Antibody Co-Administration Can Improve Systemic and Local Distribution of Antibody Drug Conjugates to Increase In Vivo Efficacy

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**Supplementary Information**

Supplementary Figures – Fig S1-S6

Supplementary Methods – Deparaffinization and antigen retrieval

Krogh Cylinder Tumor Model

* Differential Equations
* Model Parameters

Supplementary Discussion

* Selection of ADC cellular internalization rate

Supplementary Figures – Fig S8-S9

* Tumor growth curves statistical analysis and mouse body weight

Supplementary References

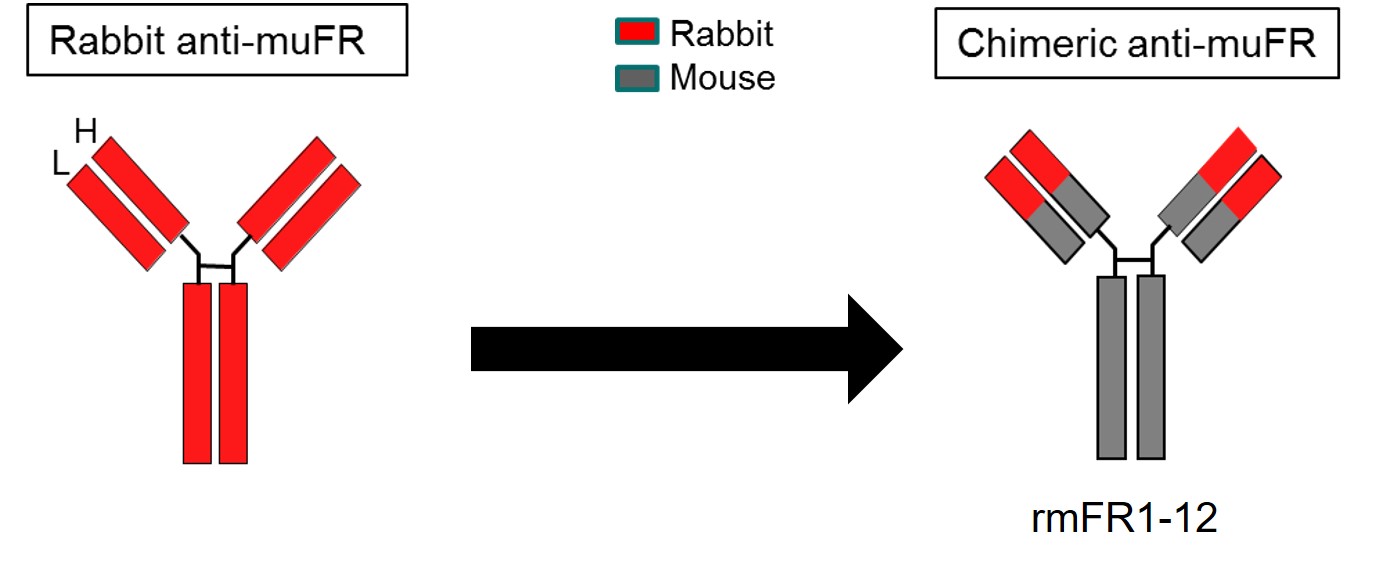
**Supplementary Figures**

A close up of a map

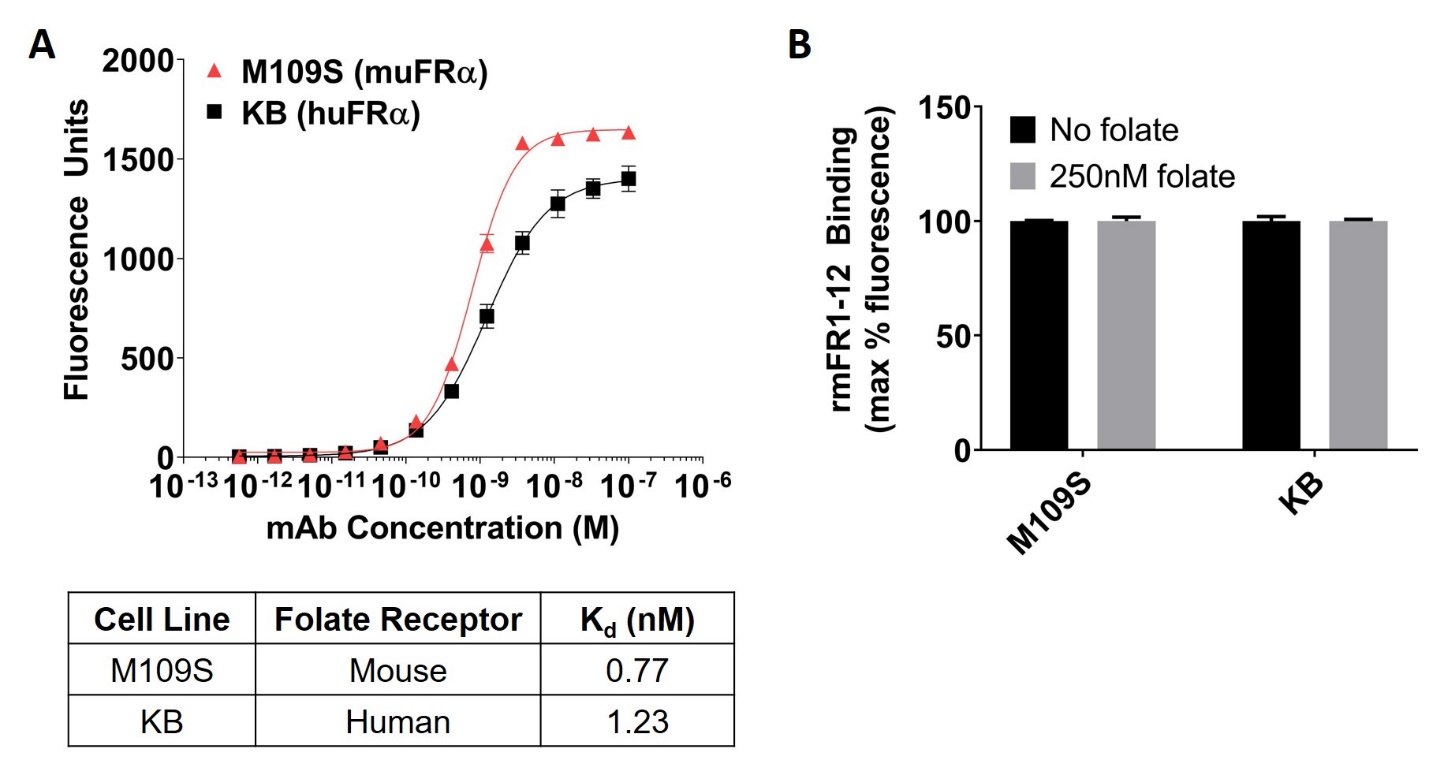
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**Fig. S1**. **Impact of receptor expression and payload potency on toxicity of cross-reactive FRα antibody-drug conjugate**. In vitro viability/toxicity assays of the cross-reactive ADC with the ‘high’ (DGN549) and ‘low’ (DM4) potency payloads in ‘high’ (KB) and ‘low’ (OV90) FRα expression systems. DGN549-ADC shows higher potency in both systems compared to DM4-ADC. Additionally, both payloads showed higher toxicity in the higher expression system (KB), indicating greater overall cellular ADC uptake due to lack of transport limitations in an *in vitro* system. The addition of an antibody carrier dose to both DGN549-ADC and DM4-ADC antagonizes ADC uptake due to competition from the excess antibody for surface receptors, resulting in 10-20-fold increase in IC50.

For DGN549, *in vitro* calculations indicate that the fraction of bound ADCs required for 50% cell death is 3-fold higher in KB cells than OV90 cells, suggesting that OV90 may be more sensitive (assuming the internalization rate is the same). This is relevant for the simulations in Fig 2B and 2D, where the absolute ADC concentration in OV90 appears to be lower than KB, despite an overall better efficacy in OV90.

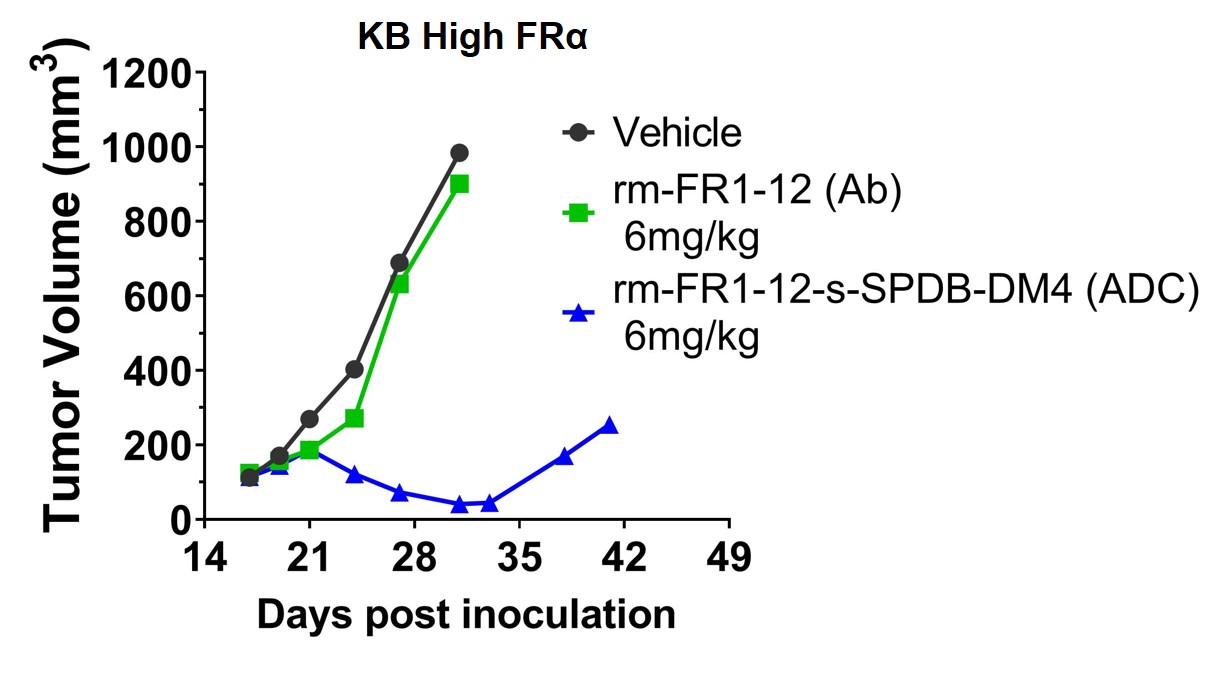


**Fig. S2.** **Generation of chimera cross-reactive antibody rmFR1-12 that recognizes both human and murine FRα**.

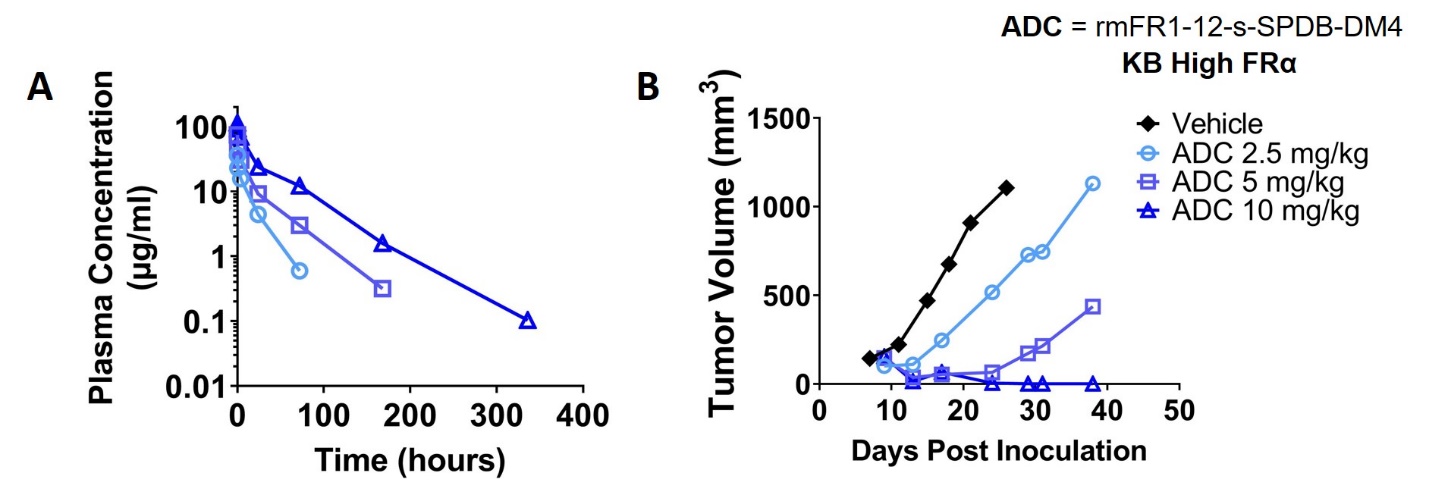


**Fig. S3. Binding of cross-reactive antibody rmFR1-12 to human and murine FRα.**

(A) In vitro assays with the cross-reactive antibody showed comparable binding affinity with both mouse FRα (M109S cell line) and human FRα (KB cell line). Binding affinity of non-cross-reactive antibody in KB cells is 0.08nM(1) Importantly, while this difference in affinity is expected to impact the in vitro IC50, the difference in affinity has a negligible impact on total tumor uptake and distribution in this range according to computational simulations and experimental measurements(2) (B) The cross-reactive antibody exhibited equivalent binding to both mouse and human FRα in the presence of a supraphysiological concentration of folate, indicating binding does not significantly compete for the same FRα epitope as folate.



**Fig. S4**. **Unconjugated rmFR1-12 antibody without cytotoxic payload shows no efficacy in KB (high FR∝) tumors when administered alone (singe dose).**



**Fig. S5.** **Dose-dependent pharmacokinetics and efficacy of cross-reactive rmFR1-12 antibody-drug conjugate**. Administration of increasing doses of ADC in KB (high FRα) tumor-bearing mice results in (A) improved pharmacokinetics indicated by reduced TMDD-mediated clearance and (B) better efficacy (single dose).

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**Fig. S6. Immunofluorescence tumor imaging to assess ADC distribution heterogeneity.** KB (high FRα) xenograft tumors harvested 48 hours post-administration from SCID mice injected with (A) *saline*, (B) *2.5mg/kg AF488-Ab*, (C) *2.5mg/kg AF488-Ab + 10mg/kg Ab*, or (D) *2.5mg/kg AF488-Ab + 25mg/kg Ab* were preserved via FFPE sectioned (5µm) and processed for immunofluorescence imaging. In addition to AF488 signal from the bound/internalized fluorescent antibody (*red*), secondary staining using AlexaFluor647 chicken anti-mouse antibody (*green*) was performed. The secondary antibody binds both fluorescent and non-fluorescent antibody, indicated by the dramatic difference in distribution and intensity between panel (B) and (C)/(D). The lack of complete overlap between red and green images might be due to incomplete epitope unmasking (which limits *ex-vivo* binding of secondary antibody) and/or AF488 signal from degraded ADC (undetectable by *ex-vivo* secondary antibody). *Ab = cross-reactive rmFR1-12 antibody*

*red - AF488 (bound & internalized fluorescent antibody), green – AlexaFluor647-chicken anti-mouse secondary (all surface antibody), gray – AlexaFluor555-CD31 (blood vessels), blue – Hoechst33342 (nucleus)*.

**Supplementary Methods**

**Tissue section deparaffinization and antigen retrieval**

Deparaffinization of tumor sections was performed by washing in the following solvents - histology-grade xylene (2x for 3 min each), followed by 50/50 xylene:100% histology-grade ethanol (3 min), followed by 100% ethanol (2x for 3 min each). The sections were then rehydrated by successive washes in 95%, 70%, and 50% ethanol (3 min each), followed by rinsing in distilled water for 5 minutes. Slides were prevented from drying out between each step to minimize non-specific immunofluorescence staining. Slides were stored in the dark in cold distilled water until antigen retrieval, which was performed using citrate buffer. Briefly, slides were submerged in 10mM anhydrous citric acid containing 0.5% Tween-20, adjusted to a pH of 6.0 using 1N NaOH. Slides were heated in a microwave until boiling point (95-100oC), then allowed to cool at room temperature for 45 minutes. Slides were washed using PBS and stored in the dark in cold distilled water until imaging.

**Krogh Cylinder Model(3)**

**Krogh Cylinder Model Equations**

1. **Free unconjugated antibody (Cmab)**
2. **Free ADC (CADC)**
3. **Free Receptor (Tfree)**
4. **Bound mAb (Bmab)**
5. **Bound ADC (BADC)**
6. **Internalized mAb (Cint,mab)**
7. **Internalized ADC (Cint, ADC)**

**Boundary Conditions**

1.

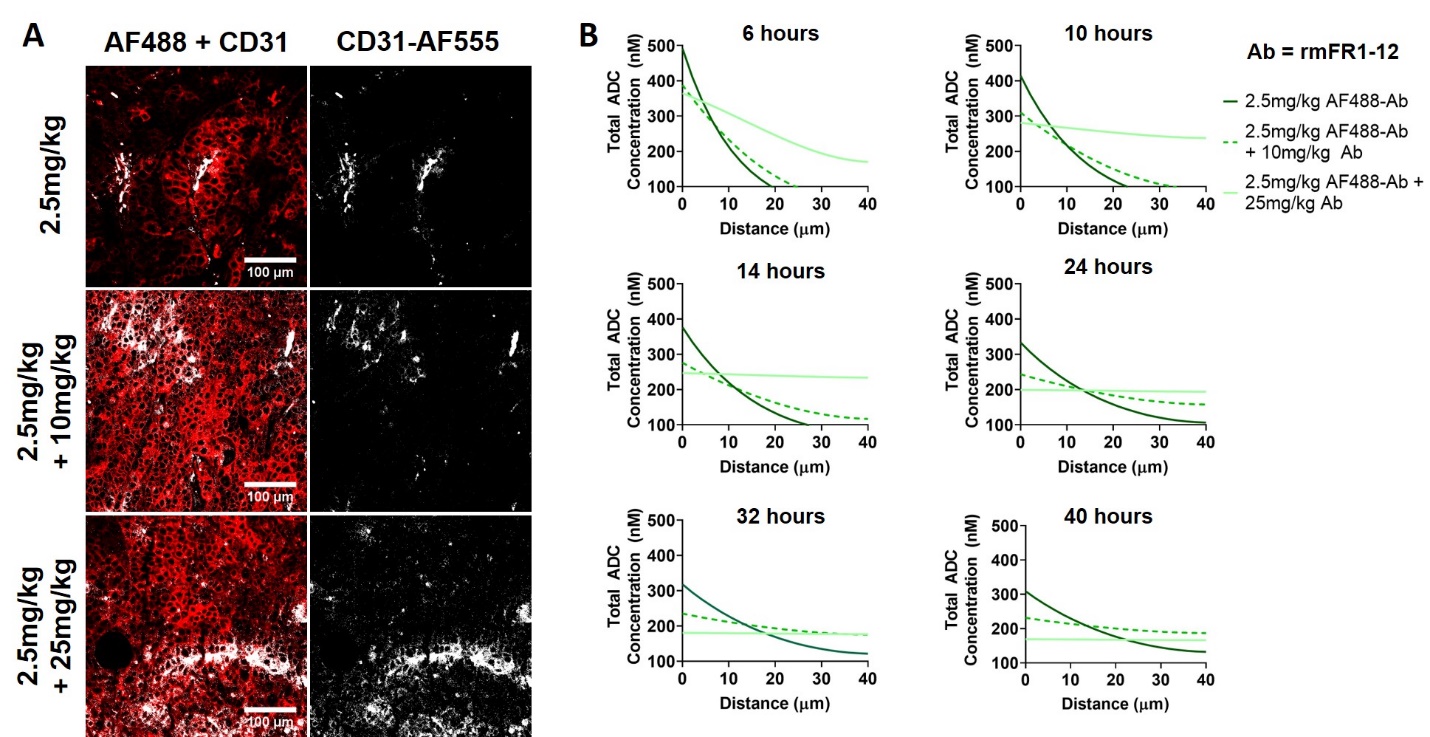
2.

3.

**Table S1. Parameters**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Parameter** | **Value** | **Unit** | **Description** | **Reference** |
| RKrogh | 40 | μm | Krogh cylinder (tumor) radius | Estimated for high vascularity (S/V=100/cm) |
| RCapillary | 8 | μm | Capillary radius | (4) |
| A | 0.6 | ND | Fraction of alpha clearance | Plasma clearance biexponential decay fit |
| kα, M | 0.0533 | hr-1 | Alpha phase clearance rate of Mirvetuximab |
| kα, rmFR1-12 | 3.466 | hr-1 | Alpha phase clearance rate of cross reactive rmFR1-12 |
| kβ, M | 0.0033 | hr-1 | Beta phase clearance rate of Mirvetuximab |
| kβ, rmFR1-12,2.5 | 0.063 | hr-1 | Beta phase clearance rate of 2.5mg/kg rmFR1-12 |
| kβ, rmFR1-12,2.5+10 | 0.0533 | hr-1 | Beta phase clearance rate of 2.5mg/kg rmFR1-12 ADC co-dosed with 10mg/kg rmFR1-12 Ab |
| kβ, rmFR1-12,2.5+25 | 0.0247 | hr-1 | Beta phase clearance rate of 2.5mg/kg rmFR1-12 ADC co-dosed with 25mg/kg rmFR1-12 Ab |
| DADC | 10 | μm2/s | Antibody/ADC diffusivity | (5) |
| PADC | 3 x10-9 | m/s | Vascular permeability of antibody/ADC | (6) |
| Q | 0.0015 | mL/g/s | Blood flow rate to tumor | (7) |
| H | 0.45 | ND | Hematocrit | (8) |
| ε | 0.24 | ND | Tumor void fraction | (9) |
| [Ag]0 | 1.2495 | μM | Initial antigen concentration  KB (High FRα) | (10) |
| [Ag]0 | 0.0333 | μM | Initial antigen concentration  OV90 (Low FRα) |  |
| kon, Ab | 1x105 | M-1s-1 | Antibody/ADC binding rate | Estimated |
| koff, Ab | 8x10−6 | s-1 | Antibody/ADC dissociation rate | Calculated |
| Kd | 0.08 | nM | Antibody/ADC dissociation constant | (1) |
| ke | 0.6x10−5 | s-1 | Antibody/ADC internalization rate | Estimated, see supplementary discussion |
| Rs | ke\*[Ag0] | μM/s | Antigen recycle rate | - |
| kdeg | 8.0x10−6 | s-1 | ADC lysosomal degradation rate | Assumed half-life 24 hours(10,11) |

**Selection of ADC cellular internalization rate**



**Fig. S7**. Distribution analysis of cross-reactive rmFR1-12 to select cellular internalization rate. (A) Immunofluorescence histology analysis shows differential distribution of 2.5mg/kg cross-reactive antibody rmFR1-12 (*red, AlexaFluor488*) without and with carrier dosing (10mg/kg and 25mg/kg) relative to blood vessels (*gray, CD31-AlexaFluor555*). (B) Krogh cylinder simulations showing radial distribution of bound and internalized fluorescent antibody with and without carrier dosing using literature reported range of FRα internalization rates*. Simulation run time is 48 hours to correspond to tumors in (A) harvested 48 hours post-injection*.

**Internalization Rate**

Distribution heterogeneity of ADCs is a result of the ‘binding site-barrier’ effect where antibody-binding to receptors outcompetes intratumoral antibody diffusion, resulting in concentration of the drug in perivascular cells. A less appreciated but equally critical factor that contributes to this heterogeneity is the rate of cellular internalization of the antibody-receptor complex, which irreversibly traps the drug in cells. Therefore, ADC cellular internalization rate is an important parameter required to predict the intratumoral distribution of M-s-SPDB-DM4 in Fig. 2. However, literature analysis reveals a wide range of reported folate receptor internalization rate, from 6-40 hours(12) with antibodies (versus folate ligands) typically on the order of 24 to 40 hrs. In order to identify the most representative internalization rate for the system reported here, we performed a qualitative comparison between the observed distribution of 2.5mg/kg AlexaFluor488-rmFR1-12 antibody co-dosed with and without a carrier, non-fluorescent dose of rmFR1-12 (10mg/kg, 25mg/kg) and predictive simulations(3) differing in only internalization half-life ranging from 6-40 hours. Since rmFR1-12 is a cross-reactive antibody, we accounted for TMDD effects by estimating individual plasma clearance rates for each of the three dosing schemes As expected, the fraction of alpha clearance (A) and alpha phase clearance rate (kα) remained consistent across the dosing schemes, and only the beta phase clearance (kβ) changed, with 2.5mg/kg showing the fastest clearance due to TMDD and 2.5mg/kg + 25mg/kg with the slowest clearance due to saturation of receptor sinks in healthy tissue (Table S1.).

Immunofluorescence imaging shows the relative difference in tissue penetration (*red, AlexaFluor488*) for the three dosing schemes relative to blood vessels (*gray, CD31-AlexaFluor555*). Cross-reactive fluorescence antibody when administered by itself distributes heterogeneously, but when co-administered with unlabeled carrier dose (10mg/kg and 25mg/kg) exhibits more homogeneous distribution (Fig. S7A). Using this as the “expected” simulation outcome for total cellular ADC (surface bound and internalized), various internalization rates were tested in the model (Fig. S7B). For the simulations with 6 hours internalization half-life, both 2.5mg/kg and 2.5+10mg/kg doses show highly perivascular distribution of the total cellular ADC (AUC ~ 6000), while 2.5+25mg/kg shows homogeneous distribution with improved total ADC binding and uptake (AUC~10,000). This does not match the observed behavior of the ADC in the histology images and suggests that a predicted internalization half-life of 6 hours is too fast. Iterative simulations helped converge to an internalization half-life of 30-40 hours, which showed relatively heterogeneous distribution of the 2.5mg/kg only dose, and similarly homogeneous distribution of the carrier-dosed tumors, similar that observed in the histology images. Additionally, in this internalization rate range, area under curve (AUC) analysis shows increased ADC binding and uptake of ADC going from 2.5mg/kg to 2.5+10mg/kg dosing scheme, but a subsequent decrease for the 2.5+25mg/kg dosing scheme, representative of the efficacy trends observed in Fig. 6A. Therefore, we selected 32 hours as the predicted internalization half-life of the ADC and performed subsequent simulations for maximum ADC internalization in KB and OV90 tumors (Fig. 2B & 2D) using this value.

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**Fig. S8**. Mean and standard deviation of tumor volumes, statistical analysis (unpaired t-test), and corresponding weight changes for DM4 ADC treated mice. (*ns = not significant, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001*)

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**Fig. S9**. Mean and standard deviation of tumor volumes , statistical analysis, and corresponding weight changes for DGN549 ADC treated mice. (*ns = not significant, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001*)

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