**Supplemental Data for: Valency of HER2 targeting antibodies influences tumor cell internalization and penetration**

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**Supplemental Table 1: Plasma PK Parameters of HER2 targeting antibodies in KPL-4 tumor bearing mice.**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Group** | **N** | **Dose**  **(mg/kg)** | **Cmax**  **(μg-eq/mL)** | **Cmax/Dose**  **(kg\*μg-eq/mL)** | **AUC(0-7)**  **(μg-eq/mL\*day)** | **AUC(0-7) / Dose**  **(μg-eq/mL\*day\*kg/mg)** | **CL**  **(mL/day/kg)** | **Vss**  **(mL/kg)** | **t1/2**  **(d)** |
| anti-gD | 4 | 5 | 68 ± 2 | 14 | 6965 ± 220 | 1393 | 8 | 86 | 7 |
| anti-gD/HER2 | 13 | 1 | 15 ± 1 | 15 | 693 ± 58 | 693 | 22 | 152 | 5 |
| anti-gD/HER2 | 13 | 10 | 184 ± 29 | 18 | 7340 ± 469 | 734 | 23 | 126 | 4 |
| anti-HER@/HER2 | 13 | 0.5 | 9 ± 0.3 | 17 | 414 ± 10 | 827 | 23 | 98 | 3 |
| anti-HER@/HER2 | 13 | 5 | 110 ± 6 | 22 | 5515 ± 238 | 1103 | 16 | 80 | 3 |

**Supplemental Table 1: Plasma PK Parameters of HER2 targeting antibodies in KPL-4 tumor bearing mice.** SCID.bg mice bearing KPL-4 tumors in mammary fat pads were injected with a single IV bolus of 125I- and 111In-labeled monovalent HER2 binding (anti-gD/HER2, 1 or 10 mg/kg), bivalent HER2 binding (anti-HER2/HER2, 0.5 or 5 mg/kg) or non-binding control (anti-gD/gD, 5 mg/kg) antibodies. Sparse blood samples were collected from 0 to 7 days. Plasma concentrations of 125I radiolabeled and 111In radiolabeled antibodies were represented as %ID/mL and converted to nominal values based on total antibody concentrations of dosing materials. PK parameters were calculated using Phoenix WinNonLin (Certara).

**Supplemental Table 2: Intact antibody tissue distribution statistics (p-values)**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **6h** | | | | | | |
|  | **Blood** | **Heart** | **Liver** | **Kidney** | **Spleen** | **Muscle** | **Tumor** |
| **anti-gD/HER2 @ 1 mg/kg vs. anti-gD/HER2 @ 10 mg/kg** | \*0.01 | \*0.01 | 0.06 | \*0.02 | \*0.003 | 0.93 | 0.84 |
| **anti-HER2/HER2 @ 0.5 mg/kg vs. anti-HER2/HER2 @ 5 mg/kg** | 0.12 | 0.95 | 0.07 | 0.57 | 0.14 | 0.83 | 0.75 |
| **anti-gD/HER2 @ 1 mg/kg vs. anti-HER2/HER2 @ 0.5 mg/kg** | 0.39 | 0.06 | 0.96 | 0.06 | 0.41 | 0.96 | 0.43 |
| **anti-gD/HER2 @ 10 mg/kg vs. anti-HER2/HER2 @ 5 mg/kg** | 0.49 | 0.61 | 0.81 | 0.54 | \*0.007 | 0.79 | 0.97 |
|  | **1d** | | | | | | |
|  | **Blood** | **Heart** | **Liver** | **Kidney** | **Spleen** | **Muscle** | **Tumor** |
| **anti-gD/HER2 @ 1 mg/kg vs. anti-gD/HER2 @ 10 mg/kg** | 0.68 | 0.46 | 0.97 | 0.79 | 0.55 | 0.34 | \*0.04 |
| **anti-HER2/HER2 @ 0.5 mg/kg vs. anti-HER2/HER2 @ 5 mg/kg** | 0.71 | 0.59 | 0.67 | 0.92 | 0.89 | 0.33 | 0.28 |
| **anti-gD/HER2 @ 1 mg/kg vs. anti-HER2/HER2 @ 0.5 mg/kg** | 0.80 | 0.95 | 0.38 | 0.92 | 0.92 | 0.19 | \*0.01 |
| **anti-gD/HER2 @ 10 mg/kg vs. anti-HER2/HER2 @ 5 mg/kg** | 0.41 | 0.94 | 0.49 | 0.98 | 0.56 | 0.47 | 0.07 |
|  | **7d** | | | | | | |
|  | **Blood** | **Heart** | **Liver** | **Kidney** | **Spleen** | **Muscle** | **Tumor** |
| **anti-gD/HER2 @ 1 mg/kg vs. anti-gD/HER2 @ 10 mg/kg** | 0.01 | 0.46 | 0.88 | 0.34 | 0.49 | 0.39 | 0.73 |
| **anti-HER2/HER2 @ 0.5 mg/kg vs. anti-HER2/HER2 @ 5 mg/kg** | 0.32 | 0.15 | 0.16 | 0.17 | 0.15 | 0.18 | 0.80 |
| **anti-gD/HER2 @ 1 mg/kg vs. anti-HER2/HER2 @ 0.5 mg/kg** | 0.06 | 0.08 | 0.43 | 0.13 | 0.95 | 0.30 | 0.69 |
| **anti-gD/HER2 @ 10 mg/kg vs. anti-HER2/HER2 @ 5 mg/kg** | 0.49 | 0.51 | 0.34 | 0.35 | 0.22 | 0.86 | 0.66 |

**\***Statistically significant (p < 0.05)

**Supplemental Table 2: Intact antibody tissue distribution statistics (p-values).** SCID.bg mice bearing KPL-4 tumors in mammary fat pads were injected with a single IV bolus of 125I- and 111In-labeled monovalent HER2 binding (anti-gD/HER2, 1 or 10 mg/kg), bivalent HER2 binding (anti-HER2/HER2, 0.5 or 5 mg/kg) or non-binding control (anti-gD/gD, 5 mg/kg) antibodies. Tissue enrichment of antibodies is expressed as %ID/g. Intact antibodies were determined from the measurement of 125I. The amount of catabolized antibody (open bars) is calculated as 111In-labeled antibody %ID/g minus 125I-labeled antibody %ID/g (Intact + Catabolized - Intact). Intact antibody distribution and catabolism were measured at 6 h, 1 day and 7 days post dose. Catabolized antibody was measured in tumor over the course of the study. p-values were obtained by Student’s t-test for each group (n=4). All significant p values were reported as \*, P < 0.05.

**Supplemental Table 3: Fitted parameters and model discrimination results for base model and variants.**

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**Supplemental Table 3: Fitted parameters and model discrimination results for base model and variants.** A global optimization algorithm (22) was used to estimate model parameters and the Akaike Information Criteria (21) is calculated as *AIC = -2\*LogLikelihood + 2Np*, where Np is the number of fitted parameters. Note that estimates of *C*1, *C*2, and *C*3 are unreliable and likely numerically unidentifiable when simultaneously fitted to data (Variant 7); numerical precision of each estimated parameter can be found in the Supplemental file ‘fitted\_parameters\_and\_stats.xlsx’. Additional details regarding the model and parameter optimization methodology can be found in Supplemental files ‘in\_vitro\_simbiology\_model.sbproj’, ‘in\_vitro\_data\_for\_simbiology.csv’ and ‘modelEquations.html’.

**Supplemental Figure 1: Impact of HER2 targeting antibody valency on systemic exposure in KPL-4 tumor bearing mice**

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**Supplemental Figure 1: Impact of HER2 targeting antibody valency on systemic exposure in KPL-4 tumor bearing mice.** SCID.bg mice bearing KPL-4 tumors in mammary fat pads were injected with a single IV bolus of 125I- and 111In-labeled antibodies with monovalent HER2 affinity (anti-gD/HER2, 1 or 10 mg/kg), bivalent HER2 affinity (anti-HER2/HER2, 0.5 or 5 mg/kg) or non-binding control (anti-gD/gD, 5 mg/kg). Sparse blood samples were collected 0 to 7 days. Whole blood concentrations of **(A)** 125I radiolabeled and **(B)** 111In radiolabeled antibodies are represented as %ID/mL. Exposures (AUC ± SEM) were calculated using GraphPad Prism and significance was determined using a Student’s t-test. Whole blood concentrations of **(C)** 125I radiolabeled and **(D)** 111In radiolabeled antibodies are represented as µg Antibody/mL. Plasma concentrations of **(E)** 125I radiolabeled and **(F)** 111In radiolabeled antibodies are represented as µg Antibody/mL.

**Supplemental Figure 2: HER2 targeting antibody valency impact on tissue distribution and uptake in KPL-4 tumor bearing mice**

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**Supplemental Figure 2: HER2 targeting antibody valency impact on tissue distribution and uptake in KPL-4 tumor bearing mice.** SCID.bg mice bearing KPL-4 tumors in mammary fat pads were injected with a single IV bolus of 125I- and 111In-labeled antibodies with monovalent HER2 affinity (anti-gD/HER2, 1 or 10 mg/kg), bivalent HER2 affinity (anti-HER2/HER2, 0.5 or 5 mg/kg) or non-binding control (anti-gD/gD, 5 mg/kg). Tissue enrichment of antibodies are expressed as %ID/g. Intact antibodies (solid bars) were determined from the measurement of 125I, while the amount of catabolized antibody (open bars) is 111In-labeled antibody %ID/g minus 125I-labeled antibody %ID/g (Intact + Catabolized – Intact). Intact antibody distribution and catabolism were measured at **(A)** 6 h, **(B)** 1 day and **(C)** 7 days post dose. Catabolized antibody was measured in tumor over the course of the study and expressed as µg Antibody/g Tumor **(D)**.

**Supplemental Figure 3: Whole body radioactivity in SPECT-CT imaged KPL4 tumor bearing mice.**

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**Supplemental Figure 3: Whole body radioactivity in SPECT-CT imaged KPL4 tumor bearing mice.** SCID.bg mice bearing KPL-4 tumors in mammary fat pads received 111In-labeled antibodies, representing distribution of both intact and catabolized antibodies. Mice dosed with 5 mg/kg anti-gD/gD, 10 mg/kg anti-gD/HER2 and anti-HER2/HER2 received a single IV bolus of ~700 µCi of 111In-labeled antibody and imaged at 6 h, day 1, 2 and 7 using SPECT-CT.

**Supplemental Figure 4. Observed *in vivo* antibody kinetics and comparison of HER2 concentrations, KD’s and doses.**

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**Supplemental Figure 4. Observed *in vivo* antibody kinetics and comparison of HER2 concentrations, KD’s and doses.** Monovalent anti-gD/HER2 mAb uptake **(A)**, bivalent anti-HER2/HER2 mAb uptake **(B)**, nonbinding anti-gD/gD mAb uptake **(C)**, and comparison of projected HER2 concentrations over the study time course with compartmental model based *KD*’s and initial mAb concentrations in media **(D).**

**Supplemental Figure 5. Model structure with equations.**

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**Supplemental Figure 5. Model structure with equations.** Model equations are shown, where units of all compartments are nanomole and units of all mass transport equations are nanomole/hour. The model is *simultaneously* fitted to all *in vitro* data, where *C*1, *C*2 and *C*3 (Eqns 4, 5 and 11) are fixed to 1 when fitting the model to data describing monovalent mAb kinetics and are either fixed to 1 or estimated (depending on the model variant) when fitting the model to data from bivalent mAb studies. See the full model equations and model data file in the Supplementary Material for details. Parameteters *C*1, *C*2 and *C*3 facilitate exploration of various hypothesis regarding increased rates of bivalent mAb/HER2 complex internalization, degradation and recycling. Note that the reaction volume (*V*) for binding equations is fixed to 1 mL. Parameter definitions can be found in Table 1 of the main article.