

## Supporting Information

### **iBodies: Modular Synthetic Antibody Mimetics Based on Hydrophilic Polymers Decorated with Functional Moieties**

*Pavel Šácha<sup>+</sup>, Tomáš Knedlík<sup>+</sup>, Jiří Schimer, Jan Tykvart, Jan Parolek, Václav Navrátil, Petra Dvořáková, František Sedlák, Karel Ulbrich, Jiří Strohalm, Pavel Majer, Vladimír Šubr,\* and Jan Konvalinka\**

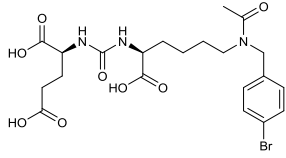
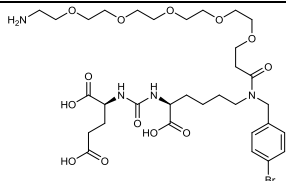
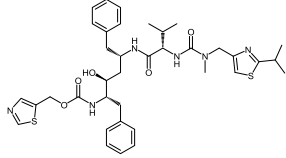
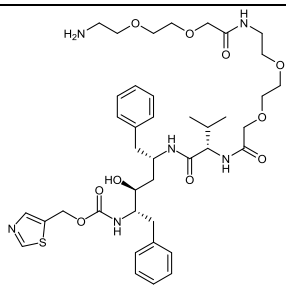
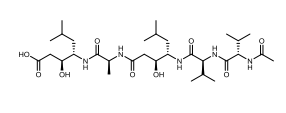
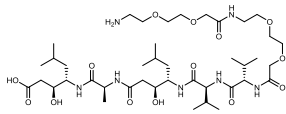
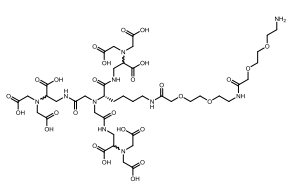
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## Supplementary Table S1

Summary of low-molecular-weight compounds.

Name	M <sub>r</sub>	Formula	Targeting	K <sub>i</sub> [pM]	Modification
compound <b>22a</b> <sup>[1]</sup>	530		GCPII	45 ± 5.7 <sup>[1]</sup>	-
compound <b>1</b>	780		GCPII	2,030 ± 430	PEG linker
ritonavir	721		HIV-1 protease	15 ± 2 <sup>[2]</sup>	-
compound <b>2</b>	815		HIV-1 protease	12 ± 1	PEG linker
acetyl-pepstatin	644		aspartic proteases	116,000 ± 5,000 <sup>[2]*</sup>	-
compound <b>3</b>	892		aspartic proteases	623,000 ± 1,710*	PEG linker
compound <b>4</b>	1,159		His-tag sequence	ND	PEG linker

\* K<sub>i</sub> values were determined for wild-type HIV-1 protease.

## Supplementary Table S2

The composition and full characteristics of the prepared iBodies

Conjugate	M <sub>n</sub> (precursor)	Đ (precursor)	M <sub>w</sub> (precursor)	M <sub>n</sub> (conjugate)	Đ (conjugate)	M <sub>w</sub> (conjugate)	Inhibitor (wt%)	ATTO488 (wt%)	Biotin (wt%)
iBody 1	79,000	1.20	94,600	148,800	1.37	203,900	9.8 %	3.9 %	9.8 %
iBody 2	24,800	1.08	26,700	40,600	1.65	67,000	11.7 %	-	4.7 %
iBody 3	24,800	1.08	26,700	42,100	1.32	55,500	15.3 %	-	5.9 %
iBody 4	73,800	1.23	90,600	135,800	1.88	255,000	11.3 %	4.2 %	9.7 %
iBody 5	79,000	1.20	94,600	108,100	1.63	176,200	-	5.1 %	10.8 %
iBody 6	24,800	1.08	26,700	37,800	1.53	57,800	-	-	6.4 %

\* M<sub>w</sub>: weight-average molecular weight; M<sub>n</sub>: number-average molecular weight; Đ: dispersity

### Supplementary Table S3

Composition of iBodies determined by analytical methods

#		iBody 1	iBody 2	iBody 3	iBody 4	iBody 5	iBody 6
1	ATTO488 (wt%)	3.9	-	-	4.2	5.1	-
2	Biotin (wt%)	9.8	4.7	5.9	9.7	10.8	6.4
3	Targeting ligand (wt%)	9.8	11.7	15.3	11.3	-	-
4	No. of ATTO488 units	7	-	-	7	6	-
5	No. of biotin units	51	7	9	46	41	8
6	No. of targeting ligand units	19	6	7	12	-	-
7	Normalized ATTO488 content	<b>1.0</b>	-	-	<b>1.0</b>	<b>1.0</b>	-
8	Normalized biotin content	<b>7.5</b>	<b>1.1</b>	<b>1.2</b>	<b>6.9</b>	<b>6.4</b>	<b>1.0</b>
9	Normalized targeting ligand content	<b>2.8</b>	<b>1.0</b>	<b>1.0</b>	<b>1.8</b>	-	-

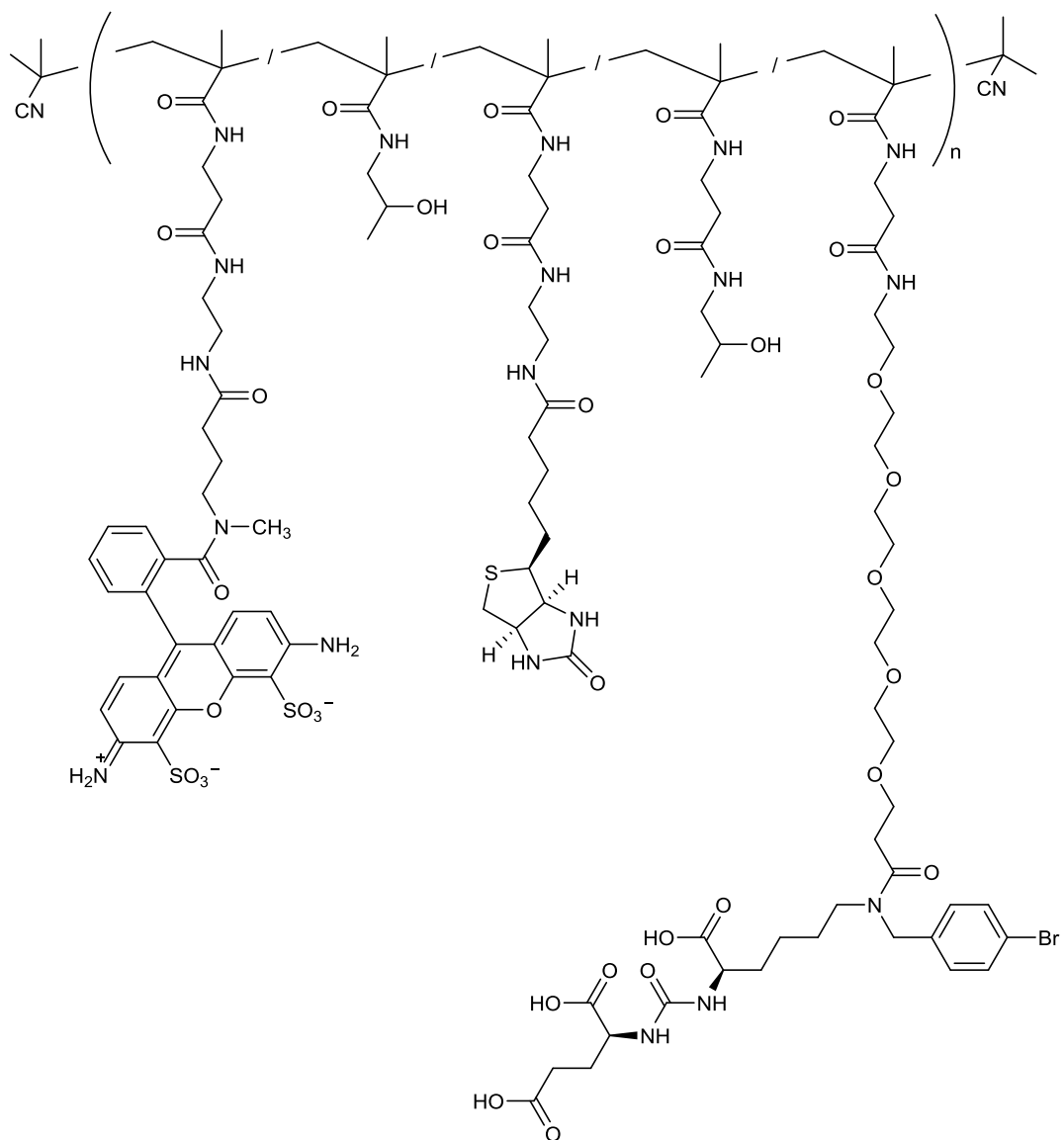
To find out whether the aminolytic reaction follows the statistical course, we calculated the composition of the iBodies by analytical methods and compared this to the theoretical composition of the iBodies calculated from the reaction stoichiometry. The values (in red) of the corresponding parameters correlate well, thus proving that the reaction proceeds statistically. The composition of iBodies, as determined by analytical methods (described in the Supporting Information, p. 29). Lines 1-3 represent mass fraction (wt%) of the individual moieties (ATTO488, biotin, targeting ligand). Lines 4-6 represent the number of units (ATTO488, biotin, targeting ligand) per conjugate. Lines 7-9 represent the ratios between the moieties. Ratios were normalized to the lowest-incorporated moiety.

### Supplementary Table S4

Theoretical composition of iBodies calculated from the reaction stoichiometry

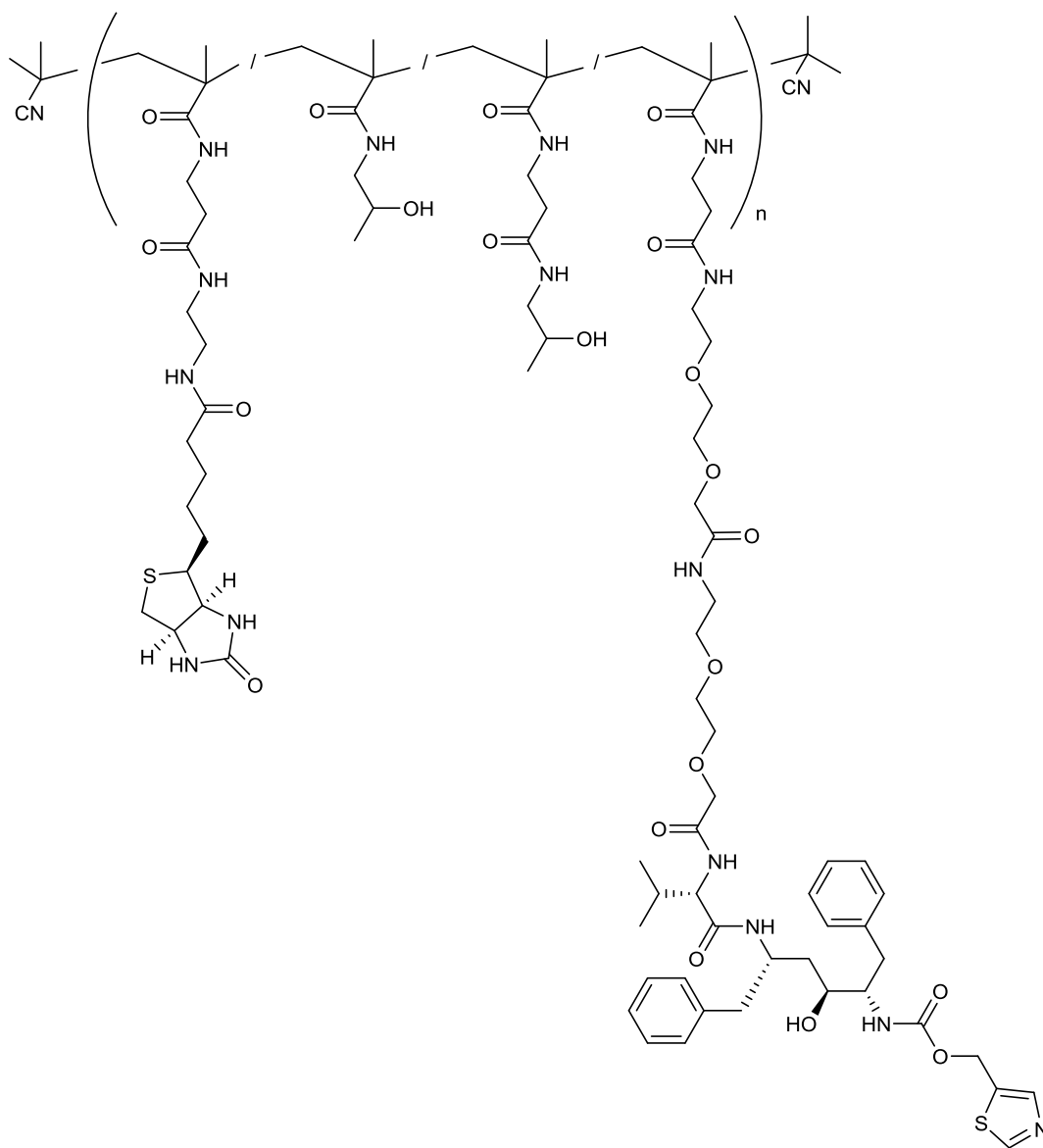
#		iBody 1	iBody 2	iBody 3	iBody 4	iBody 5	iBody 6
1	m (ATTO488; mg)	2.5	-	-	2.5	2.5	-
2	m (biotin; mg)	5.0	6.0	6.0	5.0	5.0	5.0
3	m (targeting ligand; mg)	6.2	13.0	10.0	6.0	-	-
4	n (ATTO488; $\mu\text{mol}$ )	2.9	-	-	2.9	2.9	-
5	n (biotin; $\mu\text{mol}$ )	17.5	20.9	20.9	17.5	17.5	17.5
6	n (targeting ligand; $\mu\text{mol}$ )	8.0	16.0	11.2	4.7	-	-
7	$n_{\text{norm.}}$ (ATTO488; $\mu\text{mol}$ )	<b>1.0</b>	-	-	<b>1.0</b>	<b>1.0</b>	-
8	$n_{\text{norm.}}$ (biotin; $\mu\text{mol}$ )	<b>6.0</b>	<b>1.3</b>	<b>1.9</b>	<b>6.0</b>	<b>6.0</b>	<b>1.0</b>
9	$n_{\text{norm.}}$ (targeting ligand; $\mu\text{mol}$ )	<b>2.7</b>	<b>1.0</b>	<b>1.0</b>	<b>1.6</b>	-	-

The theoretical composition of iBodies, as calculated from the reaction stoichiometry (described in the Supporting Information, p. 28-33). Lines 1-3 represent the masses of the individual moieties (ATTO488, biotin, targeting ligand) expected to be incorporated into the iBodies. Lines 4-6 represent the moles of each moiety expected to be incorporated into the iBodies. Lines 7-9 represent the ratios between the molar amounts, normalized to the lowest-incorporated moiety.



**Supplementary Figure S1**  
Structure of iBody 1 targeting GCPII.



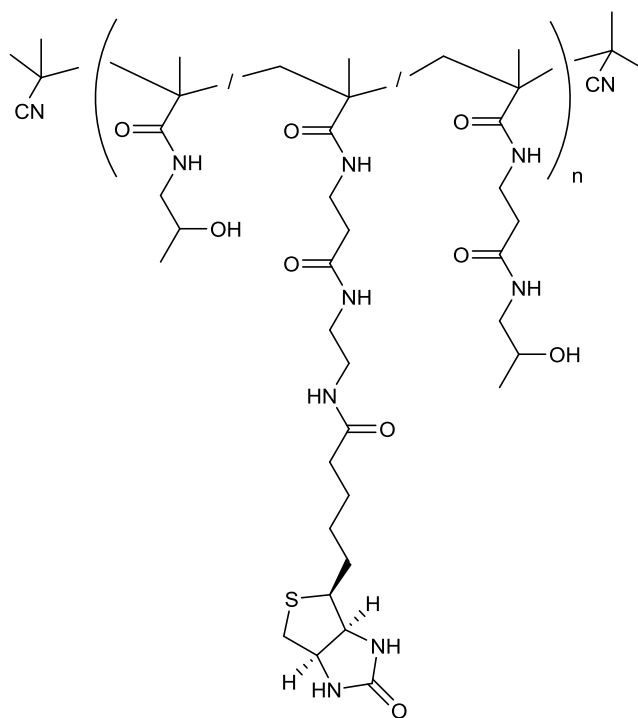


### Supplementary Figure S3

Structure of iBody 2 targeting HIV-1 protease.



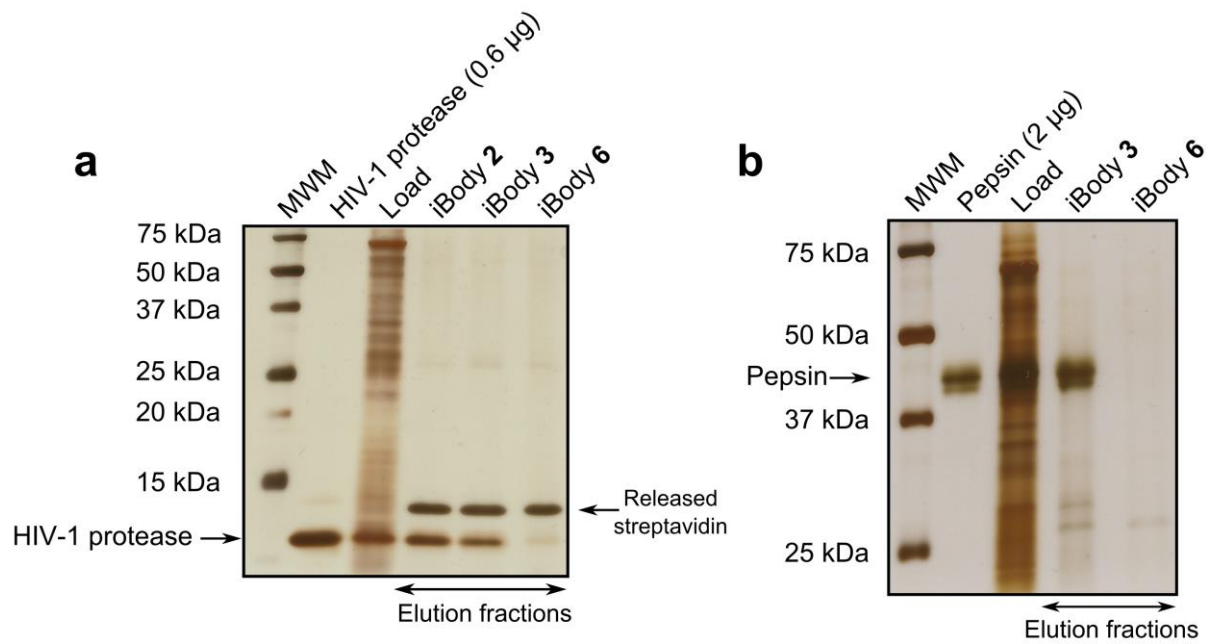




### Supplementary Figure S5

Structure of iBody 6 used as a negative control.





### Supplementary Figure S7

iBodies targeting HIV-1 protease and aspartic proteases (pepsin). a) Affinity isolation of HIV-1 protease from the HIV-1 protease-spiked LNCaP cell lysate using iBody 2 and iBody 3; iBody 6 lacking the targeting ligand was used as a negative control. b) Affinity isolation of pepsin from the pepsin-spiked cell lysate using iBody 3; as a negative control, iBody 6 was used.

## Supplementary methods

### HPLC-based GCPII inhibition assay

The inhibitory effects of all inhibitors and iBodies targeting GCPII were tested using an HPLC-based assay with recombinant extracellular GCPII, as described previously<sup>[1]</sup>. The data were processed and IC<sub>50</sub> values were obtained using GraFit v.5.0.11 (Erithacus Software Ltd.).

From the kinetic parameters of pteroyl-di-L-glutamate cleavage and by assuming a competitive mode of inhibition, the K<sub>i</sub> value was calculated using the Cheng-Prusoff equation<sup>[3]</sup>. Measurements were performed in duplicate; the K<sub>i</sub> values are presented as the mean ± standard deviation.

### Pull-down/immunoprecipitation of GCPII from LNCaP lysate

LNCaP cells endogenously expressing GCPII were lysed by sonication in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % Tween 20. The resulting cell lysate was diluted in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween 20 (TBST) to a final protein concentration of 2 mg/ml.

First, 200 µl of 30 nM iBody **1** and **5** in TBST were bound onto 30 µl Streptavidin Agarose Ultra Performance (Solulink) for 30 min at room temperature. After washing with TBST (3×1,000 µl), the resin was mixed with 200 µl of the LNCaP lysate and incubated for 1 h at room temperature. The resin was then washed with TBST (5×1,000 µl). Finally, proteins were eluted from Streptavidin Agarose by adding 30 µl of reducing SDS sample buffer and heating to 98 °C for 10 min.

To compare iBodies with antibodies, an analogous immunoprecipitation experiment with the monoclonal antibody J591<sup>[4]</sup> was performed. First, 200 µl of 30 nM J591 was bound to 30 µl Protein G Sepharose (GE Healthcare), washed with TBST, and mixed with LNCaP lysate. After incubation, the resin was washed with TBST, and proteins were eluted with reducing SDS sample buffer and heating.

Isolated GCPII was visualized on Western blot using mAb GCP-04, followed by goat anti-mouse secondary antibody conjugated with horseradish peroxidase. 10 µl of the sample was loaded into each lane.

## **Quantitative detection of GCPII using ELISA**

When iBody 1 was used in place of the detecting antibody, a 96-well Maxisorb plate (Nunc) was coated with the GCPII-specific mouse antibody 2G7<sup>[5]</sup> in borate buffer (500 ng/well; 1 h at RT). The surface was blocked with 1.1 % (w/v) casein solution in TBS (Casein Buffer 20X-4X Concentrate, SDT). Afterwards, recombinant extracellular GCPII in TBST was added (ranging from 0.5 pg – 1 ng/well) and, after washing with TBST (3×200 µl), 500 pM iBody 1 or biotinylated anti-GCPII mAb J591 in TBST was added to bind GCPII. After washing out the unbound conjugate (3×200 µl TBST), NeutrAvidin conjugated with horseradish peroxidase was added (50 ng/well; Thermo Scientific), followed by a final TBST wash (5×200 µl) and measurement of 4-iodophenol-enhanced luminol chemiluminescence on a Tecan Infinite M1000 PRO spectrophotometric reader. Measurement of each sample was performed in triplicate; values are presented as the mean ± standard deviation.

## **Confocal microscopy of fluorescently labeled cells**

LNCaP and PC3 cells were grown in 4-Chamber 35 mm Glass Bottom Dishes (In Vitro Scientific). LNCaP cells (which endogenously express GCPII) were grown in RPMI-1640 medium (Sigma-Aldrich) with addition of FBS (final concentration of 10 %), while PC3 cells (which do not express GCPII) were grown in DMEM-High Glucose medium (GE Healthcare) with addition of L-glutamine (final concentration of 4 mM) and FBS (final concentration of 10 %).

After two days, iBody solution was added to the media to a final concentration of 10 nM. If desirable, 2-(phosphonomethyl)pentanedioic acid (2-PMPA) was added to a final concentration of 500 nM. As a control, fluorescently labeled anti-GCPII monoclonal antibody 2G7<sup>[5]</sup> (2G7-ATTO488) was added to a final concentration of 100 nM. The cells were incubated in the presence of iBodies for 2 h at 37 °C. Then, the media were removed, a 0.5 µg/ml solution of Hoechst Stain Solution H34580 (Sigma) in PBS was added, and cells were incubated for 10 min at 37 °C to stain cell nuclei. Finally, the cells were washed twice with 500 µl PBS.

Confocal images (pinhole 1 Airy unit) of cells in each chamber were taken with a Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy) using an oil-immersion objective (Plan-Apochromat 63x/1.40 Oil DIC M27). The fluorescent images were collected at room temperature using 4.5 % of the 405 nm diode laser (max. power 30 mW) for excitation with emission collected from 410 to 585 nm (voltage on detector: 850 V) for Hoechst 34580 and 4.0 % of the 488 nm argon-ion laser (max. power 25 mW) for excitation

with emission collected from 517 to 534 nm (voltage on detector: 870 V) for ATTO488. All images were taken using the same settings. The microscope was operated and the images were processed with ZEN 2011 software (Carl Zeiss Microscopy).

### **Quantification of iBody binding to GCPII by surface plasmon resonance (SPR)**

All SPR measurements were performed at 25 °C according to a previously described protocol<sup>[1, 6]</sup>. In a typical experiment, a 7:3 mixture of HS-(CH<sub>2</sub>)<sub>11</sub>-PEG<sub>4</sub>-OH and HS-(CH<sub>2</sub>)<sub>11</sub>-PEG<sub>6</sub>-O-CH<sub>2</sub>-COOH alkanethiols (Prochimia) at a final concentration of 0.2 mM was incubated with an SPR chip (provided by the Institute of Photonics and Electronics, Prague) for 1 h at 37 °C. The chip was then washed with UV ethanol and deionized water and dried with a flow of nitrogen gas. Finally, the chip was mounted to the prism on the SPR sensor.

Activation of carboxylic terminal groups on the sensor surface was performed *in situ* by injecting a 1:1 mixture of 11.51 mg/ml *N*-hydroxysuccinimide (NHS) and 76.68 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) in deionized water (Biacore) for 5 min at a flow rate of 20 µl/min. The next part of the experiment was performed at a flow rate of 30 µl/min. Then, a mixture of the antibody D2B<sup>[7]</sup>, which recognizes native GCPII (20 ng/µl), and BSA (20 ng/µl) in 10 mM sodium acetate, pH 5.0, was loaded for 8 min. Next, a high ionic strength solution (PBS with 0.5 M NaCl) was used to wash out noncovalently bound molecules, followed by addition of 1 M ethanolamine (Sigma-Aldrich) for deactivation of residual activated carboxylic groups.

An 8 ng/µl solution of recombinant extracellular GCPII in TBS (Avi-GCPII<sup>[8]</sup>) was used for Avi-GCPII immobilization onto the D2B/BSA layer on the golden chip to saturate all binding sites. Finally, at a flow rate of 60 µl/min, 4 different concentrations (1, 2, 4, and 8 nM) of iBody 1 in TBS were applied for approximately 10 min (association), followed by injection of TBS alone (dissociation).

Kinetic curves of binding were exported and subsequently fitted using the One-To-One interaction model in TraceDrawer v.1.5 (Ridgeview Instruments AB) to obtain  $k_{on}$  and  $k_{off}$  parameters.

### **SDS-PAGE and Western blotting**

Protein samples were resolved by SDS-PAGE, and gels were electroblotted onto a nitrocellulose or PVDF membrane (wet blotting: 100 V/1 h). Membranes were blocked with 1.1 % (w/v) casein solution in PBS (Casein Buffer 20X-4X Concentrate, SDT) at room temperature for 1 h. Afterwards, the blots were incubated with the primary antibody GCP-

04<sup>[9]</sup> at 4 °C for 12 h (200 ng/ml; diluted in 0.55 % casein solution), washed three times with PBS containing 0.05 % Tween 20 (PBST), and incubated with goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Thermo Scientific; diluted 1:25,000 in 0.55 % casein solution). For polyhistidine sequence (His-tag) detection, either anti-polyhistidine-peroxidase antibody (Sigma Aldrich, #A7058-1VL) (1:2,000 dilution in PBST) or iBody 4 (5 nM in PBST, loaded with nickel ions) followed by NeutrAvidin-HRP conjugate (dilution 1:2,000 in PBST) was used.

Finally, the blots were washed three times with PBST to remove free antibodies, and SuperSignal West Dura/Femto Chemiluminescent Substrate (Thermo Scientific) was applied. Chemiluminescence was captured with a ChemiDoc-It™ 600 Imaging System (UVP).

### **Preparation of a fluorescently labeled anti-GCPII monoclonal antibody 2G7 (2G7-ATTO488)**

An antibody against human native GCPII (2G7<sup>[5]</sup>) was labeled with a fluorophore ATTO488 using ATTO488 NHS ester (Sigma Aldrich, #41698). First, 77 µl of a 2.5 mg/ml solution of the antibody 2G7 in PBS was mixed with 8.6 µl of 100 mM bicarbonate buffer, pH 8.3. ATTO488 NHS ester was dissolved in dry DMSO at 10 mg/ml. Afterwards, 1 µl of the ATTO488 NHS ester solution was mixed with the antibody solution and incubated for 1 h at room temperature. Finally, the fluorophore-antibody conjugate (2G7-ATTO488) was separated from the free fluorophore by gel permeation chromatography using Sephadex G-25 column. Fractions containing 2G7-ATTO488 were pooled and concentrated using Amicon Ultra 30 kDa centrifugal filter (Millipore).

### **Flow cytometry**

PC3 and LNCaP cell lines were grown on a 100 mm dish to 90 % confluence in DMEM and RPMI medium, respectively; both supplemented with 10 % FBS and 4 mM L-glutamine. The medium was removed, and cells were rinsed with PBS and subsequently incubated in 1.5 ml trypsin/EDTA solution for 3 min to release adherent cells from the dish surface. Cells were resuspended and transferred into 8 ml of DMEM or RPMI medium, centrifuged at 250×g for 2 min, and washed with 5 ml PBS. Then, 500 µl of 10 % fetal bovine serum in PBS was added to block the cell surface. The final concentration of the cell suspension was 4×10<sup>6</sup> cells/ml.

Afterwards, 50 µl of cell suspension (containing 2×10<sup>5</sup> cells) was placed into wells of a polypropylene 96-well plate (round bottom) and incubated with 10 nM solutions of iBodies



and 400 nM 2G7-ATTO488 for 1 h at 37 °C. Cells were washed twice with 200 µl of 10 % fetal bovine serum in PBS. Finally, the cell suspension was diluted with 200 µl of 10 % fetal bovine serum in PBS, and a single cell suspension was analyzed with a BD LSR Fortessa™ cell analyzer (Becton, Dickinson and Company).

The gates on the side scatter and forward scatter were set to ensure measurement of viable cells, and 10,000 events were measured for each well. All experiments (all staining agents and both cell lines) were performed in triplicates. Analysis was performed using BD FACSDiva™ Software. A histogram showing a representative measurement for each sample is presented. The number in the top corner of each histogram represents the percentage of the treated cell population having a higher MFI signal than the highest 1 % of the untreated cell population. The number was calculated for each measurement and is shown as the mean ± standard deviation.

### **Inhibition of HIV-1 protease activity with inhibitors and iBodies**

The inhibition analyses were performed by spectrophotometric assay using the chromogenic peptide substrate KARVNle\*NphEANle-NH<sub>2</sub> as previously described<sup>[10]</sup>.

The 1 ml reaction mixture contained 100 mM sodium acetate, 300 mM NaCl, pH 4.7, 6.8 pmol of HIV-1 protease<sup>[10]</sup> and inhibitor in concentrations ranging between 2 and 130 nM. Substrate was added to a final concentration of 16 µM. Afterwards, the hydrolysis of substrate was followed as a decrease in absorbance at 305 nm using a UNICAM UV500 UV-VIS spectrophotometer (Thermo, Cambridge, MA). The data were analyzed using the equation for competitive inhibition according to Williams and Morrison<sup>[11]</sup>. The mechanism of inhibition was determined by analysis of Lineweaver-Burk plots.

### **Pull-down of HIV-1 protease from spiked LNCaP lysate**

LNCaP cells grown on two 100 mm Petri dishes were lysed by sonication (3×5 min, in water bath) in 2 ml of 50 mM Tris-HCl, 150 mM NaCl, 1 % Tween 20, pH 7.4.

First, 200 nM iBody **2** or **3** in 20 mM Tris-HCl, 150 mM NaCl, 0.1 % Tween 20, pH 7.4, was bound to 30 µl Streptavidin Agarose Ultra Performance (Solulink) at room temperature for 1 h. iBody **6**, which lacks the targeting ligand, was used as a negative control. To block unoccupied biotin binding sites, the resin was incubated with 1 ml of 2 mM biotin, 20 mM Tris-HCl, 150 mM NaCl, pH 7.4. Then, the resin was washed three times with 1 ml of 100 mM sodium acetate, 300 mM NaCl, 0.1 % Tween 20, pH 4.7. The washed resin was mixed with 200 µl of LNCaP cell lysate spiked with HIV-1 protease<sup>[10]</sup> (12 ng/µl, total protein

concentration 1 mg/ml) in 100 mM sodium acetate, 300 mM NaCl, 0.1 % Tween 20, pH 4.7, and incubated for 30 min at room temperature. The resin was washed four times with 1 ml of 100 mM sodium acetate, 300 mM NaCl, 0.1 % Tween 20, pH 4.7. Finally, bound HIV-1 protease was eluted from Streptavidin Agarose by adding 30  $\mu$ l reducing SDS sample buffer and heating to 98 °C for 10 min. Ten microliters of the samples was loaded onto the gel.

### **Pull-down of pepsin from spiked LNCaP lysate**

The experiment was performed analogously to the above described pull-down of HIV-1 protease.

200 nM iBody **3** was bound to 30  $\mu$ l Streptavidin Agarose (Solulink) at room temperature for 1 h; iBody **6** lacking the targeting ligand was used as a negative control. Biotin binding sites were blocked with biotin and after washing with the acetate buffer, the resin was mixed with LNCaP cell lysate spiked with pepsin (100 ng/ $\mu$ l, Worthington Biochemical Corporation; total protein concentration 1.5 mg/ml) in 100 mM sodium acetate, 300 mM NaCl, 0.1 % Tween 20, pH 4.7, and incubated for 5 min at room temperature. The resin was washed with the acetate buffer and bound pepsin was eluted from Streptavidin Agarose by adding 30  $\mu$ l reducing SDS sample buffer and heating to 98 °C for 10 min. 5  $\mu$ l of the samples was loaded onto the gel.

### **Expression and purification of His-tagged DNA damage protein 1**

DNA coding for full-length DNA-damage protein 1 (Ddi1) of *Leishmania major* was synthesized by GenScript and subcloned into pET16b vector for N-terminal hexahistidine tagged fusion protein production (Ddi1-HisTag). Ddi1-HisTag was expressed in *E. coli* BL21(DE3)RIL host cells, subsequently resuspended in 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0 and lysed by three passages through EmulsiFlex-C3 high pressure homogenizer (Avestin, Canada) at 1200 bar. Purification was performed using nickel affinity chromatography using Ni-NTA Superflow resin (Qiagen) with isocratic elution with 250 mM imidazole. Afterwards, pooled elution fractions were dialyzed overnight against 50 mM HEPES, pH 7.4, 150 mM NaCl, 10 % glycerol. The dialyzed sample was applied onto a size-exclusion chromatography column HiLoad 16/60 Superdex 200 (GE Healthcare). Individual fractions were analyzed by SDS-PAGE and fractions containing Ddi1-HisTag were pooled, aliquoted and stored at -80 °C until further use.

### **Quantification of iBody binding to Ddi1-HisTag by surface plasmon resonance (SPR)**

The experiment was performed analogously to quantitation of iBody 1 binding to GCPII.

Activation of carboxylic terminal groups on the sensor surface was performed *in situ* by injecting a 1:1 mixture of 11.51 mg/ml *N*-hydroxysuccinimide (NHS) and 76.68 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) in deionized water (Biacore) for 5 min at a flow rate of 20  $\mu$ l/min. The next part of the experiment was performed at a flow rate of 30  $\mu$ l/min. Then, a 20 ng/ $\mu$ l solution of NeutrAvidin (Thermo Scientific) in 10 mM sodium acetate, pH 5.0, was loaded for 8 min. Next, a high ionic strength solution (PBS with 0.5 M NaCl) was used to wash out noncovalently bound molecules, followed by addition of 1 M ethanolamine (Sigma-Aldrich) for deactivation of residual activated carboxylic groups. Afterwards, 1  $\mu$ M solution of iBody 4 in TBS was used for iBody 4 immobilization onto the NeutrAvidin layer; the bound iBody 4 was subsequently charged with nickel ions using 100 mM NiCl<sub>2</sub>. Finally, four different concentrations of Ddi1-HisTag (100, 200, 400 and 800 nM) in 50 mM HEPES, 150 mM NaCl, pH 7.4 were applied for approximately 10 min (association), followed by injection of the HEPES buffer alone (dissociation).

Kinetic curves of binding were exported and subsequently fitted using the One-To-One interaction model in TraceDrawer v.1.5 (Ridgeview Instruments AB) to obtain  $k_{on}$  and  $k_{off}$  parameters.

### **Pull-down of Ddi1-HisTag from an *E. coli* lysate**

200 nM iBody 4 in 20 mM Tris-HCl, 150 mM NaCl, 2 mM NiCl<sub>2</sub>, 0.1 % Tween 20, pH 7.4 was bound to 30  $\mu$ l Streptavidin Agarose (Solulink) at room temperature for 1 h. iBody 5 lacking the targeting ligand and blank Streptavidin Agarose were used as negative controls. To block biotin binding sites, the resin was then incubated with 1 ml of 2 mM biotin, 100 mM Tris-HCl, 150 mM NaCl, pH 7.2. Afterwards, the resin was washed three times with 1 ml of 20 mM Tris-HCl, 150 mM NaCl, 0.1 % Tween 20, pH 7.4 (TBST). The resin was then mixed with 1 ml of Ddi1-HisTag *E. coli* lysate (diluted in TBST to final protein concentration of 325 ng/ $\mu$ l), and incubated at room temperature for 1 h. The resin was washed five times with 1 ml of 25 mM imidazol in TBST. Bound proteins were eluted from Streptavidin Agarose by adding 30  $\mu$ l 1 M imidazol in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4), and incubated for 15 min at 37 °C.

### **Inductively coupled plasma atomic emission spectroscopy (ICP-OES)**

The ICP-OES measurements were performed with the SPECTRO ARCOS optical emission spectrometer (SPECTRO Analytical Instruments, Kleve, Germany) with radial plasma observation. The SPECTRO ARCOS features a Paschen-Runge spectrometer mount; the wavelength range between 130 and 770 nm can be analyzed simultaneously. An air-cooled ICP-generator, based on a free-running 27.12 MHz system, is installed. For sample introduction, a Modified-Lichte nebulizer with a cyclonic spray chamber was used.

For calibration, commercially available Br and S standard solutions (Analytika) were used. 0.5 % (v/v) Triton X-100 and 2 % HNO<sub>3</sub> solution were used for organic matrix matching strategy and improvement of LOD. Before analysis, the analyzed polymer conjugate solutions were diluted with 0.5 % Triton X-100 solution, as well as all calibration standard solutions. All used chemicals were suprapur quality. All calibrations and sample solutions were prepared or controlled by weighing on an analytical balance. Calibration solutions with concentration 0.2, 1.0, 2.0, 5.0, and 10.0 mg/kg and blank solution Br and S were prepared. For organic matrix background structure compensation the Spectro-SmartBg function was used.

Each sample aliquot (200 – 400 µl) was weighted on analytical balance and then diluted with 0.5 % Triton X-100 and 2 % HNO<sub>3</sub> solution to 5 ml (weight controlled), mixed and then analyzed via the described method. Then the concentrations of original samples were calculated.

Calibration parameters:

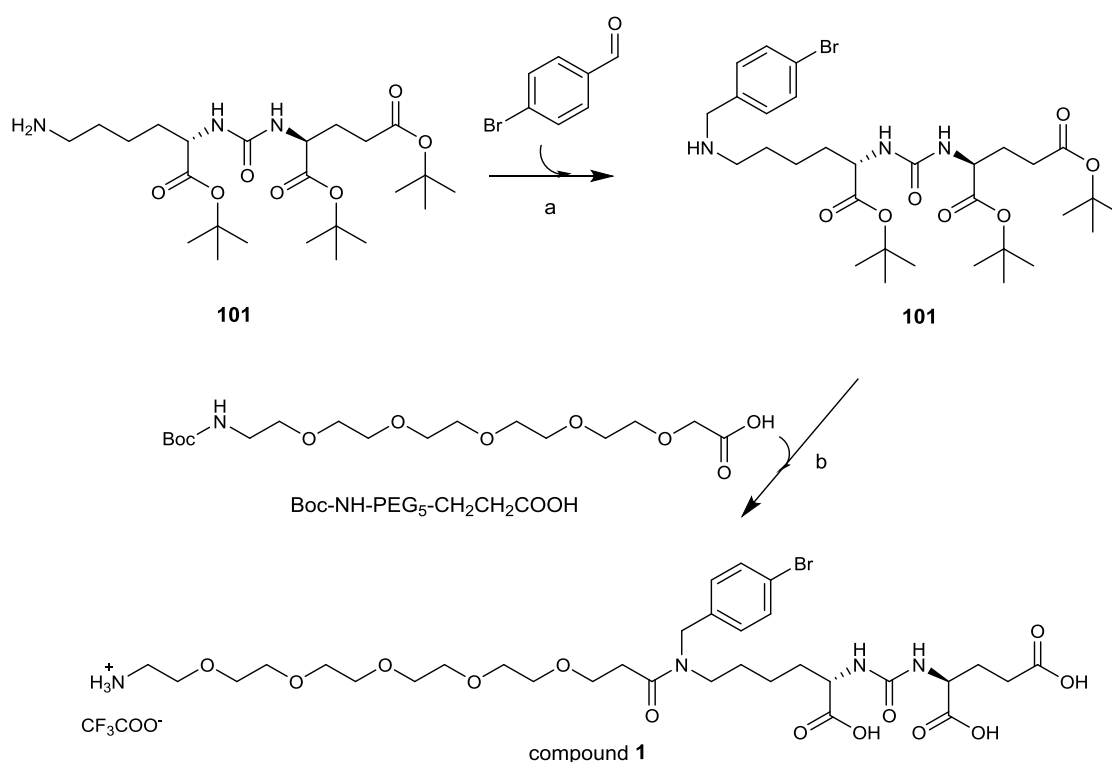
Element line	LOD (mg/kg)	Corr. Coefficient
Br 148.845	0.018	0.9996
Br 153.174	0.060	0.9994
Br 154.065	0.024	0.9996
S 142.731	0.121	0.9998
S 180.731	0.004	0.9998
S 182.034	0.003	0.9999

## Synthesis of low-molecular-weight compounds

All chemicals were purchased from Sigma-Aldrich, unless stated otherwise. All inhibitors tested in the biological assays were purified using preparative scale HPLC Waters Delta 600 (flow rate 7 ml/min, gradient shown for each compound - including RT), with column Waters SunFire C18 OBD Prep Column, 5  $\mu\text{m}$ , 19 $\times$ 150 mm. The purity of compounds was tested on analytical Jasco PU-1580 HPLC (flow rate 1 ml/min, invariable gradient 2-100 % ACN in 30 minutes,  $R_T$  shown for each compound) with column Watrex C18 Analytical Column, 5  $\mu\text{m}$ , 250 $\times$ 5 mm. The final inhibitors were all at least of 99 % purity. Structure was further confirmed by HRMS at LTQ Orbitrap XL (Thermo Fisher Scientific) and by NMR (Bruker Avance I<sup>TM</sup> 500 MHz equipped with Cryoprobe). All interaction constants are in Hertz units.

## Synthesis of compound 1

Compound 1 was synthesized according to the scheme depicted below:



- a) NaBH<sub>3</sub>CN, MeOH + 1%AcOH  
b) 1)TBTU, DIEA, DMF; 2)TFA

### Compound **102**:

Di-tert-butyl 2-(3-(6-((4-bromobenzyl)amino)-1-(tert-butoxy)-1-oxohexan-2-yl)ureido)pentanedioate, compound **102**: 300 mg (0.615 mmol, 1.0 eq) of di-tert-butyl 2-(3-(6-amino-1-(tert-butoxy)-1-oxohexan-2-yl)ureido)pentanedioate (compound **101**, prepared as described in Murelli *et al.*<sup>[12]</sup>) and 120 mg (0.646 mmol, 1.05 eq) of 4-bromobenzaldehyde was dissolved in 5 ml of methanol in a round-bottom flask. 50  $\mu$ l of glacial acetic acid was added and, upon fast stirring, 120 mg (1.85 mmol, 3.0 eq) of sodium cyanoborohydride was added in one portion. After 12 hours, the reaction mixture was quenched by addition of 10 ml of water. The reaction mixture was further diluted after 10 minutes by 50 ml of water and was extracted 3 times by EtOAc (3 $\times$ 25 ml). The organic phase was dried and evaporated and crude product was purified by chromatography on silica (mobile phase: EtOAc + 1 % of saturated ammonia in water, TLC  $r_f$  = 0.55). 395 mg of pure product was isolated (yield = 48 %). Analytical HPLC (grad 2-100 %, 30 min)  $R_T$  = 23.4 min. HRMS (ESI+): calculated for C<sub>31</sub>H<sub>51</sub>O<sub>7</sub>N<sub>3</sub>Br [M]<sup>+</sup> 656.29049. Found 656.29062. <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>): 7.47 (m, 2H, *m-Ph*), 7.27 (m, 2H, *o-Ph*), 6.29 (d, 1H, J = 8.5, **HN-Glu-2**), 6.24 (d, 1H, J = 8.4, **HN-Lys-2**), 4.02 (btd, 1H, J<sup>1</sup> = 8.6, J<sup>2</sup> = 5.1, **Glu-2**), 3.96 (td, 1H, J<sup>1</sup> = 8.1, J<sup>2</sup> = 5.4, **Lys-2**), 3.62 (s, 2H, **CH<sub>2</sub>-Ph**), 2.41 (t, 2H, J = 7.1, **Lys-6**), 2.25 (ddd, 1H, J<sup>1</sup> = 16.6, J<sup>2</sup> = 8.8, J<sup>3</sup> = 6.8, **Glu-4b**), 2.18 (ddd, 1H, J<sup>1</sup> = 16.6, J<sup>2</sup> = 8.8, J<sup>3</sup> = 6.1, **Glu-4a**), 1.86 (m, 1H, **Glu-3b**), 1.66 (m, 1H, **Glu-3a**), 1.57 (m, 1H, **Lys-3b**), 1.49 (m, 1H, **Lys-3a**), 1.40 (m, 2H, **Lys-5**), 1.38 (bs, 27 H, **tBu**), 1.29 (m, 2H, **Lys-4**). <sup>13</sup>C NMR (125.7 MHz, DMSO-d<sub>6</sub>): 172.50 (**Lys-1**), 172.11 (**Glu-1**), 171.63 (**Glu-5**), 157.31 (NH-CO-NH), 140.83 (*i-Ph*), 131.07 (*m-Ph*), 130.26 (*o-Ph*), 119.52 (*p-Ph*), 80.76 (**CH(CH<sub>3</sub>)<sub>3</sub>**), 80.45 (**CH(CH<sub>3</sub>)<sub>3</sub>**), 79.95 (**CH(CH<sub>3</sub>)<sub>3</sub>**), 53.18 (**Lys-2**), 52.38 (**CH<sub>2</sub>-Ph**), 52.36 (**Glu-2**), 48.49 (**Lys-6**), 32.17 (**Lys-3**), 31.07 (**Glu-4**), 29.24 (**Lys-5**), 27.93 (**CH(CH<sub>3</sub>)<sub>3</sub>**), 27.84 (**CH(CH<sub>3</sub>)<sub>3</sub>**), 27.82 (**CH(CH<sub>3</sub>)<sub>3</sub>**), 27.77 (**GLu-3**), 23.03 (**Lys-4**).

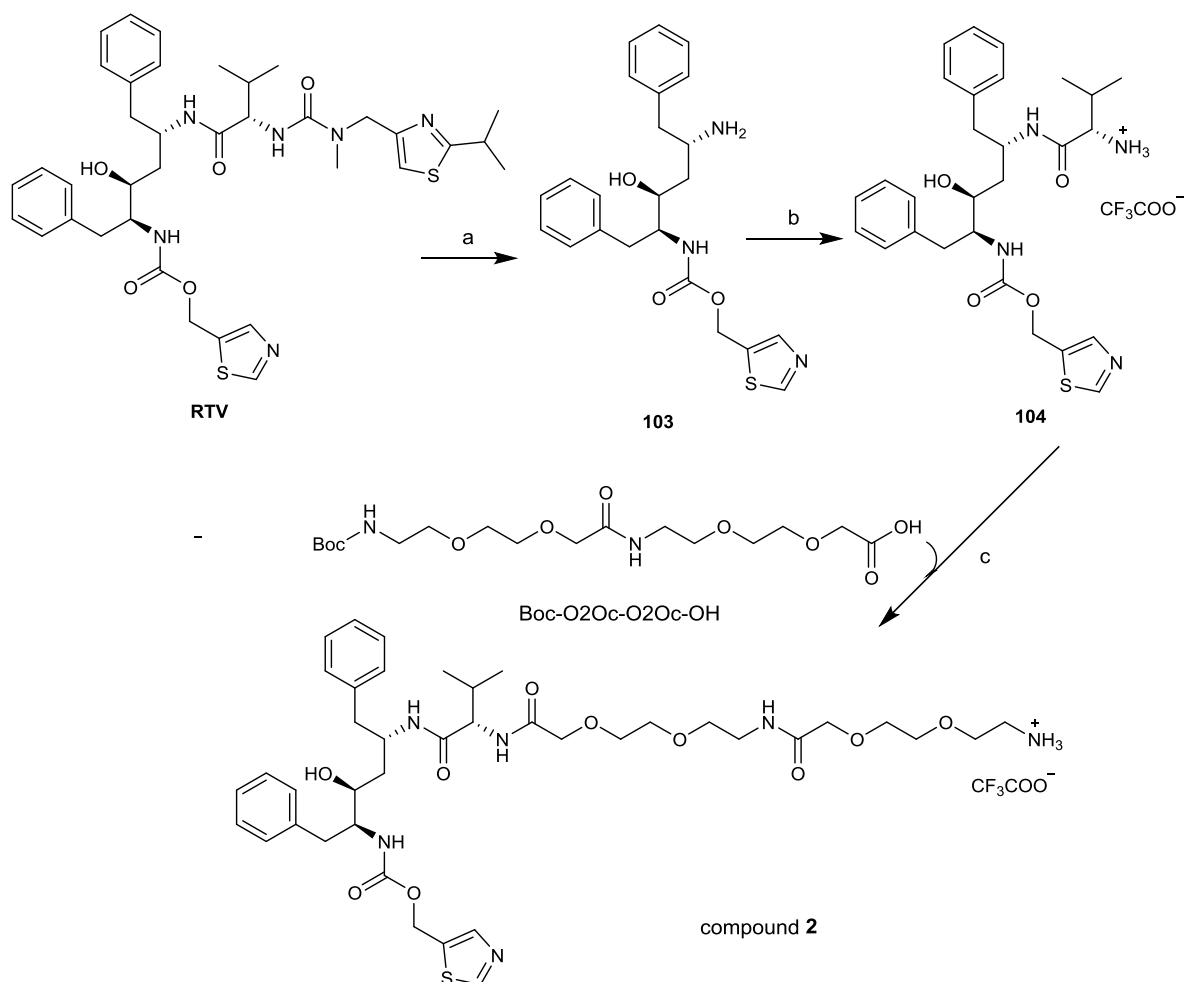
### Compound **1**:

(2*S*,28*S*)-19-(4-bromobenzyl)-24,28,30-tricarboxy-18,26-dioxo-3,6,9,12,15-pentaoxa-19,25,27-triazatriacontan-1-aminium 2,2,2-trifluoroacetate, compound **1**: 137 mg (0.34 mmol, 1.1 eq) of Boc-NH-PEG<sub>5</sub>-CH<sub>2</sub>CH<sub>2</sub>COOH (PurePEG, #432705) was dissolved in 1 ml of DMF along with 1222 mg (0.38 mmol, 1.25 eq) of TBTU. 132  $\mu$ l (0.76 mmol, 2.5 eq) of DIEA were added in one portion and the reaction mixture was left stirring for 10 minutes. 200 mg (0.30 mmol, 1.0 eq) of compound **102** dissolved in 1 ml of DMF were added in one portion and the reaction mixture was monitored by TLC analysis, until all compound **102**

disappeared (approx. 4 h). The DMF was then rotary evaporated, the crude mixture was dissolved in 20 ml of EtOAc and was extracted twice with concentrated bicarbonate, twice with 10 % KHSO<sub>4</sub> and once with brine. The organic layer was dried and rotary evaporated to complete dryness. 1 ml of TFA was then added to the oily crude product and was left sonicated for 15 minutes. TFA was removed by flow of nitrogen and the product was purified by preparative HPLC (gradient: 15-50 % ACN, R<sub>T</sub> = 33 min). 83.4 mg isolated (overall yield = 30 %). R<sub>T</sub> at analytical HPLC (grad 2-100 %, 30 min) 17.1 min. HRMS (ESI<sup>-</sup>): calculated for C<sub>32</sub>H<sub>50</sub>O<sub>13</sub>N<sub>4</sub>Br [M]<sup>-</sup> 777.25632. Found 777.25681.

## Synthesis of compound 2

Compound **2**, based on a commercially available HIV protease inhibitor drug ritonavir (RTV), was synthesized according to the below depicted scheme:



- a) 1) Dioxan/HCl, 65 °C, 20 h 2) K<sub>2</sub>CO<sub>3</sub>  
 b) 1) Boc-Val-OH, TBTU, DIEA, DMF, 2) TFA  
 c) 1) TBTU, DIEA, DMF, 2) TFA

Isolation of ritonavir (RTV) from commercially available capsules: RTV is suspended in capsules in an oily mixture of rather non-polar compounds. 50 tablets (100 mg RTV each) were cut open and the oily substance was squeezed out into a round-bottom shaped 2 l flask. 200 ml of hexan was added along with 500 ml of diethyl ether. The resulting suspension was triturated and sonicated for 3 hours until all oil turned into a white precipitate. This precipitate was filtered and again triturated/sonicated in pure diethyl ether, after which the pure RTV was filtered. 3.6 g of RTV was obtained (isolation yield 72 %). The purity of RTV was determined by HPLC and was well above 99 % (analytical HPLC  $R_T = 23.7$  min).

### Compound **103**

Partial hydrolysis of ritonavir (RTV), thiazol-5-ylmethyl ((2S,3S,5S)-5-amino-3-hydroxy-1,6-diphenylhexan-2-yl)carbamate, compound **103**: 1.00 g of RTV was dissolved in 50 ml of dioxan in a bottom-round flask. 50 ml of concentrated hydrochloric acid was added and the resulting mixture was stirred at 65 °C for 20 hours (note that different temperature and/or time lead to different cleavage products). After 20 hours the mixture was let cool down to RT. The reaction mixture was neutralized by addition of  $K_2CO_3$  until the resulting mixture showed basic pH. The solvents were concentrated using rotary evaporater to roughly 50 ml and diluted by 150 ml of water and washed 3 times by 100 ml of EtOAc. The water phase was discarded and organic phase was dried and evaporated. 885 mg of crude product was obtained and was used in the next step without further purification (purity roughly 70 % - HPLC determination). For spectral determination, 50 mg was purified using preparative HPLC (gradient: 20-50 % ACN in 40 minutes.  $R_T = 15$  min). Analytical HPLC  $R_T = 17.3$  min. HRMS (ESI+): calculated for  $C_{23}H_{28}O_3N_3S$   $[M]^+$  426.18459. Found 426.18454. NMR measured for trifluoroacetate salt.  $^1H$  NMR (500 MHz, DMSO- $d_6$ ): 9.06 (d, 1H,  $^4J = 0.8$ , N-**CH-S**), 7.84 (q, 1H,  $^4J = 0.8$ , S-C-**CH-N**), 7.81 (bs, 3H,  $NH_3^+$ ), 7.32-7.15 (m, 10H, **Ph**-), 7.20 (bs, 1H, **NH**), 5.50 (bs, 1H, **OH**), 5.15 (dd, 1H,  $J_{gem} = 13.2$ ,  $^4J = 0.8$ , O-**CH<sub>2</sub>**), 5.11 (dd, 1H,  $J_{gem} = 13.2$ ,  $^4J = 0.8$ , COO-**CH<sub>2</sub>**), 3.69 (m, 1H, HO-**CH**), 3.67 (m, 1H, HO-CH-**CH-NH**), 3.50 (bm, 1H,  $NH_3^+$ -**CH**), 2.87 (dd, 1H,  $J_{gem} = 14.0$ ,  $^3J = 6.4$ ,  $NH_3^+$ -CH-**CH<sub>2</sub>-Ph**), 2.80 (dd, 1H,  $J_{gem} = 14.0$ ,  $^3J = 7.3$ ,  $NH_3^+$ -CH-**CH<sub>2</sub>-Ph**), 2.79 (dd, 1H,  $J_{gem} = 13.7$ ,  $^3J = 3.7$ , NH-CH-**CH<sub>2</sub>-Ph**), 2.79 (dd, 1H,  $J_{gem} = 13.7$ ,  $^3J = 10.5$ , NH-CH-**CH<sub>2</sub>-Ph**), 1.58 (bs, 2H, OH-CH-**CH<sub>2</sub>-CH**).  $^{13}C$  NMR (125.7 MHz, DMSO- $d_6$ ): 155.39 (O-C-N), 155.77 (N-**CH-S**), 143.23 (S-C-**CH-N**), 139.52 (**Ph**), 136.37 (**Ph**), 134.14 (S-C-CH-N), 129.61 (**Ph**), 129.18 (**Ph**), 128.81 (**Ph**), 128.23 (**Ph**), 127.07 (**Ph**), 126.12 (**Ph**), 69.81 (HO-**CH**), 57.49 (COO-**CH<sub>2</sub>**), 56.94 (HO-CH-



**CH-NH**), 50.87 ( $\text{NH}_3^+$ -**CH**), 38.71 ( $\text{NH}_3^+$ -**CH-CH}\_2\text{-Ph}**), 35.69 (**NH-CH-CH}\_2\text{-Ph}**), 34.66 (**CH-CH}\_2\text{-CH}**).

#### Compound **104**

Thiazol-5-ylmethyl ((2S,3S,5S)-5-((S)-2-amino-3-methylbutanamido)-3-hydroxy-1,6-diphenylhexan-2-yl)carbamate, compound **104**: 526 mg (1.64 mmol, 1.0 eq) of TBTU was added to 356 mg (1.64 mmol, 1.0 eq) Boc-Val dissolved in 1.5 ml of DMF along with 690  $\mu\text{l}$  of DIEA (3.94 mmol, 2.4 eq). The crude hydrolysate of RTV (700 mg, 1.64 mmol, 1.0 eq), dissolved in 1 ml of DMF, was added after 5 minutes of stirring in one portion. The reaction was left overnight and DMF was rotary evaporated. The reaction mixture was dissolved in 50 ml of EtOAc and washed two times by saturated  $\text{NaHCO}_3$ , two times with 10 %  $\text{KHSO}_4$  and once with brine. The organic mixture was dried, evaporated and the product was purified using Flash chromatography (TLC analysis: EtOAc,  $R_f = 0.65$ ). Product was further dissolved in 5 ml of hot EtOAc and 5 ml of diethyl ether was added. The resulting gel was filtrated and dried to give very pure (>99 %, HPLC) 250 mg of product (yield = 25 %). The product was then treated with TFA (approx. 1 ml) for 15 minutes, alternately sonicated and stirred. The remaining TFA was then removed by flow of nitrogen. The oily product was dissolved in water/ACN and was lyophilisated. Analytical HPLC  $R_T = 17.4$  min. HRMS (ESI+): calculated for  $\text{C}_{28}\text{H}_{37}\text{O}_4\text{N}_4\text{S}$   $[\text{M}]^+$  525.25300. Found 525.25292.  $^1\text{H}$  NMR (500 MHz, DMSO- $d_6$ ): 9.06 (d, 1H,  $^4J = 0.8$ , N-**CH-S**), 8.24 (d, 1H  $J = 8.2$ , -**NH-CO**), 8.00 (bd, 3H,  $J = 5.2$ , -**NH}\_3^+**), 7.85 (q, 1H,  $^4J = 0.8$ , S-C-**CH-N**), 7.28-7.13 (m, 10H, **Ph-**), 6.94 (d,  $J = 9.4$ , 1H, **NH-CO-O**), 5.12 (d, 2H,  $^4J = 0.8$ , O-**CH}\_2**), 4.16 (m, 1H, **CH-NH-CO**), 3.78 (m, 1H, **CH-NH}\_3^+**, partial overlap with water residual peak), 3.58 (td, 1H,  $J = 6.8$ ,  $J = 2.0$ , **CH-OH**), 3.48 (m, 1H, Ph-**CH}\_2\text{-CH-NH}**), 2.72-2.67 (m, 4H, 2x**CH-CH}\_2\text{-Ph}**), 2.00 (m, 1H, **CH-(CH}\_3)\_2**), 1.50 (m, 1H, OH-**CH-CH}\_2**), 1.43 (m, 1H, OH-**CH-CH}\_2**), 0.89 (d, 3H,  $J = 6.8$  -**CH}\_3**), 0.84 (d, 3H,  $J = 6.8$  -**CH}\_3**).  $^{13}\text{C}$  NMR (125.7 MHz, DMSO- $d_6$ ): 167.33 (**CO Val**), 158.33(q,  $J_{C,F} = 34.4$ ,  $\text{CF}_3\text{COO}^-$ ), 155.79 (O-C-N), 155.71 (N-**CH-S**), 143.23 (S-C-**CH-N**), 139.50 (**Ph**), 138.55 (**Ph**), 134.23 (S-C-**CH-N**), 129.56 (**Ph**), 129.17 (**Ph**), 128.30 (**Ph**), 128.25 (**Ph**), 126.26 (**Ph**), 126.09 (**Ph**), 116.44 (q,  $J_{C,F} = 294.8$ ,  $\text{CF}_3\text{-COO}^-$ ) 68.90 (HO-**CH**), 57.56 (CO-**CH-NH}\_3**), 57.44 (COO-**CH}\_2**), 55.74 (HO-**CH-CH-NH**), 47.98 (CONH-**CH**), 39.75 (NH-**CH-CH}\_2\text{-Ph}**), 37.77 (-**CH}\_2\text{-CH-CH-}**), 37.33 (Ph-**CH}\_2\text{-CH-NH}**), 30.04 (**CH(CH}\_3)\_2**), 17.26 and 18.69 (2x**CH}\_3**).

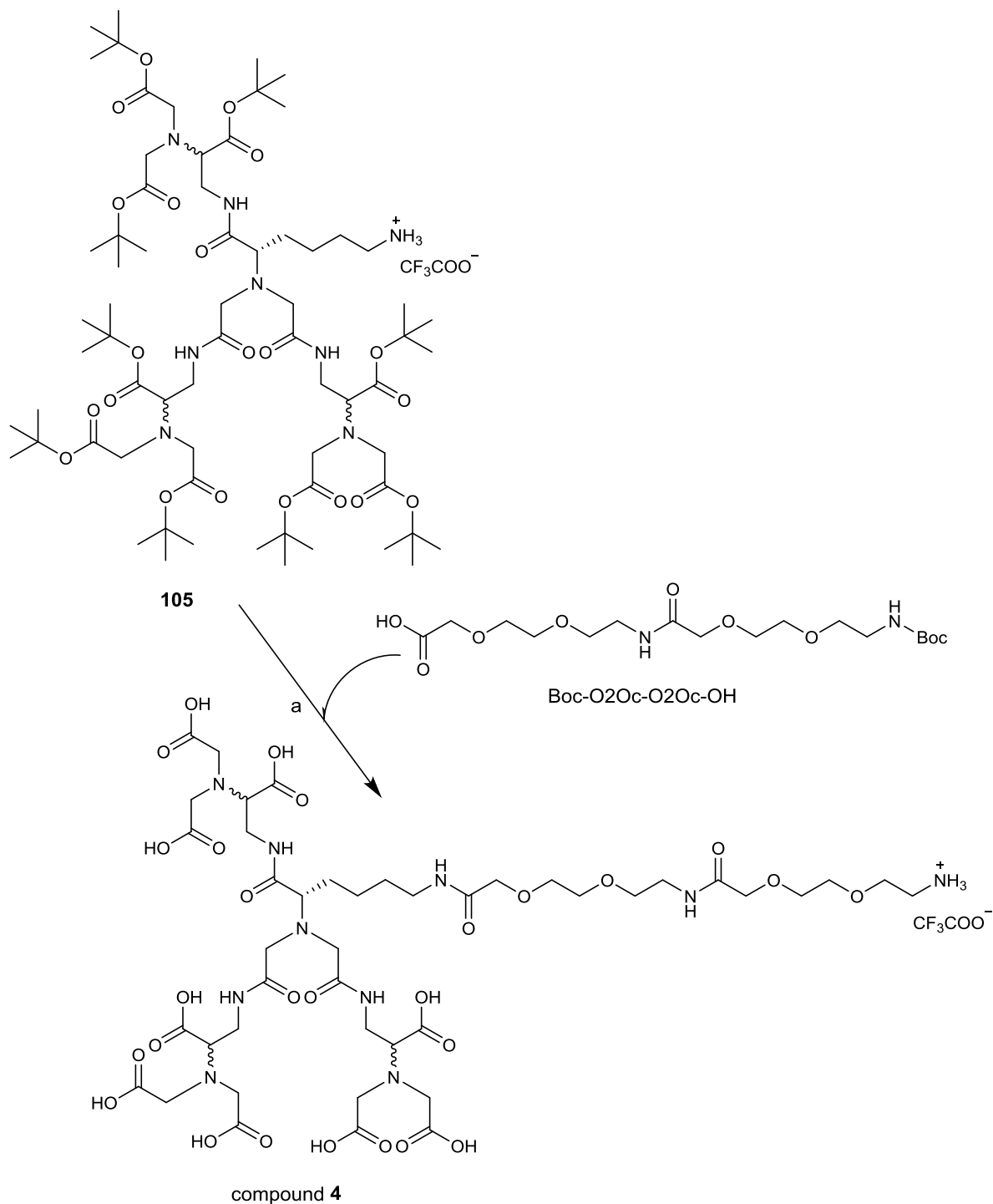
## Compound 2

(5S,6S,8S,11S)-5,8-dibenzyl-6-hydroxy-11-isopropyl-3,10,13,22-tetraoxo-1-(thiazol-5-yl)-2,15,18,24,27-pentaoxa-4,9,12,21-tetraazanonacosan-29-aminium 2,2,2-trifluoroacetate, compound **2**: 64 mg (157  $\mu\text{mol}$ , 1.0 eq) of Boc-O2Oc-O2Oc-OH (Iris-Biotech, #BAA1485) was dissolved in 1 ml of DMF along with 51 mg (157  $\mu\text{mol}$ , 1.0 eq) of TBTU and 95  $\mu\text{l}$  (558  $\mu\text{mol}$ , 3.5 eq) of DIEA and the whole reaction mixture was stirred for 15 minutes. 100 mg (157  $\mu\text{mol}$ , 1.0 eq) of compound **104** (dissolved in 0.5 ml of DMF) was added into the mixture in one portion. After 3 hours all volatiles were evaporated, the crude product was dissolved in 25 ml of EtOAc and was washed two times with saturated  $\text{NaHCO}_3$ , two times with 10 %  $\text{KHSO}_4$  and once with brine. The organic layer was dried and evaporated. The Boc-protecting group was then removed by stirring in 1 ml of TFA for 15 minutes. The product was purified using preparative HPLC (gradient: 15-50 % ACN in 40 minutes.  $R_T = 31$  min). Analytical HPLC  $R_T = 17.7$  min. HRMS (ESI+): calculated for  $\text{C}_{40}\text{H}_{59}\text{O}_{10}\text{N}_6\text{S}$   $[\text{M}]^+$  815.40079. Found 815.40096.  $^1\text{H}$  NMR (500 MHz, DMSO- $d_6$ ): 9.05 (d, 1H,  $J = 0.8$ , N-**CH-S**), 7.96 (d, 1H,  $J = 8.7$ , **NH-CO-Val**), 7.85 (q, 1H,  $J = 0.8$ , S-C-**CH-N**), 7.81 (vbs, 3H, -**NH}\_3^+**), 7.79 (bt, 1H,  $J = 5.8$ , Linker **NH-CO**), 7.31 (d, 1H,  $J = \text{NH-Val-2}$ ), 7.24-7.08 (m, 10H, 2x**Ph**), 6.92 (d, 1H,  $J = 9.4$ , **NH-COO-CH}\_2\text{-thiazol}**), 5.16 (dd, 1H,  $J_{\text{gem}} = 13.2$ ,  $^4J = 0.8$ , **NH-COO-CH}\_2\text{-thiazol}**), 5.12 (dd, 1H,  $J_{\text{gem}} = 13.2$ ,  $^4J = 0.8$ , **NH-COO-CH}\_2\text{-thiazol}**), 4.13 (m, 1H, **CH-NH-CO-Val**), 4.13 (dd, 1H,  $^2J = 9.3$ ,  $^3J = 6.8$ , **Val-2**), 3.92-3.89 (m, 4H, linker 2x**NH-CO-CH}\_2\text{-}**), 3.82 (m, 1H, **CH-NH-COO-CH}\_2\text{-thiazol}**), 3.62-3.51 (m, 12H, linker, **OH-CH**), 3.46 (bt, 2H, **O-CH}\_2\text{-CH}\_2\text{-NH-CO-CH}\_2\text{-}**), 3.29 (bt, 2H, **O-CH}\_2\text{-CH}\_2\text{-NH-CO-CH}\_2\text{-}**), 2.98 (m, 2H, **CH}\_2\text{-NH}\_3^+**), 2.71-2.65 (m, 2H **Ph-CH}\_2\text{-CH-NH-Thiazol}**, 1H **Ph-CH}\_2\text{-NH-Val}**), 2.58 (dd, 1H,  $J_{\text{gem}} = 13.6$ ,  $^3J = 8.4$ , **Ph-CH}\_2\text{-NH-Val}**), 1.84 (o, 1H,  $J = 6.8$ , **Val-3**), 1.46 (m, 2H, **OH-CH-CH}\_2\text{-}**), 0.76 (d, 3H,  $J = 6.8$ , **Val-4**), 0.74 (d, 3H,  $J = 6.8$ , **Val-4**).  $^{13}\text{C}$  NMR (125.7 MHz, DMSO- $d_6$ ): 170.04 (**Val-1**), 169.56 (**NH-CO-Linker**), 168.90 (**Val-NH-CO-**), 158.31 (q,  $J = 34.4$ ,  $\text{CF}_3\text{COO}^-$ ), 155.82 (**COO-CH}\_2\text{-thiazol}**, **S-CH-N**), 143.24 (**S-C-CH-N**), 139.60 (*i*-**Ph**), 138.92 (*i*-**Ph**), 134.30 (**S-C-CH-N**), 129.47 (*o*-**Ph**), 129.25 (*o*-**Ph**), 128.20 (*m*-**Ph**), 128.08 (*m*-**Ph**), 126.04 (*p*-**Ph**), 126.03 (*p*-**Ph**), 116.46 (q,  $J = 293.5$ ,  $\text{CF}_3\text{COO}^-$ ), 70.44 (linker), 70.17 (linker), 70.03 (linker), 69.83 (linker), 69.66 (linker), 69.48 (linker), 69.22 (linker), 69.11 (**CH-OH**), 66.85 (linker), 57.40 (**O-CH}\_2\text{-thiazol}**), 57.07 (**Val-2**), 55.64 (**CH-COO-CH}\_2\text{-thiazol}**), 47.45 (**CH-NH-Val**), 39.90 (**CH}\_2\text{-CH-NH-Val}**), 38.74 (**CH}\_2\text{-NH}\_3^+**), 38.44 (**OH-CH-CH}\_2\text{-}**), 38.23 (**CH}\_2\text{-NH-COO-CH}\_2\text{-}**), 37.41 (**OCO-NH-CH}\_2\text{-Ph**), 31.19 (**Val-3**), 19.48 (**Val-4**), 18.10 (**Val-4**).



## Synthesis of compound 4

Compound 4 was synthesized according to the scheme depicted below:



a) 1) Boc-NH-PEG<sub>5</sub>-COOH, DCC, DMF; 2) TFA

### Compound 105

NH<sub>2</sub>-tris-NTA(o-tBu)<sub>9</sub>, compound 105: The synthesis was performed as described previously<sup>[13]</sup> with an only minor variation in one step (for the reaction between 3 monomers

of NTA and lysine tricarboxylic acid derivative TSTU was used as an activation reagent, instead of NHS/DCC, because it gave much better yields).

#### **Compound 4**

Compound **105** (52 mg, 38  $\mu\text{mol}$ , 1.0 eq, purified by HPLC before this step) was dissolved in 1 ml of DMF and 15 mg (38  $\mu\text{mol}$ , 1.0 eq) of the linker Boc-O2Oc-O2Oc-OH (Iris-Biotech, #BAA1485) was added in one portion. To this reaction mixture was added 16 mg (76  $\mu\text{mol}$ , 2.0 eq) of DCC and the reaction was left for 24 hours stirring at room temperature. The solvents were evaporated and the crude mixture was treated with 1 ml of pure TFA, and the reaction mixture was alternately stirred and sonicated for 3 hours. The TFA was removed by flow of nitrogen and the final product was purified by preparative scale HPLC (gradient: 2-30 % ACN in 50 minutes,  $R_T = 35$  min). 14 mg isolated (overall yield = 32 %)  $R_T$  at analytical HPLC (grad 2-100 %, in 30 min) 12.0 min. HRMS (ESI+): calculated for  $\text{C}_{43}\text{H}_{71}\text{O}_{27}\text{N}_{10}$   $[\text{M}]^+ 1159.44846$ . Found 1159.44849.

## **Synthesis of monomers, polymer precursors and polymer conjugates**

### **Synthesis of HPMA copolymer conjugates (iBodies)**

Generally, the HPMA copolymer conjugates (iBodies **1–6**) were prepared by reaction of the polymer precursor poly(HPMA-co-Ma- $\beta$ -Ala-TT) with the affinity anchor *N*-(2-aminoethyl)biotinamide hydrobromide (biotin-NH<sub>2</sub>) or with a combination of fluorophore (ATTO488-amine), affinity anchor (biotin-NH<sub>2</sub>), and targeting ligand (compounds **1–4**). To achieve statistical representation of all three ligands in the conjugate, the aminolysis of the statistical TT groups-containing polymer precursor<sup>[14]</sup> with the -CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub> group-bearing compounds was performed simultaneously in one step. Yield of the aminolytic reaction was approximately 60–70 % for all three compounds. The compounds were dissolved in DMSO, and *N,N*-diisopropylethylamine (DIPEA) was added. The reaction mixture was stirred at room temperature, and the unreacted thiazolidine-2-thione groups were quenched with 1-aminopropan-2-ol. Polymer conjugates were isolated by precipitation into an acetone:diethyl ether mixture (3:1), filtered off, washed with acetone and diethyl ether, and dried *in vacuo*. Finally, the conjugates were purified on a Sephadex LH-20 chromatography column with methanol as the mobile phase, precipitated into diethyl ether, filtered off, and dried *in vacuo*.

The synthesis of all iBodies, polymer precursors and monomers is described in more detail below.

### **Determination of the composition (i.e. the number of each moiety) of the iBodies**

Content of ATTO-488 was determined using spectrophotometry ( $\epsilon_{502\text{nm}} = 90,000 \text{ l}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ , water). For polymer conjugates containing only biotin and/or targeting group, content of biotin was determined spectrophotometrically using HABA/avidin reagent according to manufacturer's instruction (Sigma-Aldrich). For polymer conjugates containing both biotin and ATTO488, content of biotin was determined using inductively coupled plasma atomic emission spectroscopy (ICP-OES; sulphur detection), since HABA/avidin method was not applicable due to ATTO488 absorption at 500 nm. Content of targeting groups (compounds **1-4**) was determined in the sample hydrolyzate (6N-HCl, 115 °C, 16 h) using HPLC-based *o*-phthaldialdehyde pre-column derivatization method on the Chromolith C18 column with a fluorescence detector (Ex. 229 nm, Em. 450 nm). As standards, the corresponding low-molecular-weight inhibitors were hydrolyzed and derivatized in the same way.

### **Determination of molecular weight of polymer conjugates**

The weight-average molecular weights ( $M_w$ ), number average molecular weights ( $M_n$ ), and dispersities ( $D$ ) of the polymer precursor and conjugates were determined using HPLC Shimadzu system equipped with a UV detector, an Optilab<sup>®</sup> rEX differential refractometer and multi-angle light scattering DAWN<sup>®</sup> 8<sup>TM</sup> (Wyatt Technology, USA) detector and size-exclusion chromatography TSKgel G4000SW column. The  $M_w$ ,  $M_n$  and  $D$  were calculated using the Astra V software. The refractive index increment  $dn/dc = 0.167 \text{ ml/g}$  was used for calculation. For these experiments, a 20 % 300 mM acetate:80 % methanol (v/v) buffer was used. The flow rate was 0.5 mL/min. The data are summarized in Table S2.

### **Synthesis of monomers**

*N*-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized by reaction of methacryloyl chloride with 1-aminopropan-2-ol in dichloromethane in the presence of sodium carbonate<sup>[15]</sup>.

3-(3-Methacrylamidopropanoyl)thiazolidine-2-thione (Ma- $\beta$ -Ala-TT) was prepared by reaction of 3-methacrylamidopropanoic acid and 4,5-dihydrothiazole-2-thiol (H-TT) (4.37 g, 37 mmol) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride

(EDC). Briefly, Ma- $\beta$ -Ala-OH (4.0 g, 28 mmol), H-TT (3.5 g, 29 mmol), and a catalytic amount of 4-dimethylaminopyridine (DMAP) were dissolved in dichloromethane (50 ml), and EDC (6.9 g, 36 mmol) was added. The reaction was carried out at room temperature for 2 h. The reaction mixture was then extracted with distilled water (3 $\times$ 20 ml), 2 wt% aqueous NaHCO<sub>3</sub> (20 ml), and distilled water (20 ml). The organic layer was dried with Na<sub>2</sub>SO<sub>4</sub>, dichloromethane was evaporated, and Ma- $\beta$ -Ala-TT was crystallized from ethyl acetate. The crystals were filtered off, washed with diethyl ether, and dried *in vacuo*. Yield: 4.6 g (64 %).

### Synthesis of iBody 1

Monomers *N*-(2-hydroxypropyl)methacrylamide (HPMA) and 3-(3-methacrylamidopropanoyl)thiazolidine-2-thione (Ma- $\beta$ -Ala-TT) were synthesized as described earlier<sup>[14]</sup>. Polymer precursor poly(HPMA-co-Ma- $\beta$ -Ala-TT) was prepared by reversible addition-fragmentation chain transfer (RAFT) copolymerization<sup>[15-16]</sup>. 0.659 g of mixture of HPMA (85 % mol, 500 mg), Ma- $\beta$ -Ala-TT (15 % mol, 159 mg dissolved in 0.8 ml dimethyl sulfoxide), 1.22 mg 2-cyano-2-propyl benzodithioate and 0.45 mg 2,2'-azobis(2-methylpropionitrile) was dissolved in 3.8 ml tert-butanol and the solution was introduced into polymerization ampule. The mixture was bubbled with argon for 10 min and ampule was sealed. Polymerization was carried out at 70 °C for 16 h. Polymer precursor was isolated by precipitation into mixture of acetone:diethyl ether (3:1), filtered off, washed with acetone and diethyl ether and dried in vacuum. Terminating dithiobenzoate group was removed as described by Perrier<sup>[17]</sup>. The polymer precursor poly(HPMA-co-Ma- $\beta$ -Ala-TT) with molecular weight  $M_n = 79,000$  g/mol,  $M_w = 94,600$  g/mol, dispersity  $\mathcal{D} = 1.20$  and content of reactive thiazolidine-2-thione groups 13.4 mol% was obtained.

Polymer precursor poly(HPMA-co-Ma- $\beta$ -Ala-TT) (45 mg;  $M_n = 79,000$  g/mol,  $M_w = 94,600$  g/mol,  $\mathcal{D} = 1.20$ ; 13.4 mol% TT), compound **1** (6.2 mg) and *N*-(2-aminoethyl)biotinamid hydrobromid (biotin-NH<sub>2</sub>) (5 mg) were dissolved in 0.2 ml DMSO. ATTO-488-NH<sub>2</sub> (2.5 mg) was dissolved in 0.1 ml DMSO and added to the solution. Then 2.5  $\mu$ l of *N,N*-diisopropylethylamine (DIPEA) was added. Reaction was carried out for 4 h at room temperature and then 5  $\mu$ l of 1-aminopropan-2-ol was added and the reaction was stirred for 10 min. Polymer conjugate poly(HPMA-co-Ma- $\beta$ -Ala-Compound**1**-co-Ma- $\beta$ -Ala-ATTO488-co-Ma- $\beta$ -Ala-NH-biotin) was isolated by precipitation into mixture of acetone:diethyl ether (3:1), filtered off, washed with acetone and diethyl ether and dried in vacuum. Polymer conjugate was purified on chromatography column Sephadex LH-20 in

methanol, precipitated into diethyl ether, filtered off and dried in vacuum. Yield of the iBody 1 ( $M_n = 148,800$  g/mol,  $M_w = 203,900$  g/mol,  $\bar{D} = 1.37$ ) was 33 mg, content of compound 1 was 9.8 wt%, content of biotin was 9.8 wt% and content of ATTO-488 was 3.9 %.

### Synthesis of iBody 2

Polymer precursor poly(HPMA-co-Ma- $\beta$ -Ala-TT) was prepared by RAFT copolymerization as described in the Synthesis of iBody 1 by using following composition of polymerization mixture: HPMA (90 % mol; 500 mg), Ma- $\beta$ -Ala-TT (10 % mol, 100 mg), 4.29 mg 2-cyano-2-propyl benzodithioate and 1.59 mg 2,2'-azobis(2-methylpropionitrile). The polymer precursor with molecular weight  $M_n = 24,800$  g/mol,  $M_w = 26,700$  g/mol, dispersity  $\bar{D} = 1.08$  and content of reactive thiazolidine-2-thione groups 10.4 mol% was obtained.

Polymer precursor (74 mg;  $M_n = 24,800$  g/mol,  $M_w = 26,700$  g/mol,  $\bar{D} = 1.08$ ; 10.4 mol% TT) and biotin-NH<sub>2</sub> (6 mg) were dissolved in 0.4 ml DMSO and the solution was stirred for 20 min. Then 13 mg of compound 2 and 12.2  $\mu$ l of *N,N*-diisopropylethylamine (DIPEA) was added. Reaction was carried out for 4 h at room temperature and then 5  $\mu$ l of 1-aminopropan-2-ol was added and the reaction was stirred for 10 min. Polymer conjugate poly(HPMA-co-Ma- $\beta$ -Ala-Compound2-co-Ma- $\beta$ -Ala-NH-biotin) was isolated by precipitation into mixture of acetone:diethyl ether (3:1), filtered off, washed with acetone and diethyl ether and dried in vacuum. Polymer conjugate was purified on chromatography column Sephadex LH-20 in methanol, precipitated into diethyl ether, filtered off and dried in vacuum. Yield of the iBody 2 ( $M_n = 40,600$  g/mol,  $M_w = 67,000$  g/mol,  $\bar{D} = 1.65$ ) was 62 mg, content of compound 2 was 11.7 wt% and content of biotin was 4.7 wt%.

### Synthesis of iBody 3

Polymer precursor (55 mg,  $M_n = 24,800$  g/mol,  $M_w = 26,700$  g/mol,  $\bar{D} = 1.08$ ; 10.4 mol% TT) and biotin-NH<sub>2</sub> (6 mg) were dissolved in 0.32 ml DMSO and the solution was stirred for 20 min. Then 10 mg of compound 3 and 8.7  $\mu$ l of *N,N*-diisopropylethylamine (DIPEA) was added. Reaction was carried out for 4 h at room temperature and then 5  $\mu$ l of 1-aminopropan-2-ol was added and the reaction was stirred for 10 min. Polymer conjugate poly(HPMA-co-Ma- $\beta$ -Ala-Compound3-co-Ma- $\beta$ -Ala-NH-biotin) was isolated by precipitation into mixture of acetone:diethyl ether (3:1), filtered off, washed with acetone and diethyl ether and dried in vacuum. Polymer conjugate was purified on chromatography column Sephadex LH-20 in methanol, precipitated into diethyl ether, filtered off and dried in vacuum. Yield of the



iBody **3** ( $M_n = 42,100$  g/mol,  $M_w = 55,500$  g/mol,  $\bar{D} = 1.32$ ) was 35 mg, content of compound **3** was 15.3 wt% and content of biotin was 5.9 wt%.

### Synthesis of iBody **4**

Polymer precursor poly(HPMA-co-Ma- $\beta$ -Ala-TT) was prepared by RAFT copolymerization as described in the Synthesis of iBody **1** by using following composition of polymerization mixture: in 7.3 ml of tert-butanol, following compounds were dissolved: 1,000 mg of HPMA (85 % mol), 318 mg Ma- $\beta$ -Ala-TT (15 % mol, dissolved in 1.9 ml DMSO), 2.42 mg 2-cyano-2-propyl benzodithioate and 0.90 mg 2,2'-azobis(2-methylpropionitrile). The polymer precursor with molecular weight  $M_n = 73,800$  g/mol,  $M_w = 90,600$  g/mol, dispersity  $\bar{D} = 1.23$  and content of reactive thiazolidine-2-thione groups 14.6 mol% was obtained.

Polymer precursor poly(HPMA-co-Ma- $\beta$ -Ala-TT) (40 mg,  $M_n = 73,800$  g/mol,  $M_w = 90,600$  g/mol,  $\bar{D} = 1.23$ ; 14.6 mol% TT), compound **4** (6.0 mg) and *N*-(2-aminoethyl)biotinamid hydrobromid (biotin-NH<sub>2</sub>) (5 mg) were dissolved in 0.2 ml DMSO. ATTO-488-NH<sub>2</sub> (2.5 mg) was dissolved in 0.1 ml DMSO and added to the solution. Then 8.0  $\mu$ l of *N,N*-diisopropylethylamine (DIPEA) was added. Reaction was carried out for 4 h at room temperature and then 5  $\mu$ l of 1-aminopropan-2-ol was added and the reaction was stirred for 10 min. Polymer conjugate poly(HPMA-co-Ma- $\beta$ -Ala-Compound**4**-co-Ma- $\beta$ -Ala-ATTO488-co-Ma- $\beta$ -Ala-NH-biotin) was isolated by precipitation into mixture of acetone:diethyl ether (3:1), filtered off, washed with acetone and diethyl ether and dried in vacuum. Polymer conjugate was purified on chromatography column Sephadex LH-20 in methanol, precipitated into diethyl ether, filtered off and dried in vacuum. Yield of iBody **4** ( $M_n = 135,800$  g/mol,  $M_w = 255,000$  g/mol,  $\bar{D} = 1.88$ ) was 22 mg; content of compound **4** was 11.3 %, content of ATTO-488 was 4.2 % and content of biotin was 9.7 wt%.

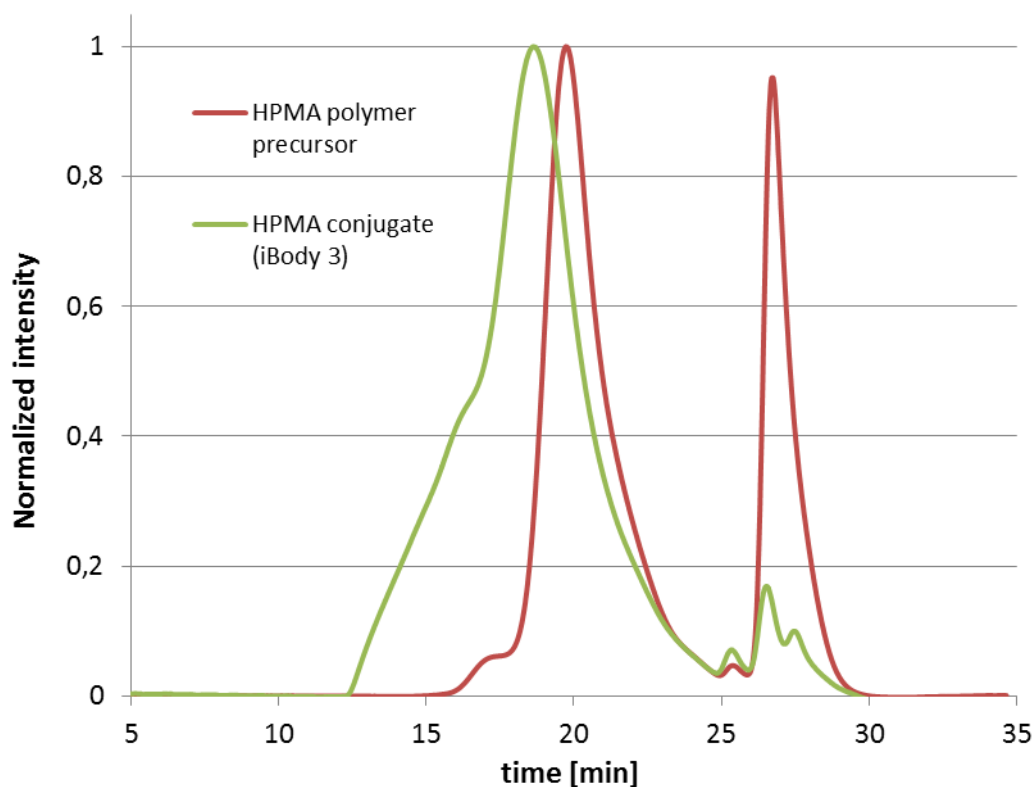
### Synthesis of iBody **5**

Polymer precursor poly(HPMA-co-Ma- $\beta$ -Ala-TT) (45 mg,  $M_n = 79,000$  g/mol,  $M_w = 94,600$  g/mol,  $\bar{D} = 1.20$ ; 13.4 mol% TT) and *N*-(2-aminoethyl)biotinamid hydrobromid (biotin-NH<sub>2</sub>) (5 mg) were dissolved in 0.2 ml DMSO. ATTO-488-NH<sub>2</sub> (2.5 mg) was dissolved in 0.1 ml DMSO and added to the solution. Then 2.5  $\mu$ l of *N,N*-diisopropylethylamine (DIPEA) was added. Reaction was carried out for 4 h at room temperature and then 5  $\mu$ l of 1-aminopropan-2-ol was added and the reaction was stirred for 10 min. Polymer conjugate poly(HPMA-co-Ma- $\beta$ -Ala-ATTO488-co-Ma- $\beta$ -Ala-NH-biotin)

was isolated by precipitation into mixture of acetone:diethyl ether (3:1), filtered off, washed with acetone and diethyl ether and dried in vacuum. Polymer conjugate was purified on chromatography column Sephadex LH-20 in methanol, precipitated into diethyl ether, filtered off and dried in vacuum. Yield of the iBody 5 ( $M_n = 108,100$  g/mol,  $M_w = 176,200$  g/mol,  $\bar{D} = 1.63$ ) was 32 mg, content of biotin was 10.8 wt% and content of ATTO-488 was 5.1 %.

### **Synthesis of iBody 6**

Polymer precursor poly(HPMA-*co*-Ma- $\beta$ -Ala-TT) (40 mg,  $M_n = 24,800$  g/mol,  $M_w = 26,700$  g/mol,  $\bar{D} = 1.08$ ; 10.4 mol% TT) and *N*-(2-aminoethyl)biotinamid hydrobromid (biotin-NH<sub>2</sub>) (5 mg) were dissolved in 0.25 ml DMSO. Then 3.0  $\mu$ l of *N,N*-diisopropylethylamine (DIPEA) was added. Reaction was carried out for 4 h at room temperature and then 5  $\mu$ l of 1-aminopropan-2-ol was added and the reaction was stirred for 10 min. Polymer conjugate poly(HPMA-*co*-Ma- $\beta$ -Ala-NH-biotin) was isolated by precipitation into mixture of acetone:diethyl ether (3:1), filtered off, washed with acetone and diethyl ether and dried in vacuum. Polymer conjugate was purified on chromatography column Sephadex LH-20 in methanol, precipitated into diethyl ether, filtered off and dried in vacuum. Yield of the iBody 6 ( $M_n = 37,800$  g/mol,  $M_w = 57,800$  g/mol,  $\bar{D} = 1.53$ ) was 28 mg, content of biotin was 6.4 wt%.



### Supplementary Figure S8

Example of a GPC chromatogram of the HPMA polymer precursor (red) and the resulting ligand-decorated HPMA copolymer conjugate (iBody **3**, green) from differential refractometer. The HPMA polymers (both precursors and conjugates) are eluted between 12 and 25 min; the peak in time between 25 and 30 min represents the buffer. The shift of the peak maximum of the HPMA copolymer conjugate corresponds to its increased molecular weight and dispersity. The small shoulder at 17 min on GPC record of polymer precursor is caused by the side reaction during RAFT polymerization (termination reaction of two polymer radicals forming dead polymer).<sup>[18]</sup>

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