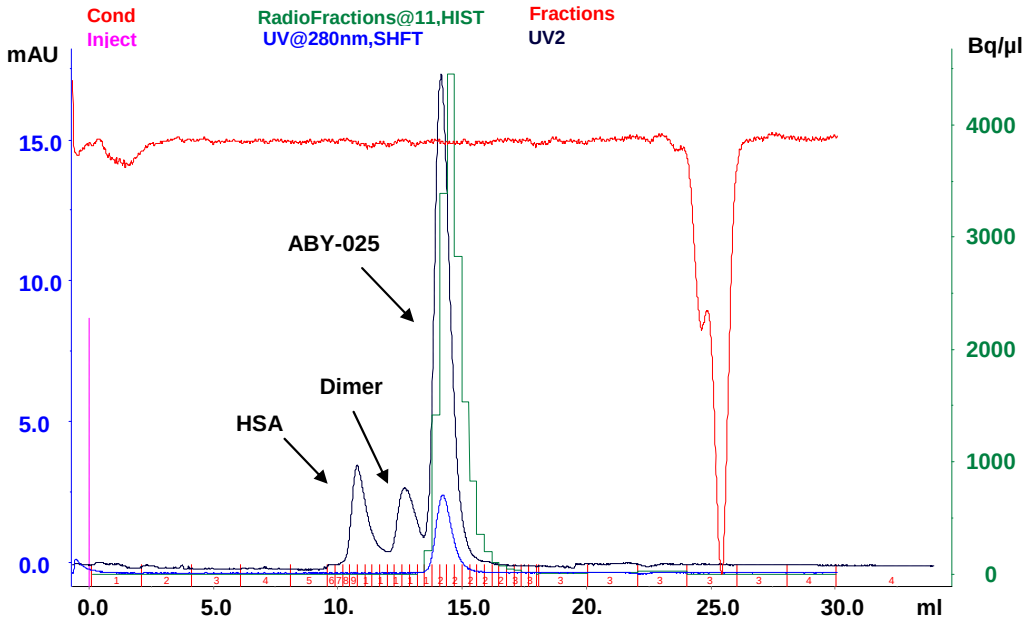
Size-exclusion HPLC analysis of ^{111}In-ABY-025

Analysis was performed using a Superdex peptide HR 10/30 column (GE Healthcare) eluted with 50 mM phosphate, pH 7.0, 150 mM NaCl at a flow rate 0.5 mL/min. Before the analytical run, the system was calibrated with a mixture of human serum albumin, ABY-025 and the tail-to-tail dimer of $Z_{HER2:2891}$-Cys.

During the analytical run, 100 µl of ^{111}In labeled ABY-025 was injected. Analysis was performed 1, 5 and 24 h after ^{111}In-labeling. Eluted fractions were collected and their radioactivity was measured using a gamma-counter. A representative chromatogram is presented in Figure S1. Only monomeric ABY-025 and monomeric ^{111}In-labeled ABY-025 were detected in all samples, using UV or radioactivity detection, respectively.



SUPPLEMENTAL FIGURE 1. Size-exclusion HPLC of ^{111}In-ABY-025. The protein was detected as the UV signal at 280 nm (solid blue line), and fractions analyzed for radioactivity to determine the presence of ^{111}In-labeled ABY-025 (green rectangles overlaid). The control containing monomeric ABY-025, dimeric Affibody molecule control and human serum albumin (HSA) is indicated by the black dashed line.

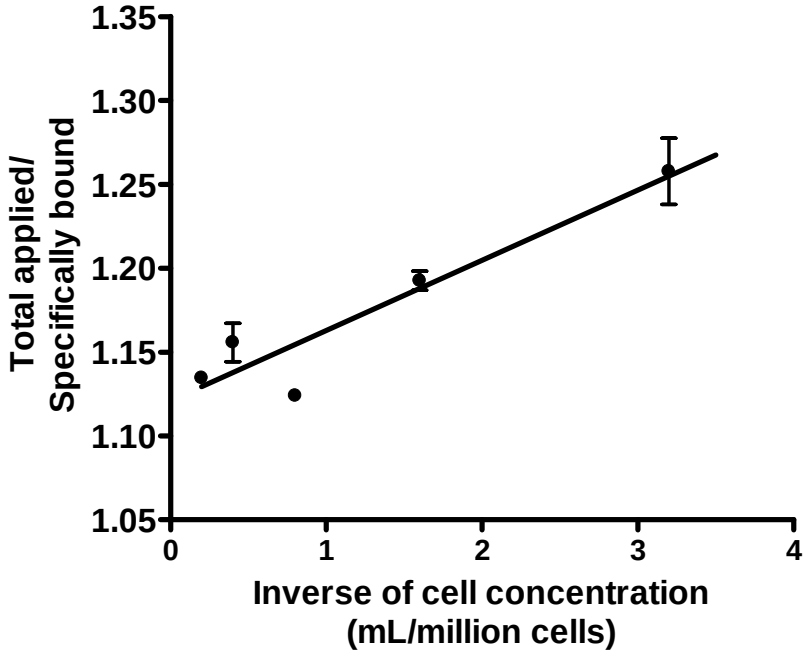
Determination of antigen binding capacity of ^{111}In-ABY-025

Extrapolated binding of ^{111}In-ABY-025 to an infinite excess of antigen (according to Lindmo (*23*))

SKOV-3 cells were scraped from the surface of a cell culture flask in order to preserve receptors expression. After re-suspension in cell-culture medium, the cells were counted in an automated cell counter and pelleted in Eppendorf tubes by gentle centrifugation, 10 x10^6 cells in each tube.

One mL PBS solution containing 28.5 μg of non-labeled ABY-025 was added to a designated specificity control tube in order to saturate receptors. One mL of PBS was added to three other tubes. The cells in all tubes were re-suspended and series of six dilutions (1:2) were prepared from each tube. ^{111}In-ABY-025 solution in PBS (8.72 ng (1.25 pmol) in 0.5 mL PBS) was added to each tube. Cells were incubated at 4 °C for 4 h and 0.5 ml of the supernatant was collected after vigorous centrifugation. The radioactivity of both samples (cell pellet + 0.5 ml medium, A, and 0.5 ml medium, B) was measured.

The bound fraction (BF) was calculated as BF = (A-B)/(A+B), i.e. the ratio of cell bound radioactivity to totally added radioactivity. Non-specific binding determined in the same manner for every data point was subtracted from the total binding, providing corrected bound fraction (BF_{corr}). The data were plotted as double inverse plot, i.e. the applied radiolabeled Affibody molecule conjugate over the specific binding ($1/BF_{corr}$) as a function of the inverse cell concentration (mL/million cells). Linear regression analysis was performed using GraphPad Prism. The theoretical value of $1/BF_{inf}$ in the case of infinite excess of receptors is found as the intercept with the Y axis. This value, corresponding to the antigen binding capacity for ^{111}In-ABY-025 was determined to 89.2 ± 0.6% (mean ± SD).



SUPPLEMENTAL FIGURE 2. Lindmo plot of ^{111}In-ABY-025 binding to HER2-expressing SKOV-3 cells.

Binding of ^{111}In-ABY-025 to 100-fold excess of antigen (according to Engfeldt (*24*))

SKOV-3 cells were scraped from the surface of a cell culture flask in order to preserve receptors expression. After re-suspension in cell-culture medium, the cells were counted in automated cell counter and pelleted in Eppendorf tubes by gentle centrifugation, 13 x10^6 cells in each tube. Analyses were performed in triplicates.

Supernatants were thoroughly removed from the Eppendorf tubes and 1 ml medium containing labeled conjugate was added. The ^{111}In-ABY-025 concentration was 0.26 pmol/mL, corresponding to a ratio of 1 Affibody molecule conjugate per 100 HER2 receptors. Cells were re-suspended and incubated for 4 h at 4 ˚C under gentle shaking. After incubation the cells were pelleted by centrifugation and 0.5 ml supernatant was taken to a separate Eppendorf tube. The amount of radioactivity in both samples (cell pellet + 0.5 ml medium, A, and 0.5 ml medium, B) was determined and the antigen binding capacity (ABC) was calculated as ABC = (A-B)/(A+B), yielding 89.8 ± 0.5 % (Average ± SD).

Biodistribution of ^{111}In-ABY-025 in BALB/c *nu / nu* mice bearing SKOV-3 xenografts (detailed data)

SUPPLEMENTAL TABLE 1

Biodistribution of ^{111}In-ABY-025 in BALB/c *nu / nu* mice bearing SKOV-3 xenografts 0.5 to 144 h after injection.*

	Uptake (%IA/g)†						
Tissue	**0.5 h**	**1 h**	**2 h**	**4 h**	**24 h**	**72 h**	**144 h**
Blood	2.0±0.2	1.1±0.1	0.31±0.04	0.18±0.02	0.039±0.004	0.03±0.02	ND
Lung	2.63±0.08	1.8±0.2	0.6±0.1	0.37±0.03	0.16±0.04	0.13±0.04	0.002±0.003
Liver	1.64±0.05	1.9±0.2	1.4±0.4	1.5±0.3	1.0±0.1	0.9±0.1	0.38±0.05
Spleen	1.1±0.1	0.8±0.2	0.40±0.05	0.47±0.09	0.25±0.08	0.31±0.07	0.10±0.02
Pancreas	0.6±0.2	0.4±0.1	0.19±0.07	0.24±0.05	0.10±0.4	0.14±0.02	0.001±0.001
Stomach	1.5±0.2	1.0±0.1	0.40±0.09	0.4±0.1	0.15±0.4	0.11±0.01	ND
Small intestine	0.9±0.4	0.7±0.1	0.22±0.09	0.52±0.53	0.03±0.04	0.05 (n=1)	ND
Large intestine	1.4±0.4	1.0±0.1	0.4±.01	0.4±0.1	0.19±0.05	0.14±0.03	ND
Kindeys	171±23	186±24	163±56	163±16	144±13	137±13	58±9
Salivary gland	1.1±0.2	0.8±0.3	0.4±0.1	0.48±0.01	0.28±0.04	0.24±0.03	0.02±0.02
Tumor	13±2	17±2	13±4	15±3	11±4	9.9±0.5	4±1
Muscle	0.5±0.2	0.36±0.05	0.15±0.06	0.16±0.05	0.05±0.01	0.040±0.007	0.002±0.003
Bone	0.9±0.1	0.5±0.2	0.3±0.1	0.3±0.1	0.05±0.04	0.07±0.04	ND
Carcass†	15±1	11±1	6±1	6±1	3.4±.07	2.5±0.1	1.1±0.1

* Data are presented as an average from four animals ± SD except in the case indicated with n=1, where a detectable level of radioactivity was only found in the sample from one of the animals. ND = not detectable levels of radioactivity. The uptake is calculated as %IA/g.

† Data for carcass are presented as %IA per whole sample

SUPPLEMENTAL TABLE 2

Tumor-to-organ ratios from the biodistribution of ^{111}In-ABY-025 in BALB/c *nu / nu* mice bearing SKOV-3 xenografts 4 h after injection.

Tumor-to-organ ratios*

Tissue	0.5 h	1 h	2 h	4 h	24h	3 days	6 days
Blood	6±1	15±3	42±18	88±15	275±113	397±232	ND
Lung	4.8±0.8	9.5±0.9	22±10	42±9	66±11	83±22	ND
Liver	8±1	8.7±0.6	9±3	10±1	10±2	11±2	10±1
Spleen	12±2	20±3	31±8	33±2	43±13	33±9	40±8
Pancreas	25±7	42±12	68±7	65±8	111±28	73±8	ND
Stomach	9±2	17±3	33±13	43±5	74±16	95±13	ND
Small intestine	16±7	25±5	60±7	45±22	155 (n=1)	474±528	ND
Large intestine	10±2	16±2	34±10	39±9	54±5	76±16	ND
Kidney	0.07±0.01	0.090±0.004	0.08±	0.09±0.02	0.07±0.02	0.073±0.008	0.065±0.006
Salivary gland	11±3	25±13	33±9	32±7	37±8	42±7	ND
Muscle	27±7	47±7	90±37	99±10	229±49	255±51	ND
Bone	13.3±0.8	38±16	47±15	48±9	351±315	211±184	ND

* Please see footnote of Supplemental Table 1.

Pharmacokinetics of ^{111}In-ABY-025 in rats and macaques

Pharmacokinetic studies were performed in outbred WI (Han) rats from Charles River Ltd, and macaques (*Macaca fascicularis*) from Bioculture Co. Ltd, or Bioda Co. Ltd (MITIUS). Three animals of each gender were included in either study. The studies were conducted under GLP by Covance Laboratories Ltd. (Harrogate) as part of a toxicokinetic analysis of ^{111}In-ABY-025. In both studies the animals received a single i.v. administration of 0.424 mg/kg ^{111}In-ABY-025 (2.0 kBq/µg for rats and 1.6 kBq/µg for macaques), and blood samples were taken after 2, 4, 7, 10, 20, 40, 60, 120 and 240 minutes. A portion of whole blood was removed for gamma counting and the remainder was allowed

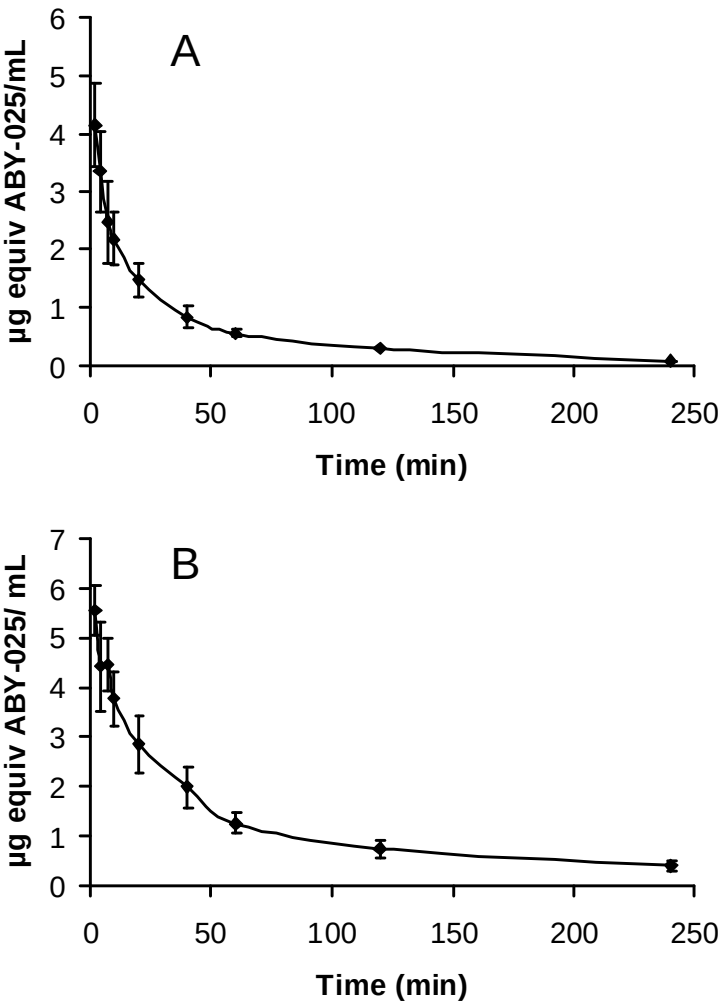
to clot, and serum collected after centrifugation. Portions of both whole blood and serum were subjected to gamma counting. Half-life values were calculated from the relevant portion of the semi-logarithmic plots of concentration/time curves having first performed linear regression analysis using the method of least squares.

Pharmacokinetic analysis in rats and cynomolgus macaques

The blood kinetics after i.v. administration of ^{111}In-ABY-025 showed a rapid, biphasic decline of concentration in males and females of both rats and cynomolgus macaques (Supplemental Figure 3). The initial and terminal half-life was calculated both from serum and blood sample data for each animal and the average calculated for each group of three individuals.

In rats, the mean initial half-life was in the range of 9-14 minutes and the mean terminal half-life in the range of 52-58 minutes. Analysis of serum and blood samples yielded an average initial half-life (± SD) of 14 ± 6 and 9.2 ± 0.7 minutes for males, and 11 ± 2 and 12.8 ± 0.8 minutes for females, respectively. The average terminal half-life was 56 ± 19 and 52 ± 21 minutes for males (serum and blood, respectively), and 52 ± 4 and 58 ± 1 minutes for females (serum and blood, respectively).

In macaques, the mean initial half-life was in the range of 12-19 minutes and the mean terminal half-life in the range 104-123 minutes. Analysis of serum and blood samples yielded an average initial half-life (± SD) of 19 ± 1 and 17 ± 5 minutes for males, and 12 ± 4 and 19 ± 6 minutes for females, respectively. The terminal half-life values were 115 ± 37 and 123 ± 41 minutes for males, and 106 ± 18 and 104 ± 6 minutes for females.



SUPPLEMENTAL FIGURE 3. The pharmacokinetic profile in blood after i.v. administration of ^{111}In-ABY-025 (0.424 mg/kg) to rats (**A**) and cynomolgus macaques (**B**). The mean of the entire groups containing three animals of each gender are plotted ± SD.