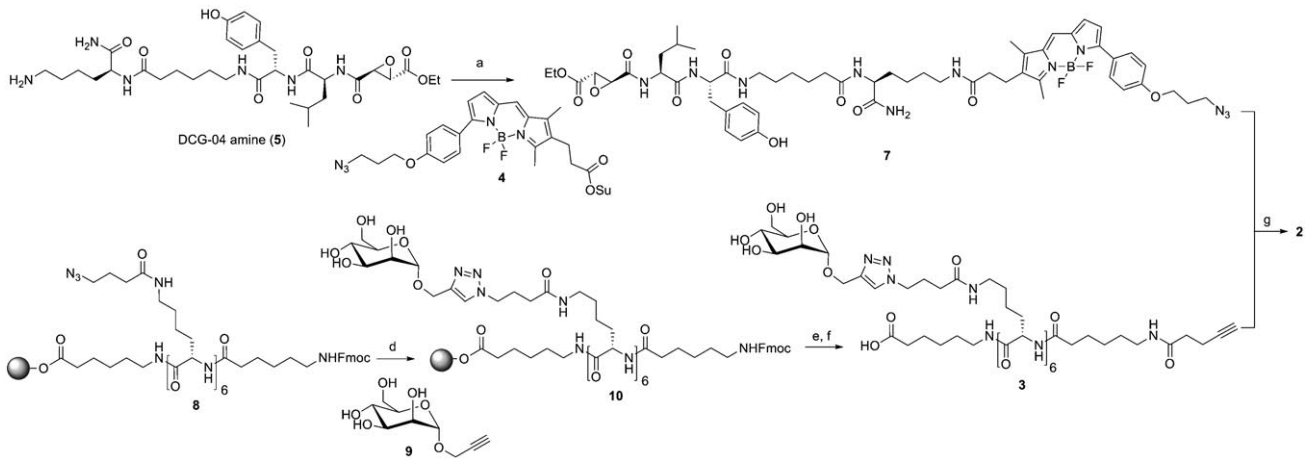

Scheme 1. Synthesis of ABP **2**. Reagents and conditions a) DMF, dipea, over night, room temperature (93%); b) Fmoc-Ahx-OH, DIC, 4-DMAP, DCM, 2 h, room temperature; c) 1. 20% piperidine in NMP, 20′, room temperature (7×); 2. α-Fmoc-ε-(4-azidobutyryl)lysine (**6**; 6×) or Fmoc-Ahx-OH (1×), BOP, dipea, NMP, 4 h, room temperature; d) **8** (80 equiv), $CuSO_4$ (8 equiv), sodium ascorbate (8 equiv), *t*BuOH:DMF:H_2O (2:1:1), 43 h, room temperature; e) 1. 20% piperidine in NMP, 20′, room temperature; 2. pent-4-ynoic acid, BOP, dipea, NMP, over night, room temperature; f) TFA/H_2O (1:4), 3×5′, room temperature, (85%); g) $CuSO_4$ (10 mol%), sodium ascorbate (15 mol%), *t*BuOH:H_2O (1:1.7), 48 h, room temperature, then DMF (1.7), 80°C, 2 h (14%). dipea = *N*,*N*-diisopropylethylamine, DIC = 5-(3,3-dimethyl-1-triazenyl)-1H-imidazole-4-carboxamide, 4-DMAP = 4-(dimethylamino)pyridine, DCM = dichloromethane, NMP = *N*-methyl-2-pyrrolidinone, BOP = 1-benzotriazolyl)oxy tris(dimethylamino) phosphonium [PF_6], TFA = trifluoroacetic acid, 20′ = 20 min, 5′ = 5 min, Su = succinimidyl.

phages by means of mannose receptor (MR) mediated uptake.

The synthesis of epoxysuccinate **5** was accomplished by standard solid-phase peptide synthesis (SPPS) methods following essentially the strategy reported by Bogyo and coworkers in their work on the design of biotinylated and BODIPYlated epoxysuccinate ABPs (Scheme 1).[9] Condensation of **5** with N_3-BODIPY(TMR)-OSu **4** (prepared as previously reported[10]) gave intermediate **7** for ensuing ligation to mannose cluster **3** by means of a copper(I)-catalyzed Huisgen [2+3] cycloaddition reaction.[11]

The synthesis of **3** was accomplished as follows. Standard Fmoc-based SPPS starting from Tentagel S HMPB resin and employing first Fmoc-aminohexanoic acid, then α-Fmoc-ε-(4-azidobutyryl)lysine **6** (six peptide coupling-Fmoc cleavage cycles; Fmoc = 9H-fluoren-9-ylmethoxy)carbonyl) and finally Fmoc-aminohexanoic acid (Fmoc-Ahx-OH) gave immobilized heptapeptide **8** featuring six azide moieties. The key step in our synthetic efforts was the at this stage simultaneous introduction of six mannose units on solid support using a copper(I) catalyzed [1,3]-dipolar cycloaddition reaction.

To this aim, intermediate **8** was treated with a large excess of 2-propynyl α-D-mannopyranoside (**9**) in the presence of sodium ascorbate and copper(II) sulfate, to give, after 43 h reaction time, in a clean conversion, mannose cluster **10**. Subsequent introduction of pent-4-ynoic acid and final cleavage from resin using mild acidic conditions gave access to hexavalent mannoside cluster **3**. Click ligation of **3** to azido-BODIPY-DCG-04 (**7**) afforded target construct **2** after HPLC purification.[12]

Incubation of rat liver lysate with **2**, followed by SDS-PAGE resolution of proteins and direct in-gel fluorescence scanning, revealed a similar labeling profile (Figure 2A) as earlier reported for fluorescent DCG-04 derivatives.[9] Proteins labeled with **2** were up-shifted by circa 4 kD which nicely corresponds with the molecular weight of this ABP. Treatment of cell lysates from immature murine bone-marrow derived dendritic cells (BM-DC) with increasing concentra-

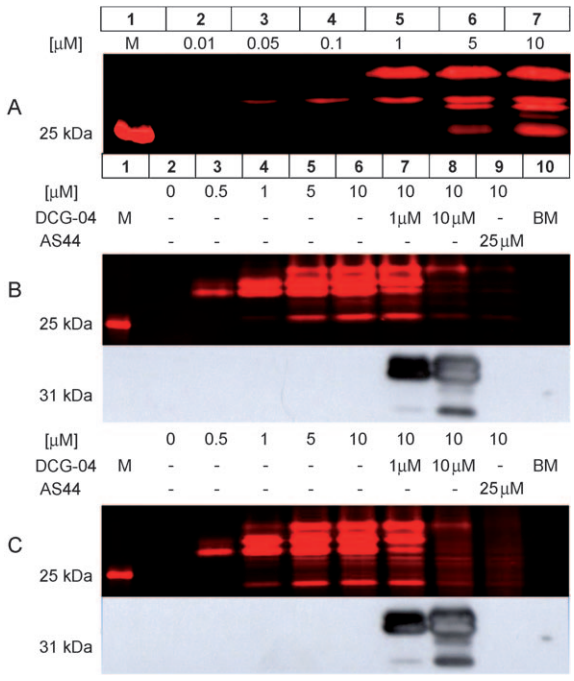

Figure 2. Activity profiling of **2** and **7**. Reagents and conditions: lysates in 25 mM MES pH 5.0 were incubated for 1 h at 37°C with ABPs, resolved on 12.5% SDS-PAGE gels, with fluorescent in-gel or luminescent western blot readout (M: Marker, dual color, BioRad) A) rat liver lysate 40 μg protein/lane, with increasing **2** concentrations; B) BM-DC lysate 10 μg protein/lane with increasing **2** concentrations, and where indicated pre-incubation with inhibitors for 1 h at 37°C; lower panel Strep-HRP western against DCG-04. C) analogue as in (B) with **7** as ABP.

tions of **2** (Figure 2B lanes 2–6)) showed concentration dependent labeling. Pre-incubation of BM-DC lysate with DCG-04 (lanes 7,8) or **AS44** (lane 9; Supporting Information), a truncated DCG-04 derivative, abolished labeling. Western blot analysis with streptavidin-HRP against the biotin tag of DCG04 showed an inversely proportional relationship to the fluorescence intensity of **2** (lane 7,8 Figure 2B lower panel). Probe **7**, lacking the oligomannose cluster, gave a similar labeling profile as **2**, except that the bands run lower on the gel owing to the lower molecular weight of **7** (Figure 2C). These results demonstrate that **2**, **7**, and **AS44** bind to the same cysteine cathepsins as DCG-04 and, importantly, that the poly-lysine mannoside moiety does not interfere with cathepsin binding.

To determine the magnitude and the mechanism of fluorescent ABP uptake, we performed pulse-chase experiments monitored by flow cytometry analysis. Figure 3A shows normalized flow cytometry results from immature BM-DCs and bone-marrow derived macrophages (BM-MΦ), both expressing the mannose receptor. Cells pulsed for 1 h with 10 μM of **2** at 4 °C, washed and chased at 4 °C (Figure 3A, lane 1) reveal no fluorescence uptake whereas cells treated at 37 °C do (Figure 3B, lane 2). The 4 °C pulse, 37 °C chase experiment (Figure 3A, lane 3) shows an intermediate uptake indicating that **2** endocytosis proceeds by an active and temperature-dependent mechanism. Apparently, at 4 °C, the extracellular **2**-mannose receptor complex is stable enough to withstand the washing step and is endocytosed at 37 °C but not at 4 °C. The fact that ligand binding to the mannose receptor is Ca^{2+} ion dependent[13] provides an alternative means to investigate the uptake mechanism of mannose clustered **2**. Thus, when we used a phosphate buffered saline (PBS) buffer containing 4 mM EDTA (ethylenediaminetetraacetate) to harvest cells prior to flow cytometry analysis, we found lowered fluorescence signal in cells chased at 4 °C but not in cells chased at 37 °C. At 4 °C the EDTA in the buffer sequesters the Ca^{2+} ions from the mannose receptor upon which mannose clustered **2** is released, prohibiting endocytosis at elevated temperatures. Adding EDTA to cells treated with **2** at 37 °C has, as expected, less effect on the fluorescence levels, as at this temperature mannose receptors clustered to **2** are already internalized.

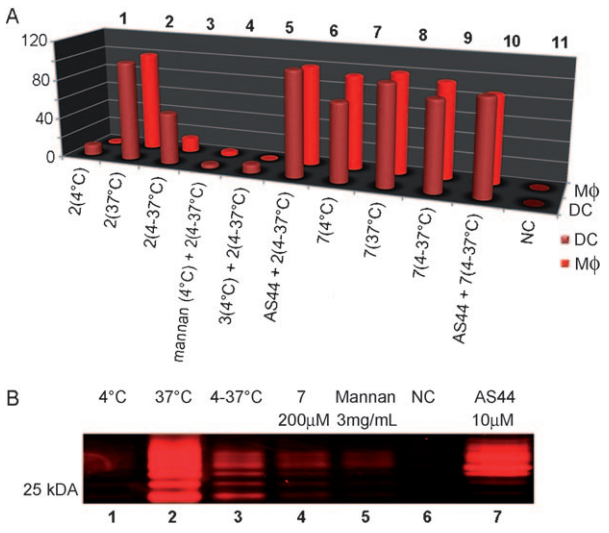

Figure 3. Cellular uptake mechanism of **2** and **7** in BM-DCs and BM-MΦ. Reagents and conditions: A) Live cells (DCs and MΦ) normalized flow cytometry analysis of **2** and **7** pulse-chase experiments: lane 1–3 and 7–9 temperature dependent, lanes 4–5 MR blockers, lanes 7 and 10 cathepsin inhibition; NC = non-treated cells; B) SDS-PAGE (12.5%) in-gel fluorescence analysis of BM-DC lysates at pH 7.5 from lanes 1–6 of Figure 3A (10 μg protein/lane).

We found that uptake of **2** is blocked by the established MR ligand, mannan (poly-β-1-6-mannose, Figure 3A lane 5) as well as hexalysine mannoside **3** (Figure 3A, lane 4). This result again strongly points towards the endocytosis of **2** in a MR-dependent manner. Preincubation of cells with the cathepsin blocker **AS44** on the other hand did not inhibit **2** uptake (Figure 3A, lane 6) and demonstrates that MR-mediated uptake is, again as expected, independent of cathepsin activity. Additionally, internalization of **7** is not temperature (Figure 3A, lanes 7,8,9) or **AS44**-inhibition (Figure 3A, lane 10) dependent. This result indicates, in agreement with the microscopy data, that **7** crosses the plasma membrane by passive diffusion.

In parallel with the flow cytometry analysis, we lyzed some of the cells at pH 7.5 (to inactivate cathepsins and prevent post-lysis tagging) and analyzed cathepsin labeling by SDS-PAGE and in-gel fluorescence (Figure 3B). In agreement with the flow cytometry results, the absence of cathepsin labeling at 4 °C (Figure 3B, lane 1) and in the presence of MR blockers (Figure 3B, lane 4 and 5) demonstrates that **2** is not cell permeable and is taken up through a MR-mediated process. Moreover, a lipophilic compound, such as **7**, readily labels cathepsins at low temperature, strengthening the point above. Cathepsin labeling with **2** was observed at 37 °C (Figure 3B, lane 2) and was reduced to 25% in the 4 °C to 37 °C pulse-chase experiment (Figure 3B lane 3) confirming the flow cytometry data. Pre-incubation with **AS44** (Figure 3B, lane 7) blocked more than 50% of the fluorescent labeling of cysteine cathepsins, but it did not inhibit the cellular uptake of **2** as seen by flow cytometry.

In conclusion, we designed and synthesized a fluorescently tagged clustered mannoside DCG-04 analogue and established that its uptake by antigen presenting cells (APCs) and subsequent cathepsin labeling proceeds in a mannose-receptor dependent manner. Compound **2** may thus find use in the study of cysteine protease activities in the relevant subcellular compartments of APCs and in the context of antigen processing and (cross)presentation. The modular approach to the assembly of **2** is flexible and in principle allows variation in both the mannose cluster and the electrophilic trap. Thus high-end fluorescent probes targeting other receptors (for instance, members of the Toll-like receptor family) and intracellular targets (for instance, any of the broad family of lysosomal hydrolase activities) may be considered and we feel that our modular strategy may find application in areas of physiological relevance quite unrelated to the subject at hand.

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