

Reducing hydrophobicity of homogeneous antibody-drug conjugates improves pharmacokinetics and therapeutic index

Robert P Lyon¹, Tim D Bovee¹, Svetlana O Doronina¹, Patrick J Burke¹, Joshua H Hunter¹, Haley D Neff-LaFord², Mechthild Jonas³, Martha E Anderson⁴, Jocelyn R Setter¹ & Peter D Senter¹

The *in vitro* potency of antibody-drug conjugates (ADCs) increases with the drug-to-antibody ratio (DAR); however, ADC plasma clearance also increases with DAR, reducing exposure and *in vivo* efficacy. Here we show that accelerated clearance arises from ADC hydrophobicity, which can be modulated through drug-linker design. We exemplify this using hydrophilic auristatin drug linkers and PEGylated ADCs that yield uniform, high-DAR ADCs with superior *in vivo* performance.

Much of the recent success of ADCs in the clinic stems from advancements in drug-linker technologies and an improved understanding of the role of pharmacokinetics in targeted drug delivery¹. From this work, the DAR has emerged as a key attribute, owing to its impact on ADC biodistribution and pharmacokinetics^{2,3}. ADCs with higher DAR values exhibit greater potency *in vitro* but are often inferior *in vivo* owing to the faster plasma clearance of highly loaded antibodies². Most investigators have therefore limited the average DAR to a range of 2–4, conjugating to native cysteine or lysine residues through methods that produce heterogeneous drug-loading distributions in which the biological activities of each component within the mixture are variable^{2,4,5}. Antibody engineering approaches that address this heterogeneity have recently been described^{6–9}, but the DARs of such ADCs have been limited to 2 or 4. Homogeneous ADCs with a DAR of 8 can be readily generated using the native cysteine residues of a human IgG1, but this approach, with a conventional drug-linker, results in accelerated plasma clearance². We sought an understanding of the underlying cause of accelerated ADC clearance to aid the design of homogeneous ADCs with improved therapeutic profiles.

To understand the mechanism behind accelerated clearance, we first studied an ADC with a DAR of 8 (also referred to as an 8-loaded ADC) prepared with the anti-CD70 antibody h1F6 and monomethyl auristatin

F (MMAF) using the protease cleavable valine-citrulline-*p*-aminobenzyl-carbamate (val-cit-PABC) linker (**1**) (**Fig. 1a**). This ADC was prepared by reduction with excess *tris*-carboxyethylphosphine (TCEP), resulting in highly homogeneous drug loading (**Supplementary Fig. 1**). In a pharmacokinetic study, clearance was much faster for the h1F6-1 ADC than for unconjugated h1F6. (**Fig. 1b**). In an immunohistochemistry (IHC)-based biodistribution study, the livers of rats dosed with h1F6-1 displayed diffuse staining of the entire hepatic sinusoidal endothelium coupled with intense, punctate staining of Kupffer cells, whereas minimal staining was observed in animals dosed with unconjugated h1F6 (**Supplementary Fig. 2a**). This pattern suggests selective uptake by these nonparenchymal cell populations of the liver, contributing to the accelerated clearance. A kinetic study of this distribution pattern revealed the rapidity of hepatic ADC uptake (**Supplementary Fig. 2b**).

One hypothesis for this accelerated clearance is that the reduction of the interchain disulfides to generate free thiols for conjugation resulted in destabilization of the IgG structure. However, there was no pharmacokinetic impact when h1F6 was fully reduced and conjugated with *N*-(2-aminoethyl)-maleimide (**Supplementary Fig. 3**), suggesting that accelerated clearance did not result from the absence of the disulfides but from the nature of the drug linker. The val-cit-PABC motif adds considerable hydrophobicity to drug linkers, evidenced by hydrophobic interaction chromatography (HIC)². ADCs with higher DARs have longer HIC retention times, reflecting greater hydrophobic character. An h1F6 ADC prepared with the noncleavable drug linker mcMMAF (**2**), which lacks the val-cit-PABC motif, eluted earlier from the HIC column than h1F6-1, consistent with diminished hydrophobicity (**Fig. 1c**). Notably, h1F6-2 also exhibited slower plasma clearance than h1F6-1 (**Fig. 1b**), leading us to postulate that ADC pharmacokinetics are correlated with hydrophobicity. To evaluate this, we designed a hydrophilic drug linker (compound **3**) in which the phenylalanine of MMAF was replaced with threonine and linked directly to a hydrophilic cleavable peptide, eliminating the need for the val-cit-PABC motif. Unlike the h1F6 conjugates with **1** and **2**, h1F6-3 exhibited pharmacokinetics similar to that of unmodified antibody (**Fig. 1b**), which is consistent with their HIC profiles (**Fig. 1c**). These experiments demonstrate the relationship between ADC hydrophobicity and plasma clearance, and they illustrate that hydrophilic drug-linker designs allow for high DAR values without inducing a pharmacokinetic penalty.

In vivo experiments were undertaken to determine if the correlative relationship between hydrophobicity and pharmacokinetics also affected antitumor activity. For hydrophobic h1F6-1, activity was greater at DAR 4 than at DAR 8 at the same antibody dose (**Fig. 1d**), and these observations are similar to previous reports². The opposite effect was observed for the hydrophilic h1F6-3, with the activity improving substantially as the DAR was increased from 4 to 8. These results illustrate the benefit of high drug loading on ADC

¹Department of Chemistry, Seattle Genetics, Bothell, Washington, USA. ²Department of Pharmacology and Toxicology, Seattle Genetics, Bothell, Washington, USA. ³Department of Translational Research, Seattle Genetics, Bothell, Washington, USA. ⁴Department of Preclinical Research, Seattle Genetics, Bothell, Washington, USA. Correspondence should be addressed to R.P.L. (rlyon@seagen.com).

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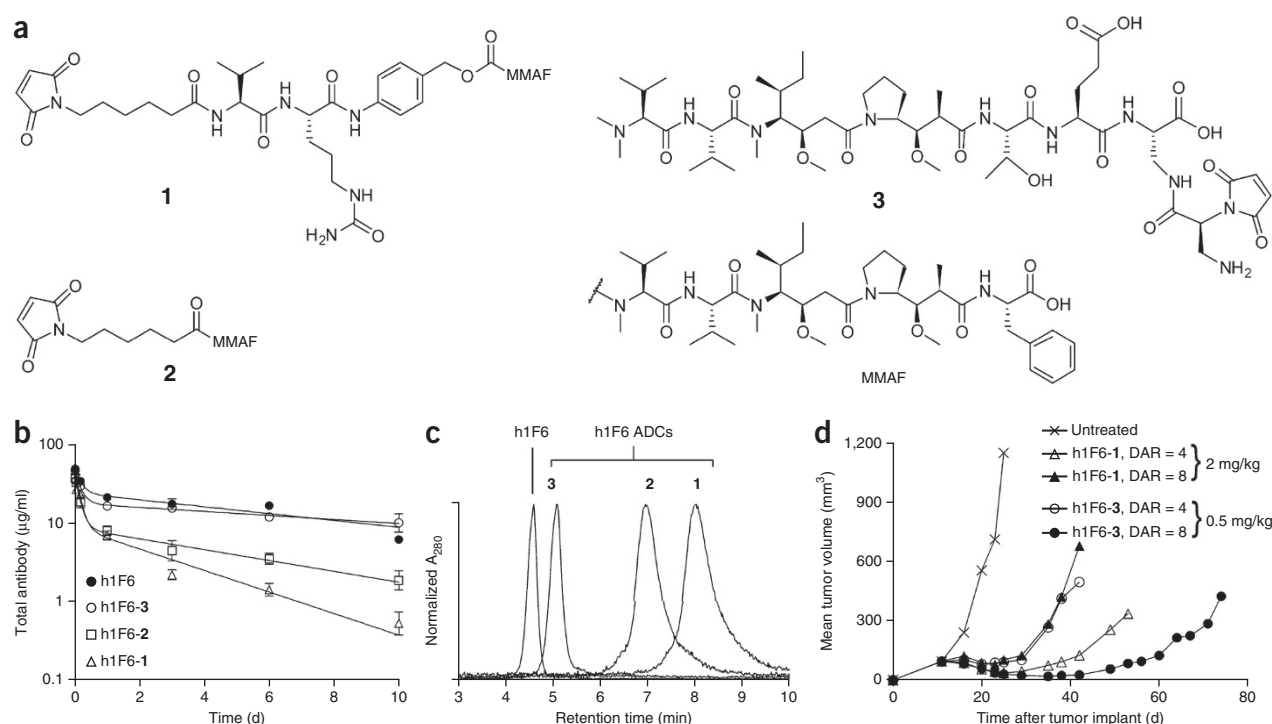


Figure 1 Structures of drug linkers and characterization of ADCs. (a) Structures of drug linkers 1–3. (b) Plasma pharmacokinetics of h1F6 ADCs (prepared with 1–3 at a DAR of 8) and unmodified h1F6 antibody in mice (mean $\pm$ s.d.; $n = 3$ animals per time point per group). (c) Characterization h1F6 antibody and ADCs by hydrophobic interaction chromatography. (d) Effect of DAR on *in vivo* activity of h1F6-1 (dosed at 2 mg/kg) and h1F6-3 (dosed at 0.5 mg/kg) in a model of renal cell carcinoma (CD70⁺ 786-O tumor fragments implanted subcutaneously in nude mice; single dose; data are mean of $n = 6$ animals per group).

activity, provided that hydrophobicity is appropriately managed. The dominant roles that hydrophobicity and pharmacokinetics played in the activities of the ADCs *in vivo* are evident from the >4-fold increased activity of h1F6-3 over h1F6-1, despite the fact that h1F6-1 was slightly more potent *in vitro* (Supplementary Fig. 4a). Similar results were observed when drug linkers 1–3 were conjugated to the anti-CD30 antibody cAC10 (Supplementary Fig. 4b).

The results with 3 demonstrate that improved pharmacokinetics for homogeneous DAR 8 ADCs can be attained by minimizing hydrophobicity in the drug-linker. However, most drugs require hydrophobic moieties to retain their potency. As an alternative to engineering hydrophobicity out of a payload drug, we therefore sought a method to mask its inherent hydrophobicity with polyethylene glycol (PEG). PEG has been incorporated into several ADC constructs^{10–13}, generally to improve drug-linker solubility or reduce ADC aggregation. However, the impact of PEGylation on ADC pharmacokinetics has not been reported, nor has any assessment of the optimal PEG configuration. Typically, the PEG is incorporated as a spacer between the drug and the point of antibody attachment^{11–13}, which effectively places the drug on the end of a PEG tether. We postulated that this tethered configuration could in fact have detrimental effects on pharmacokinetics, as it may project the drug moiety into the aqueous environment and thereby increase its potential for nonspecific hydrophobic interactions. As an alternative, we sought a design that constrains the drug to remain close to the antibody and allows the PEG to better shield it from solvent.

To evaluate this, we used monomethyl auristatin E (MMAE) as an exemplary hydrophobic drug, paired with the previously reported glucuronide cleavable linker¹⁴ (4) (Fig. 2a). We prepared two new analogs, each using a discrete PEG₂₄ linear oligomer but differing in configuration. Compound 5 used PEG₂₄ as a tether between the antibody attachment and the cleavable linker, and 6 incorporated a branching point within

the linker to which the linear PEG₂₄ was attached. DAR 8 ADCs were prepared with each of these drug linkers and the anti-CD30 antibody cAC10, and we confirmed that PEGylation of the drug linkers had negligible impact on antigen binding of the ADCs when conjugated to the native cysteine residues (Supplementary Fig. 5). The tethered conjugate cAC10-5 eluted later on a HIC column than did the non-PEGylated cAC10-4, and the branched conjugate cAC10-6 eluted earlier (Fig. 2b). These results confirmed that PEG configuration affected apparent hydrophobicity and that tethering the drug increased overall ADC hydrophobic character. ADC pharmacokinetics paralleled the trends in overall hydrophobicity, with faster clearance of cAC10-5 and slower clearance of cAC10-6 compared to the non-PEGylated cAC10-4 (Fig. 2c). Hydrophobicity and plasma clearance also correlated with the hepatic uptake of these ADCs as assessed by IHC (Supplementary Fig. 6).

In vivo studies again demonstrated an inverse correlation between apparent hydrophobicity and antitumor activity. In a xenograft model of Hodgkin's lymphoma (CD30⁺ L540cy cells implanted subcutaneously in SCID mice), a single dose of ADC at 1 mg per kilogram of body weight (mg/kg) was completely curative with the branched linker cAC10-6 but produced only temporary and incomplete tumor regressions with non-PEGylated cAC10-4 (Fig. 2d). Similar plasma stability profiles for these two ADCs indicate that the improvement in activity is not due to enhanced stability (Supplementary Fig. 7). A single-dose tolerability study in Sprague-Dawley rats revealed a maximum tolerated dose of 10 mg/kg for both of these ADCs, suggesting an improved therapeutic index for the hydrophilic ADC on the basis of its greater antitumor activity. In contrast to the improved activity observed with the branched linker, cAC10-5 with its PEG-tethering configuration produced only modest growth delay, even at 2 mg/kg (Fig. 2d). The differences in the activities of these three ADCs correlated with

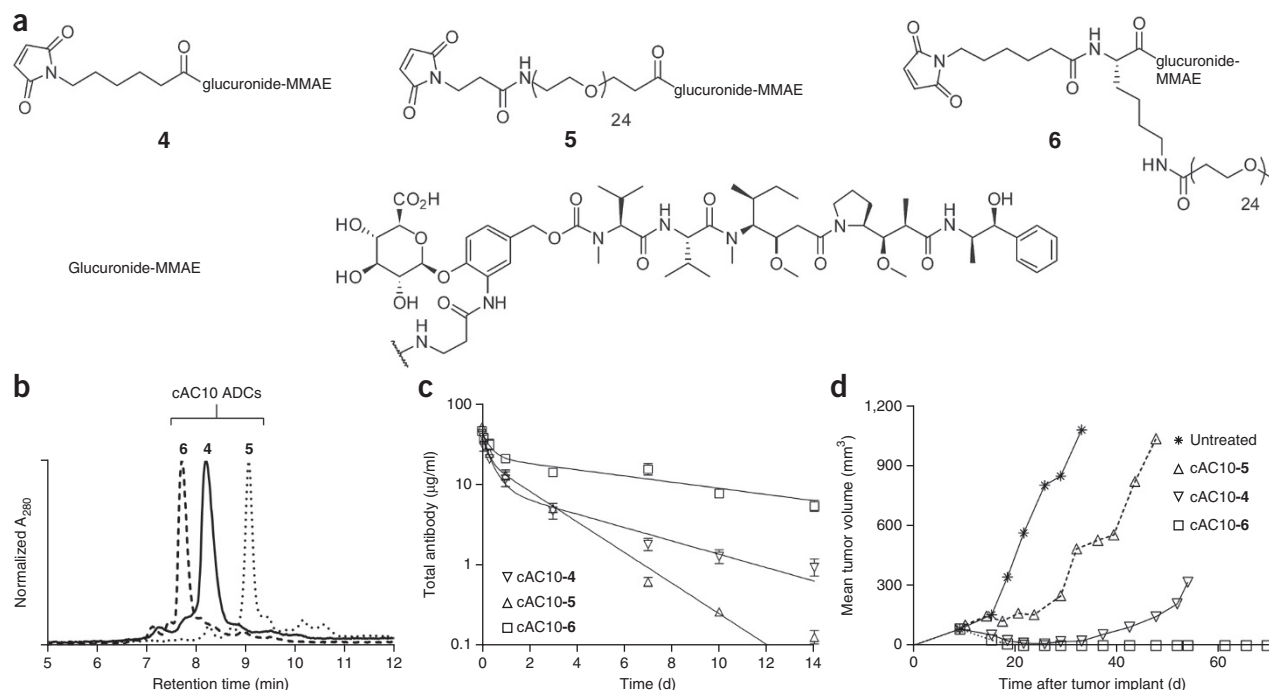


Figure 2 Structures of glucuronide-MMAE drug linkers and characterization of ADCs. **(a)** Structures of drug linkers **4–6** (top) and glucuronide-MMAE (bottom). **(b)** Characterization of DAR 8 cAC10 ADCs by hydrophobic interaction chromatography. PEGylation of drug-linker **4** increased (**5**) or decreased (**6**) apparent ADC hydrophobicity, depending upon the configuration of the PEG. **(c)** Plasma pharmacokinetics in rats of cAC10 ADCs prepared with compounds **4–6** at a DAR of 8 (data are mean $\pm$ s.d.; $n = 3$ animals per time point per group). **(d)** Antitumor activity of DAR 8 cAC10 ADCs prepared with compounds **4–6** in the Hodgkin's lymphoma model L540cy (data are means; $n = 6$ animals per group) at a single dose of 1 mg/kg (cAC10-4 and -6) or 2 mg/kg (cAC10-5).

intratumoral MMAE concentrations 3 d after dose (**Supplementary Fig. 8**), indicating that the efficiency of drug delivery was increased by appropriate PEG masking. It is noteworthy that PEG in the tethered format resulted in faster clearance and reduced antitumor activity, as this configuration has been widely employed in ADC applications^{11–13}. The advantages of the optimal linker design were further apparent when evaluating the *in vivo* activity of cAC10-6 as a function of DAR. In a xenograft model of anaplastic large-cell lymphoma (CD30⁺ Karpas-299 cells implanted subcutaneously in SCID mice), the DAR 8 conjugate outperformed a DAR 4 ADC, consistent with the finding that minimization of apparent hydrophobicity allows higher levels of drug loading and enhanced ADC potency (**Supplementary Fig. 9**).

We have demonstrated that it is possible to make homogeneous 8-loaded ADCs without impairing antibody pharmacokinetics through the use of drug linkers designed to minimize hydrophobicity. Such constructs are more uniform in composition and have improved *in vivo* potencies over lower DAR ADCs with a distribution of drug-loading levels. The finding that control of hydrophobicity can be used to manage pharmacokinetics should allow the rapid optimization of ADCs incorporating diverse drug payloads. The technology described here represents a means for the preparation of homogeneous ADCs with optimal pharmacokinetics and antitumor activities.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.P.L. designed drug linkers, prepared and characterized ADCs by HIC, designed *in vivo* studies, performed the pharmacokinetic data fitting and wrote the manuscript; T.D.B. synthesized drug linkers; S.O.D. designed and synthesized drug linkers, P.J.B. designed and synthesized PEGylated drug linkers; J.H.H. performed radiolabeling and quantification for pharmacokinetic studies; H.D.N.-L. designed and interpreted the rat toxicology studies; M.J. performed IHC analysis of ADC uptake by rat liver; M.E.A. performed *in vivo* work (activity, pharmacokinetics and biodistribution); J.R.S. performed mass spectrometry assays to characterize ADC drug loading and stability, and to quantify MMAE delivery *in vivo*; P.D.S. directed the synthetic chemistry and co-wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

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ONLINE METHODS

Materials. *N*-ethyl maleimide and *N*-aminoethyl maleimide (TFA salt) were purchased from Sigma-Aldrich. Chimeric anti-CD30 antibody cAC10 and humanized anti-CD70 antibody h1F6 were generated as described previously^{15,16}. Authenticated and mycoplasma-free cell lines L540cy (ACC 197) and Karpas-299 (ACC 31) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), and 786-O was obtained from ATCC. Authenticity of cell lines was confirmed using the Cell Check 16 panel (IDEXX Bioresearch). Maleimidocaproyl-Val-Cit-PAB-MMAF (1), maleimidocaproyl-MMAF (2), and maleimidocaproyl-glucuronide-MMAE (4) were prepared as described previously^{14,17}. Mice (CB-17 SCID, Nude, and Balb/C strains) were purchased from Harlan Laboratories. Hsd:Sprague Dawley rats were purchased from Harlan Laboratories. All animal handling and experimentation were performed under Seattle Genetics IACUC guidelines. Seattle Genetics is an AAALAC-accredited organization.

Preparation of conjugates. ADCs were prepared by reduction of antibody interchain disulfides followed by addition of a 25% excess of maleimide, as described previously⁴. The DAR of ADCs generated by this method is dictated by the extent of disulfide reduction. Full reduction to 8 thiols per antibody was carried out with excess TCEP, which was removed by passage over a PD-10 size-exclusion column (GE Healthcare) before addition of maleimide to produce DAR 8 conjugates. DAR 4 conjugates were prepared by partial reduction with 2.2 equivalents of TCEP, then conjugation to achieve an average of 4 drugs per antibody. All ADCs were purified by previously described methods⁴.

Characterization of ADCs by hydrophobic interaction chromatography was carried out as previously described⁴. Briefly, the analysis was performed with a Butyl-NPR column, 4.6 × 35 mm, 2.5 μm particle size (Tosoh Bioscience) with a linear gradient of 100% mobile phase A (1.5 M (NH₄)₂SO₄ in 25 mM potassium phosphate) to 100% mobile phase B (25 mM potassium phosphate, pH 7.0 + 25% isopropanol) in 12 min.

Antigen binding of cAC10 drug conjugates. Competition binding of the anti-CD30 IgG1, cAC10 and IgG1-drug conjugates was undertaken using CD30⁺ L540cy cells and cAC10 labeled with Alexa Fluor 647 (Molecular Probes, Invitrogen) at 1 nM. Samples were analyzed using a LSRII flow cytometer (BD Bioscience) and mean fluorescence intensity data were converted using Winlist 3D v8.0 (Verity Software House). Data were baseline corrected using GraphPad Prism v6.04 and reported as the percentage of maximum fluorescence as calculated by the sample fluorescence divided by the fluorescence of cells stained with cAC10-Alexa Fluor 647 alone.

Xenograft studies. L540cy Hodgkin's lymphoma and Karpas-299 anaplastic large cell lymphoma cells were implanted subcutaneously in female SCID mice (age 4–6 weeks). 786-O renal carcinoma tumor fragments were implanted subcutaneously in female nude mice (age 4–6 weeks). In all models, ADCs were dosed once intraperitoneally (i.p.) when tumors had grown to approximately 100 mm³. Six mice were dosed with ADC or left untreated; animals were assigned to dose groups to minimize differences in initial tumor volumes between groups. Investigators were not blinded to the dosing groups.

Pharmacokinetics. Pharmacokinetics experiments were performed using h1F6 or cAC10 antibody radiolabeled with *N*-succinimidyl propionate [propionate-2,3-³H] (Moravek Biochemicals, Cat. No.: MT 919).

Radiolabeled antibodies or ADCs were injected at 3 mg/kg in female Balb/C mice (4–6 weeks old) or Harlan Sprague-Dawley rats (7–8 weeks old) via the

tail vein (three animals per dose group, randomly assigned). Blood was drawn into K₂ EDTA tubes via the saphenous vein at various time points and processed to plasma. Plasma samples were added to Ecoscint-A liquid scintillation cocktail (National Diagnostics), and the total radioactivity was measured by liquid scintillation counting. The resulting disintegrations-per-minute values were converted to μCi, and the specific activity of the radiolabeled test articles was used to calculate the concentration of antibody at each time point.

Tolerability studies. DAR 8 ADCs with drug linkers 4 and 6 were dosed intravenously in 12- to 14-week-old female Harlan Sprague-Dawley rats at 3, 10 and 30 mg/kg (six rats per dose group, randomly assigned). These dosages were selected on the basis of prior experience with MMAE ADCs. The MTD of both ADCs was 10 mg/kg. Investigators were not blinded to the dosing groups.

Immunohistochemistry of rat liver. Female Harlan Sprague-Dawley rats (7–8 weeks old) were dosed intravenously (IV) with antibody or ADC at 6 mg/kg via tail vein. At 1 h after dose, each animal was euthanized by CO₂. A midline incision was made, a 22G butterfly catheter was placed in the portal vein, and 30 ml PBS with 10 IU/ml heparin (Hospira) was slowly infused. The descending vena cava was incised to allow blood and perfused solution to leave the body. Liver lobes were dissected and small sections of tissue were placed in formalin and processed for IHC. For the time course study, male Harlan Sprague-Dawley rats (400–450 gm; 14–16 weeks) were dosed i.v. with h1F6-1 via tail vein. At the appropriate time point each animal was anesthetized and maintained with isoflurane gas (Isoflurane Piramal Health Corp, Andhra Pradesh, India). A midline incision was made and the portal vein isolated with a single suture loop. A 22G indwelling catheter was placed and a small amount of PBS with 10 IU/ml heparin was flushed to validate placement. The suture was secured around the catheter and 60 ml of heparinized PBS slowly infused. The descending vena cava was incised to allow blood and perfused solution to leave the body. Liver lobes were dissected and small sections of tissue are placed in 10% neutral buffered formalin for 48 h and processed for paraffin embedding.

Paraffin sections were cut at 5-μm thickness. Dewaxing, target retrieval and immunostaining were performed on the Bond-Max or BondIII autostainer (Leica Microsystems). Sections were pretreated with target retrieval buffer EDTA (pH9) (Leica, #AR9640) for 20 min at 100 °C. For IHC labeling the Bond Intense R Detection Kit (Leica, #DS9263) was used. Endogenous biotin was blocked with an avidin-biotin blocking kit (Vector Laboratories). Sections were labeled with Biotin-SP-conjugated AffiniPure F(ab')₂ Fragment Goat anti-human IgG Fcγ fragment-specific (Jackson ImmunoResearch Lab #109-066-098) at 5 μg/ml for 45 min or with Biotin-SP-conjugated ChromPure Goat IgG (Jackson ImmunoResearch Lab #005-060-003) as isotype control also at 5 μg/ml. Endogenous peroxidase was blocked with PeroxAbolish Block (Biocare #PXA969) for 15 min. Biotinylated antibody was labeled with streptavidin-HRP labeling for 12 min and detected with diaminobenzidine (DAB) for 6 min. Nuclei were counterstained with hematoxylin.

Synthesis of drug linkers. Synthesis of drug linkers is described in **Supplementary Note**.

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