Supplementary Materials and Methods

Flow cytometry and immunohistology

Briefly, cells were detached from the flasks using cell dissociation buffer (Invitrogen). They were washed and suspended in PBS-1%BSA at 2×10⁶ cells/mL. Permeabilized cells were fixed with 4% paraformaldehyde for 20 minutes followed by 20-min incubation in 0.1% Triton-X (Fisher Scientific). Cells were washed each time with cold PBS to remove the treatment. Live and fixed cells were incubated with the primary antibodies (final concentration 10 µg/mL) diluted in ice cold PBS-1%BSA for 1 h on ice. Binding was revealed with the FITC-conjugated goat anti-human Fc-specific secondary antibody (Jackson Immunoresearch) for 1 h at 4 °C in the dark. After washing, samples were then processed using a FACSCalibur flow cytometer (BD Biosciences). The median fluorescence intensity (MFI) determined from a histogram of 10,000 gated events using FlowJo software (version 7.6.3; Tree Star, Inc., Ashland, OR).

Drug derivatives

Syntheses of CL2A-SN-38 (1), CL2E-SN-38 (2), and activated doxorubicin (3), were performed as described previously. LC-MS analyses were obtained on Agilent 6210 Time-of-Flight mass spectrometer and Agilent 1200 series LC, using electrospray positive ion mode of detecting molecular ion (as M+H) and associated fragment ions; Agilent XDB-C18 column (1.8 µm, 4.6 × 50 mm) and linear gradient elution of 75% ‘A’/25% ‘B’ changing to 10% A/90% B in 8 min at a flow rate of 0.9 mL/min. Solvent ‘A’ was 5% acetonitrile in water/0.01% formic acid, and ‘B’ was 90% acetonitrile in water/0.01% formic acid.

Drug release assays for SN-38 derivatives

In order to assess the stability of SN-38 to the CL2A and CL2E linkers, we first capped the maleimide group used to couple the linker to an IgG with mercaptoethanol (ME) (Sigma-Aldrich). Briefly, 8-9 mg of the maleimide-CL2A- or CL2E-SN-38 were dissolved in
dichloromethane and treated with 0.1 mL of ME. After 30 min, the reaction mixture was washed with saturated sodium chloride to remove excess reagent. The crude product was purified on a small column of silica gel (230-400 mesh) and eluted with 0-2% methanol-dichloromethane gradient; recovery was 50-60%. LC-MS analysis showed the correct mass for the product, as doubly charged ion at m/z 779.8568; ME-CL2E-SN-38: M+H at m/z 1821.9456. The samples were incubated in acetate buffer, pH 5.0, containing cathepsin B (Sigma-Aldrich) (4). Briefly, 1 mg of cathepsin B from bovine spleen (Sigma-Aldrich) was dissolved in 1 mL of 25 mM sodium acetate/1 mM EDTA, pH 5.0. A portion of this solution (0.1 mL) was activated with 0.2 mL of 30 mM DTT/15 mM EDTA for 15 min at ambient room temperature. The activated cathepsin B (0.045 mL) was diluted first with 5 mL of the pH 5 buffer, and then 2.018 mL of this dilution was added to 0.04 mL of the DMSO solution of ME-capped substrate (10 mg/mL). The mixture was incubated at 37 °C, and 0.1-mL aliquots were removed and analyzed by reversed-phase HPLC (RP-HPLC) over 46 h. Control incubations included the pH 5.0 buffer without cathepsin B mixed with the same amount of the test substrate.

**Determination of SN-38 release from CL2A- and CL2E-SN-38 conjugates in vitro**

Freshly reconstituted CL2A- or CL2E-IgG conjugates (10 mg/mL; 0.006 mL) were added to 0.2 mL of serum, and placed in a 37 °C incubator (5% CO₂). Aliquots from triplicate incubates were withdrawn periodically, mixed with a known amount of 10-hydroxycamptothecin (internal standard), and treated with acetonitrile to precipitate protein. Free SN-38 and internal standard were extracted and analyzed by RP-HPLC with in-line fluorescence detection. From the plot of SN-38/10-OH-CPT peak ratios as a function of time (generated by Prism GraphPad software) and equation for one-phase exponential association, with plots ascending to Y max with a rate constant K, half-lives were calculated as 0.69/K. *In vitro* stabilities of milatuzumab-doxorubicin in human serum and in pH 5 buffer are described in supplemental material.

**In vitro stability of hydrazone linker at lysosomally-relevant pH (pH 5.0) and in human serum at 37 °C.**

The stability of the milatuzumab-doxorubicin conjugate in serum and lysosomally relevant acidic pH 5 at 37 °C was determined by measuring the appearance of free doxorubicin over time by RP-HPLC on a Supelcosil C18 column with fluorescence detection (excitation 495 nm, emission 550 nm). Samples of the conjugate in triplicate were incubated in serum or in pH 5.0 buffer to simulate acidic conditions in the lysosome at 37 °C, and aliquots were analyzed by RP-HPLC, measuring doxorubicin and doxorubicin metabolites, and correlating the build-up of doxorubicin fluorescence over time. The rationale for this approach was that the covalently-bound doxorubicin conjugate had no fluorescence due to fluorescence quenching, but the fluorescence was restored when the drug dissociated from the conjugate. In both experiments, standard curves were established by spiking increasing amounts of free doxorubicin into either buffer alone or in serum to ascertain the efficiency of detecting doxorubicin and its metabolites.
in these settings. The data were plotted with Prism GraphPad software and fit to an equation for one-phase exponential association, with plots ascending to $Y_{\text{max}}$ at a rate constant of $K$, and with half-lives calculated as $0.69/K$. As indicated in Supplementary Fig. S3, doxorubicin is released from the milatuzumab-doxorubicin conjugate with a half-life of 98 h when incubated in serum at 37 °C, and 7-8 h when incubated at pH 5.0/37 °C. Two other hydrazine-linked antibody conjugates also were prepared and evaluated, each showing a half-life in serum of ~4 days.

References Cited


