Preclinical Evaluation of ^{225}Ac-Labeled Single Domain Antibody for the Treatment of HER2^{pos} Cancer

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SUPLEMENTAL MATERIALS AND METHODS

Sequence of the 2Rs15d

QVQLQESGGGSVQAGGSLKLTCAASGYIFNSCGMGWYRQSPGRERELVSRISGDGDTWH
KESVKGRFTISQDNVKKTLYLQMNSLKPEDTAVYFCAVCYNLETYWGQGTQVTVSS

In Vitro Assays

Binding Affinity and Specificity of [^{225}Ac]Ac-DOTA-sdAbs

In vitro specific binding of [^{225}Ac]Ac-DOTA-sdAb was evaluated via a saturation binding assay (10,18). SKOV-3 cells were seeded 24 h before the experiment in 24-well plates at a concentration of 8×10^4 cells/mL. For receptor saturation analysis, cells were incubated (4°C, 2 h) with increasing concentrations (0.02-100 nM) of [^{225}Ac]Ac-DOTA-2Rs15d, either alone or with an additional 100-fold excess of unlabeled 2Rs15d. The cells were washed twice with cold phosphate buffered-saline (PBS) and lysed with 1 M NaOH during 10 min at 37°C. The lysates were counted for radioactivity in an automatic γ-counter (Wizard 2480, Perkin-Elmer). The K_D value was determined by non-linear regression using GraphPad Prism (version 5.01).

Specificity of binding to HER2 receptor was evaluated for HER2-targeting [^{225}Ac]Ac-DOTA-2Rs15d and the control [^{225}Ac]Ac-DOTA-R3B23 on SKOV-3 (HER2^{pos}) cells that were

treated and processed as described above, except that [^{225}Ac]Ac-DOTA-sdAbs were added at 10 nM concentration only, with or without a 100-fold excess of unlabeled 2Rs15d.

Internalization Assay

Intracellular retention of [^{225}Ac]Ac-DOTA-2Rs15d in SKOV-3 cells was evaluated at different time-points. 8×10^5 cells/mL were adhered overnight and washed twice with PBS prior to incubation with 10 nM of [^{225}Ac]Ac-DOTA-2Rs15d at 4°C for 1 h. A 100-fold molar excess of unlabeled 2Rs15d was added in parallel to assess the non-specific binding. Next, the unbound fraction was washed away, cells were supplemented with fresh medium and incubated at 37°C up to 24 h. After incubation, the medium fraction was collected prior to an acid wash of 0.05 M glycine-HCl pH = 2.8, through which the membrane-bound fraction was collected. Finally, cells were lysed by the addition of 1 M NaOH (internalized fraction). All fractions were counted for radioactivity using an automatic γ-counter (Wizard 2480, Perkin-Elmer).

Clonogenic Survival Assay

SKOV-3 cells (500 cells/well) were seeded in 6-well plates and treated with (i) [^{225}Ac]Ac-DOTA-2Rs15d alone, (ii) [^{225}Ac]Ac-DOTA-2Rs15d in the presence of a 30-fold molar excess of unlabeled 2Rs15d, or (iii) non-targeting [^{225}Ac]Ac-DOTA-R3B23; all at a dose range of 0.1-120 kBq/mL, and subsequently incubated for 2 h at 37°C. Next, cells were washed twice with PBS and supplemented with fresh medium followed by incubation at 37°C for at least 11 days to allow colony formation. Next, formed colonies were washed twice with PBS, fixed with 10% formaldehyde solution, stained with Crystal Violet solution and counted using ImageJ software (NIH, LOCI, University of Wisconsin). The plating efficiency (PE) was defined as the number of grown colonies (>50 cells in a colony) in the untreated control wells divided by the number of cells seeded. The surviving fraction (SF) was calculated by dividing the number of colonies formed by the number of cells seeded and multiplying by the PE (19). The survival curve is linear on a log-linear plot. D_0 values were calculated using $lnSF = -D/D_0$ equation from linear regression using GraphPad Prism (version 5.01). Obtained results are the average of at least three independent experiments preformed in triplicate, and are presented on a graph in relation to the applied radioactivity (kBq/mL). Differences between results for each group of treated cells were tested with one-tailed Student's *t*-test.

DNA double-strand breaks (DSBs) via γH2AX phosphorylation imaging

SKOV-3 cells (2×10^5/well) were seeded onto sterilized glass coverslips placed in 6-well plates and treated with (i) [^{225}Ac]Ac-DOTA-2Rs15d alone or in combination with (ii) 60-fold molar excess of non-labeled 2Rs15d; or (iii) non-targeting [^{225}Ac]Ac-DOTA-R3B23 at three radioactive doses: 15, 125, and 625 kBq/mL. Additionally, one plate with seeded cells served as a non-treated (NT) negative control. All plates were incubated for 2 h at 37°C, followed by

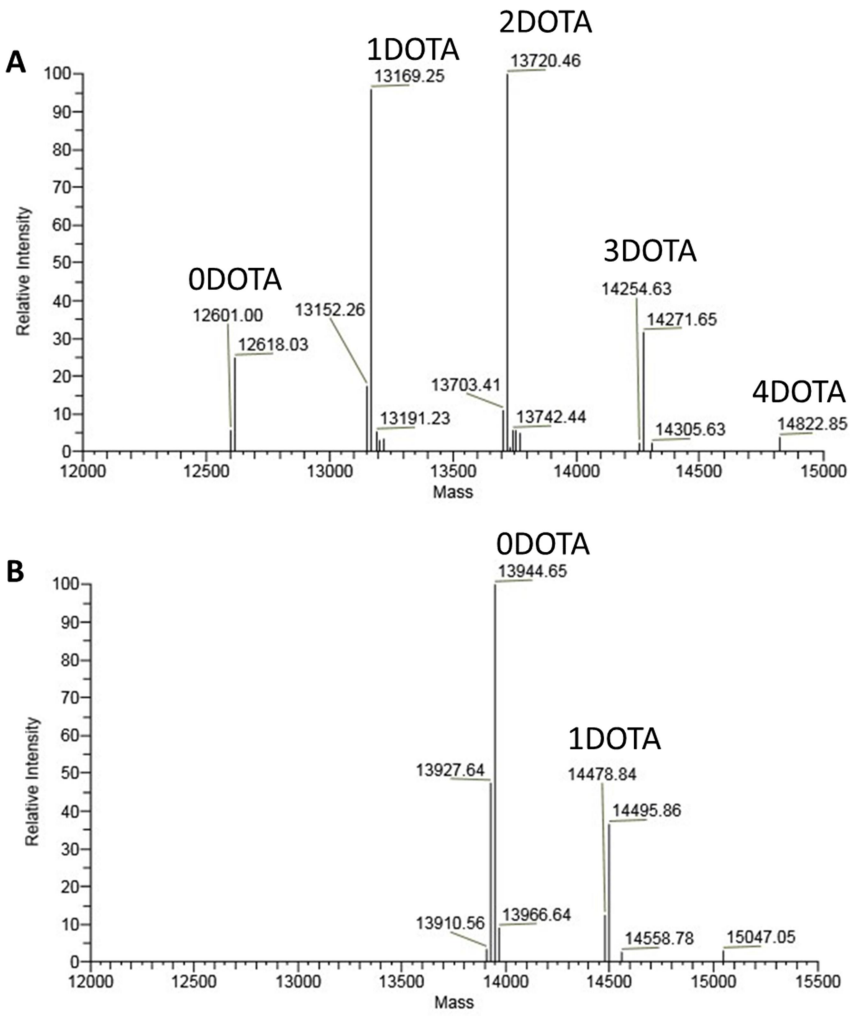
a PBS wash, subsequent addition of fresh medium, and further incubation at 37°C for 24 h. Next, cells were washed with PBS, fixed with ice-cold methanol (-20°C for 5 min) and incubated with blocking buffer (2% BSA and 10% skimmed milk in PBS) for 1 h at room temperature. Finally, cells were stained with anti-phospho-histone H2AX (Ser139, clone JBW301) antibody (Merck, Darmstadt, Germany) and AlexaFluor488 goat anti-mouse secondary antibody (Thermo Fisher Scientific, Waltham, MA). Scoring of γ-H2AX foci was done using the Zeiss Imager Z2 fluorescence microscope and the MetaSystems program. Results are presented as the mean value with a standard deviation of three independent experiments.

Histopathologic Changes - Severity Scores

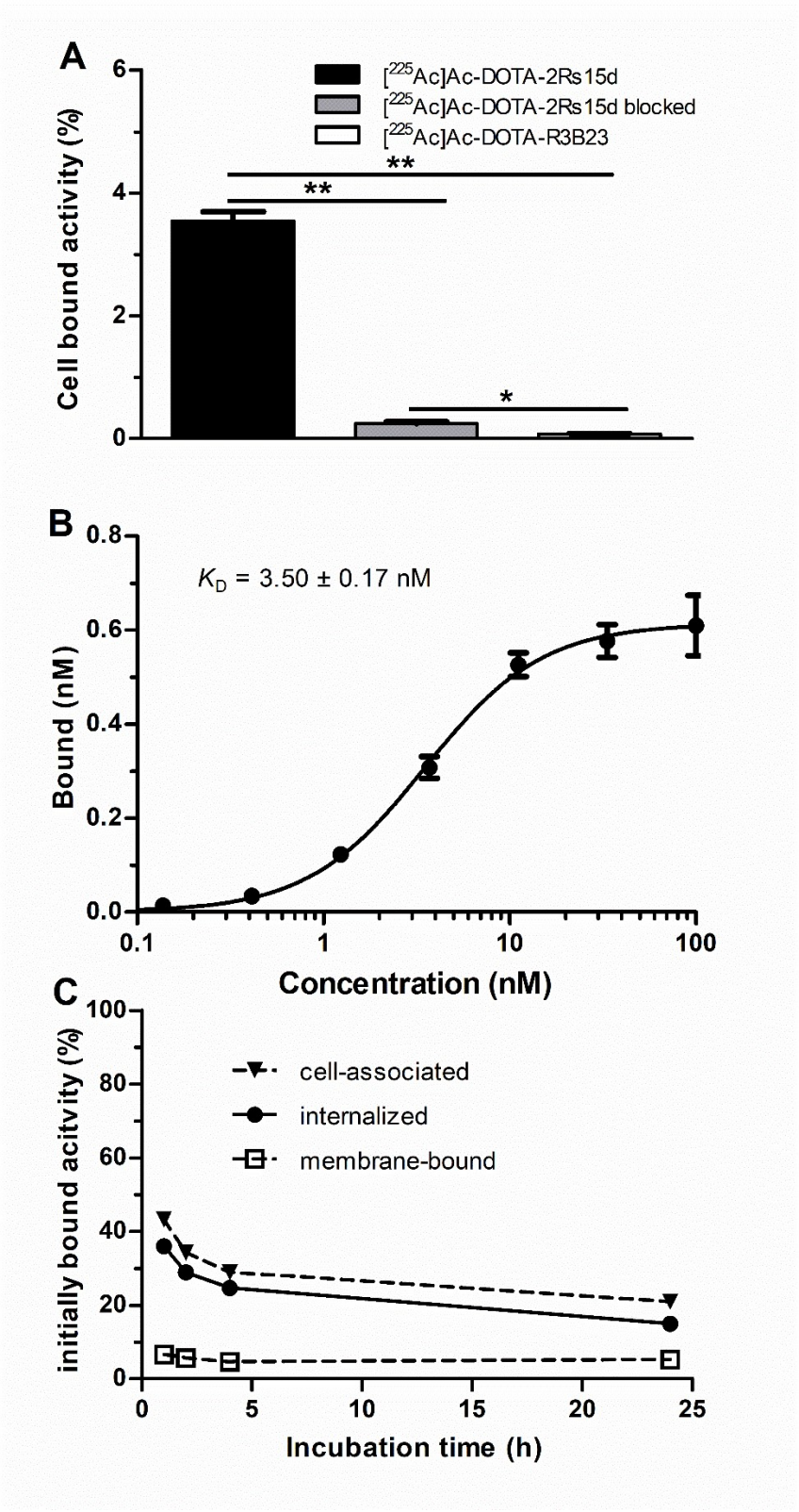
Severity scores were assigned from a scale of 1-5.

Grade 1, Minimal	This corresponds to a histopathologic change ranging from inconspicuous to barely noticeable but so minor, small, or infrequent as to warrant no more than the least assignable grade. For multifocal or diffusely-distributed lesions, this grade was used for processes where less than approximately 10% of the tissue in an average high-power field was involved.
Grade 2, Slight	This corresponds to a histopathologic change that is a noticeable but not a prominent feature of the tissue. For multifocal or diffusely-distributed lesions, this grade was used for processes where between approximately 10% and 25% of the tissue in an average high-power field was involved.
Grade 3, Moderate	This corresponds to a histopathologic change that is a prominent but not a dominant feature of the tissue. For multifocal or diffusely-distributed lesions, this grade was used for processes where between approximately 25% and 50% of the tissue in an average high-power field was involved.
Grade 4, Marked	This corresponds to a histopathologic change that is a dominant but not an overwhelming feature of the tissue. For multifocal or diffusely-distributed lesions, this grade was used for processes where between approximately 50% and 95% of the tissue in an average high-power field was involved.
Grade 5, Severe	This corresponds to a histopathologic change that is an overwhelming feature of the tissue. For multifocal or diffusely-distributed lesions, this grade was used for processes where greater than approximately 95% of the tissue in an average high-power field was involved.

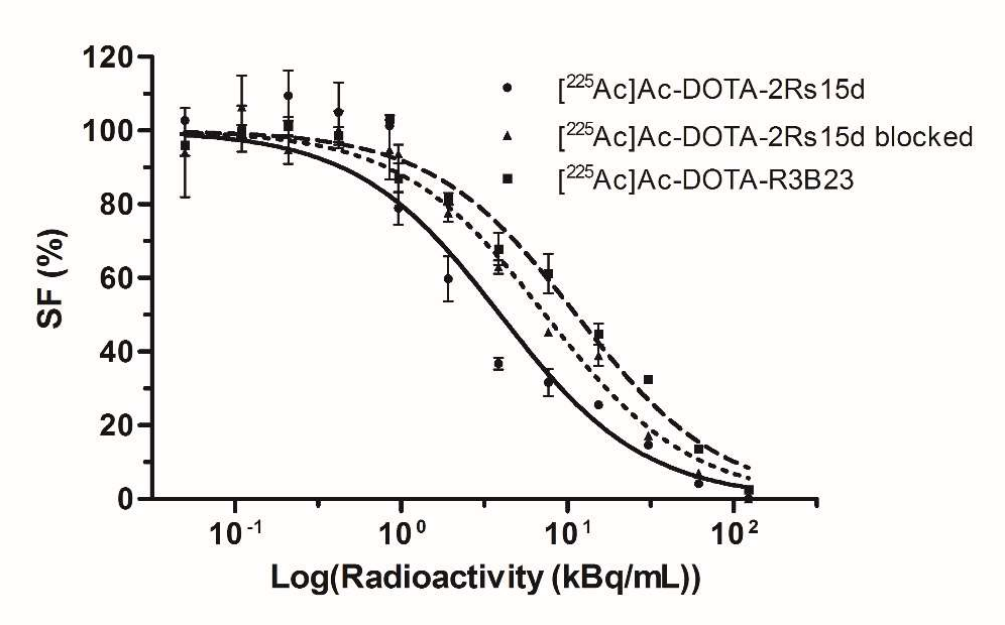
SUPPLEMENTAL RESULTS



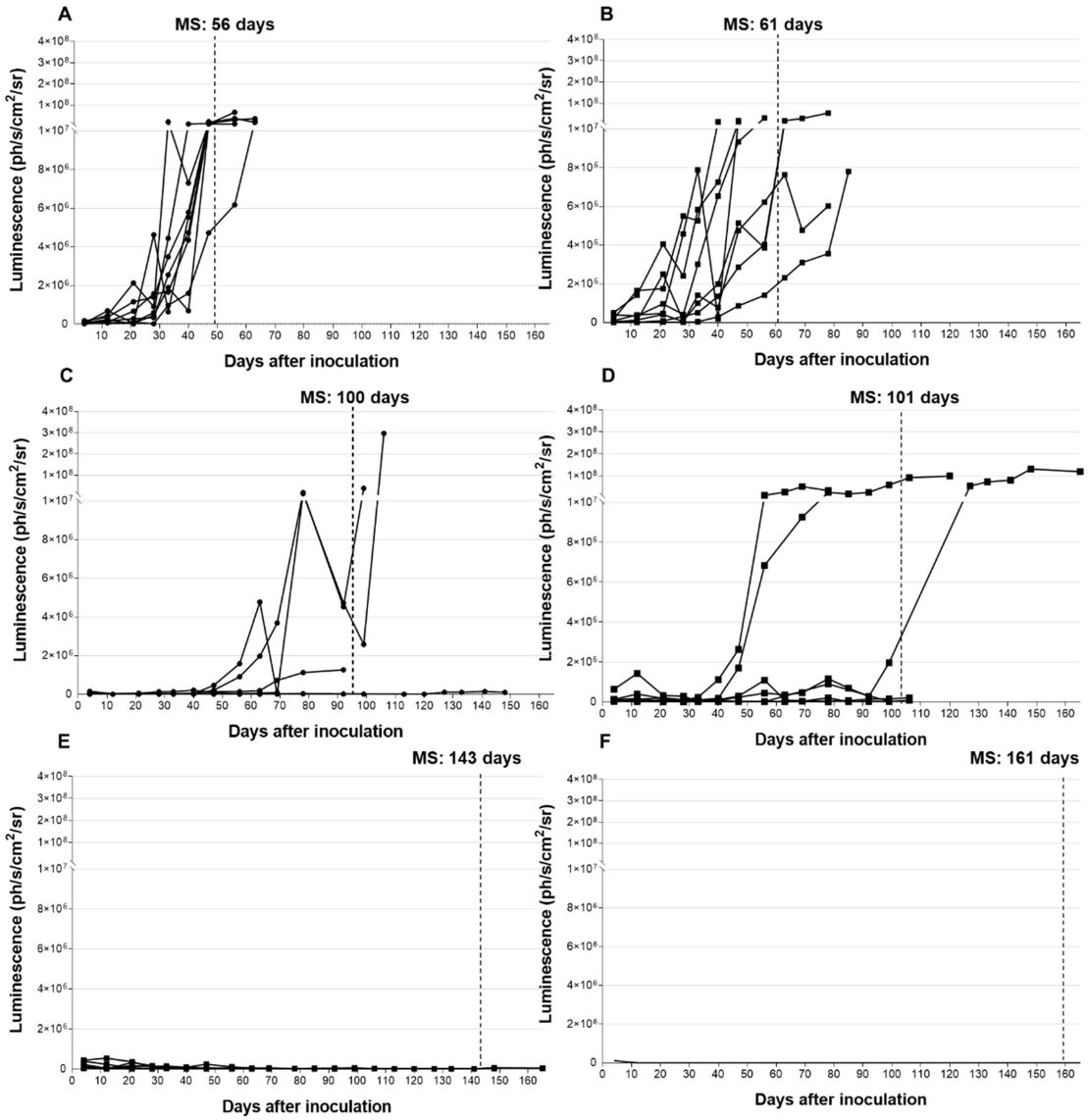
Supplemental Figure S1. The DOTA-conjugated 2Rs15d and R3B23 (control) sdAbs were obtained after the random conjugation procedure of *p*-SCN-Bn-DOTA on the lysines of the sdAb backbone. (**A**) LC-ESI-LTQ-Orbitrap-Mass Spectrometry analysis of DOTA-2Rs15d identified five peaks, representing unconjugated 2Rs15d peak (12601.00 Da), 1-DOTA conjugated peak (13169.25 Da), 2-DOTAs conjugated peak (13720.46 Da), 3-DOTAs conjugated peak (14254.63 Da) and 4-DOTAs conjugated peak (14822.85 Da). (**B**) LC-ESI-LTQ-Orbitrap-Mass Spectrometry analysis of DOTA-R3B23 identified three peaks, representing unconjugated R3B23 peak (13944.65 Da), 1-DOTA conjugated peak (14478.84 Da) and 2-DOTAs conjugated peak (15047.05 Da).



Supplemental Figure S2. *In vitro* assays: (**A**) specificity of binding, (**B**) saturation binding, and (**C**) internalization of [^{225}Ac]Ac-DOTA-2Rs15d. All studies were performed using HER2^{pos} SKOV-3 cells. (*) P=0.026; (**) P< 0.001.



Supplemental Figure S3. Survival curve fitted to the number of colonies (clonogenic assay results) for three groups: [^{225}Ac]Ac-DOTA-2Rs15d alone or blocked with cold 2Rs15d and [^{225}Ac]Ac-DOTA-R3B23.



Suplemental Figure S4. Quantification of tumor load with BLI until day 165 for all treatment groups. Bioluminescence images of animals receiving (**A**) 0.9% NaCl, (**B**) [^{225}Ac]Ac-DOTA-R3B23, (**C**) trastuzumab, (**D**) single dose of [^{225}Ac]Ac-DOTA-2Rs15d, (**E**) triple dose of [^{225}Ac]Ac-DOTA-2Rs15d and (**F**) triple dose of [^{225}Ac]Ac-DOTA-2Rs15d and trastuzumab over the complete therapy study.