**Supplementary Information**

**Enhanced Delivery of SN-38 to Human Tumor Xenografts with an Anti-Trop-2-SN-38 Antibody Conjugate (Sacituzumab Govitecan)**

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**SUPPLEMENTARY INFORMATION**

**METHODS**

*Establishment of Capan-1 and NCI-N87 xenografts in Nude Mice.*

NCI-N87 gastric tumor xenografts were established by harvesting cells from tissue culture with each mouse receiving a total of 1x107 cells in a 0.2 mL s.c. injection in the right flank. Capan-1 xenografts were established by making a tumor suspension from stock tumors and mixing with cells harvested from tissue culture. A 0.3 mL injection, containing 20% w/v tumor suspension plus 1x107 cells, was injected s.c. into the flank.

*HPLC Analysis.*

HPLC analysis was performed using a Waters Alliance 2695 HPLC system. Samples (20 µL) were loaded into an auto injector and set to run over 15 min, using a Waters 2475 in-line fluorescent detector (373 nm excitation, 540 nm detection). The area under the curve (AUC) for each peak was quantitated using Waters Empower software. Prior to each set of runs, a series of samples, representing the buffer alone to set baseline and extracts of the tissue of interest taken from mice that were not previously injected with either product (tissue blanks), were run to ascertain if any peaks co-migrated at the elution time for any of the 4 products of interest (10-HCPT, SN-38, SN-38G, and irinotecan). The final product AUC was derived by subtracting the AUC for any co-migrating peaks in the tissue blanks. For product quantitation, the AUC for the product peaks were normalized against the 10-HCPT internal standard by taking the ratio of product AUC to the 10-HCPT AUC. Standard curves were established using standards for SN-38, SN-38G, and irinotecan. The log-transformed ratio of the AUCs was plotted against the log of the standard concentration, with linear correlation coefficients for each product exceeding 0.99 over the range of 10 to 10,000 ng/mL.

 Sera from mice were assayed also by ELISA for hRS7 IgG using an anti-idiotype monoclonal antibody (designated WU) to capture the antibody on a microtiter plate, competing with the hRS7 IgG conjugated to horseradish peroxidase. This assay has a sensitivity of 1 µg/mL. A second ELISA was used to estimate the amount of IMMU-132 in the serum. This assay uses an anti-SN-38 antibody developed by Immunomedics, Inc., designated SG2, to capture IMMU-132, and then probe for binding with the anti-hRS7 idiotype antibody conjugated with horseradish peroxidase. The sensitivity of this assay is 10 ng/mL.

*In Vitro Stability Testing in Mouse Serum*

IMMU-132 (22.5 µL), freshly reconstituted with water to a concentration of 10 mg/mL, was added to 0.75 mL of pooled mouse serum and filtered through a 0.2 µ filter into a sterile vial. Aliquots (0.2 mL) were placed in 3 wells in a 96-well plate, with the rest of the wells filled with 0.2 mL of sterile PBS, and incubated at 37 C in a 5% CO2 incubator. At 4, 24, 46.5, 72, and 100.5 h, 20-µL aliquots were withdrawn and mixed with 10 µL of 2 µg/mL DMSO solution of 10-HCPT and 20 µL of the protein precipitating mixture. The mixture was vortexed and centrifuged. The clear supernatant solution was transferred to HPLC vials, and fixed volumes of the extracts were analyzed by reversed-phase HPLC, determining SN-38 by fluorescence detection of the HPLC peak. A plot of free SN-38 build-up kinetics was generated, using an equation for one-phase exponential association, starting at zero and ascending to Ymax with a rate constant K; half-life was then calculated as 0.69/K using standard Prism software.

*In Vitro Glucuronidation Assay*

An *in vitro* glucuronidation assay was performed to determine the susceptibility of SN-38 bound to IMMU-132 to be glucuronidated prior to its release. Briefly, SN-38 and IMMU-132 stock solutions prepared in dimethyl sulfoxide (DMSO) or buffer were examined separately and added to the reaction mixture at a final concentration of 500 ng/mL (1.27 µM) based on SN-38 content. In addition to SN-38 and IMMU-132, the total reaction mixture contained the following reagents at their final concentrations: uridine diphosphate glucuronic acid (5 mM) and MgCl2 (10 mM), both purchased from Sigma (St. Louis, MO), and alamethicin (25 µg/mL; Alfa Aesar, Ward Hill, MA), with the final volume adjusted to 0.2 mL with the addition of 99 µL of 0.1 M Tris). The reaction was started with the addition of UGT1A1 supersomes (Corning Life Sciences, Tweksbury, MA), and allowed to proceed for 60 min at 37oC. At the end of this incubation period, 50 µL of the reaction mixture was placed in 0.1 mL of normal human serum, which was added to facilitate precipitation by precipitating reagent, along with 10 µL of the internal standard, 10-HCPT (final SN-38 concentration in each sample was 425.3nM). After the addition of the precipitating reagent (0.16 mL), the clarified supernatant was analyzed for SN-38 and SN-38G from duplicate HPLC runs. A separate 50-µL sample from the reaction mixture containing the IMMU-132 sample was also placed in 0.1 mL of normal human serum with 10-HCPT, and this sample was acid-hydrolyzed overnight at 50 oC, neutralized and then extracted with the precipitating reagent. The acid-hydrolyzed sample assessed the amount of SN-38 [TOTAL] and SN-38G [TOTAL], whereas the former non-hydrolyzed sample measured free levels of SN-38 and SN-38G in the IMMU-132 sample, as well as in the SN-38 sample.

**RESULTS**

*Quantitative HPLC Methodological Assessments*.

Prior to initiating these studies, various aspects of the procedures used for the analysis of tissue samples were assessed to determine their suitability for measuring the products of interest. The core extraction and analysis procedure was based on those reported by Hirose et al. (1), but with certain modifications. For example, the elution gradient was modified, adding more acetonitrile to the second run buffer (i.e., 60:40 in place of 70:30 water/acetonitrile). This gradient system allowed for better separation of the 4 products of interest, particularly irinotecan (**Fig. S1A)**. Secondly, the extraction procedure by Hirose et al. (1) utilized 0.45 mL of serum with 0.15 mL of the protein precipitation reagent; however, since mouse serum was more limited in volume, and since we wanted to maximize sensitivity by minimizing the dilution of the mouse serum, we tested extraction efficiency using a smaller serum volume (0.15 mL) spiked with SN-38 and the 10-HCPT internal standard, varying the volume of the precipitation reagent (0.05 to 0.2 mL). Optimal recovery (i.e., >90%) occurred with ≥ 0.15 mL of the precipitation reagent. Therefore, our standard procedure utilized an equal volume of the sample and precipitation reagent, with the minimum sample volume of 0.15 mL. In order to have sufficient sample for at least 2 determinations without excessive dilution, an equal part of water was added to the mouse serum samples (2:1 dilution).

Tissue samples, including tumor, liver, and intestinal contents, required homogenization prior to extraction. In order to have sufficient volume for homogenization, water was added to the tissues (1 part tissue + 10 parts water; 11:1 vol/wt dilution). After homogenization, an equal amount of the precipitation reagent was added to a portion of the homogenate prior to removing sediments. This was vortexed for 15 seconds, then centrifuged, with the clarified supernatant processed by HPLC for SN-38 quantitation. Recovery assessments were made by spiking tissue samples prior to homogenization with known amounts of SN-38 and 10-HCPT. **Table S1** lists the results for the recovery of SN-38 found in one study where a single sample of mouse serum or water homogenates prepared from mouse liver and feces were spiked with SN-38, SN-38G, or irinotecan at 3 different levels, each having triplicate HPLC assessments. The % coefficient of variation (CV) was <1%, highlighting the highly reproducible results from sequential HPLC runs of the same sample. The % difference between the expected and observed values showed higher differences in the liver and feces samples when lower amounts of product were present. Similar results were observed for SN-38G and irinotecan (not shown). Overall, these studies suggested that the homogenization and extraction procedure permitted acceptable recoveries of the products.

Standard curves were established for the quantitation of the products of interest by spiking human serum with increasing amounts of SN-38, SN-38G, and irinotecan, starting at concentrations of 10 ng/mL and increasing to 10,000 ng/mL. Each sample is then spiked with a fixed amount of 10-HCPT prior to the addition of the precipitating reagent. Standard curves were derived using log-transformed values for the concentration of each product and the ratio of the AUC for the product peak divided by the AUC of the 10-HCPT peak at each product concentration tested. **Figure S1B** illustrates a typical standard curve, in this case for SN-38. Any sample value below the minimal threshold of the standard curve was reported as not detectable, whereas when samples had a value greater than maximum threshold, the tissue homogenate samples were diluted further in water, spiked with 10-HCPT, and extracted. Initial studies to determine the accuracy and precision of the HPLC procedure included an evaluation of triplicate samples of human serum spiked with SN-38, SN-38G, and the internal standard (10-HCPT) analyzed with single HPLC runs or single sample preparations analyzed using duplicate HPLC runs. **Table S2** illustrates a set of data from triplicate SN-38/SN-38G samples (acid hydrolyzed and non-hydrolyzed; see below) analyzed by a single HPLC run, with most of the % CV for observed being < 2.0% of expected. A review of the AUC peak for the internal standard in 88 separate HPLC runs of extracted samples taken from serum, liver, or tumors found a 5.9% CV, indicating a high level of accuracy and precision in the preparation and measurement of the internal standard.

When the precipitating reagent was added to serum freshly spiked with IMMU-132, only trace amounts of SN-38 were detected. These trace amounts were equal to the amount of unbound SN-38 previously determined for the lot of IMMU-132 being used, which was typically <1% of the total SN-38. This was a clear indication that the SN-38 bound to the IgG was not released during the extraction process, but instead was precipitated with the other proteins. Therefore, a method was developed to liberate all SN-38 from the conjugate by first hydrolyzing the samples overnight at 50 oC in HCl. Acid-hydrolyzed samples of serum or tissue homogenates spiked with IMMU-132 yielded the expected amount of SN-38. Thus, in order to quantify SN-38 from IMMU-132-containing samples, 2 processes were used: an acid-hydrolyzed sample measured the total amount of SN-38 or SN-38G (i.e., bound + free, referred to as SN-38 [TOTAL] or SN-38G [TOTAL]), while a non-hydrolyzed sample measured the amount of free SN-38 or SN-38G. Each of these 2 samples was then subjected to extraction with the precipitation reagent. Samples derived from animals given irinotecan were not processed by acid hydrolysis, but subjected only to the extraction procedure. Triplicate samples of serum spiked with SN-38G that were first processed by acid hydrolysis and then extracted found only 3.0 ± 0.2% of the SN-38G was converted to SN-38, indicating there was a negligible impact on the determination of SN-38G in the acid-hydrolyzed samples.

 Overall, these studies provided sufficient evidence that the procedures developed for these assessments would yield reliable and reproducible results. Since serum samples were diluted 2:1 and tissues were homogenized at a 10:1 ratio (vol:wt; dilution factor 11:1), and with the standard curve minimal acceptable value of 10 ng/mL, the minimum sensitivity of detection in serum was 20 ng/mL and in tissue homogenates 110 ng/mL.

**Supplementary Reference**

1. Hirose K, Kozu C, Yamashita K, Maruo E, Kitamura M, Hasegawa J, et al. Correlation between plasma concentration ratios of SN-38 glucuronide and SN-38 and neutropenia induction in patients with colorectal cancer and wild-type UGT1A1 gene. Oncol Lett. 2012;3:694-8.

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| **Table S1. SN-38 and SN-38G standard curves using triplicate dilutions of each concentration with single HPLC run*a.*** |
| Expected | Non-hydrolyzed (Free) | Acid hydrolyzed (TOTAL) |
| SN-38 | SN-38G | SN-38 [TOTAL] | SN-38G {TOTAL] |
| Obs | SD | % CV | Obs | SD | % CV | Obs | SD | % CV | Obs | SD | % CV |
| 10 | 9.7 | 0.1 | 1.2 | 10.1 | 0.1 | 1.0 | 10.3 | 0.2 | 1.8 | 10.5 | 0.4 | 3.7 |
| 50 | 50.4 | 0.4 | 0.8 | 48.0 | 0.7 | 1.5 | 48.2 | 0.4 | 0.8 | 48.0 | 1.2 | 2.4 |
| 100 | 100.3 | 0.2 | 0.2 | 105.7 | 8.6 | 8.1 | 99.5 | 0.7 | 0.7 | 96.8 | 1.9 | 2.0 |
| 500 | 508.0 | 0.1 | 1.2 | 485.4 | 6.8 | 1.4 | 494.1 | 1.7 | 0.3 | 504.8 | 14.1 | 2.8 |
| 1000 | 989.7 | 1.4 | 0.1 | 981.2 | 5.0 | 0.5 | 990.2 | 10.7 | 1.1 | 1033.5 | 7.3 | 0.7 |
| 5000 | 4982.3 | 105.0 | 2.1 | 5021.8 | 114.0 | 2.3 | 5015.4 | 23.2 | 0.5 | 4972.3 | 38.2 | 0.8 |
| 10,000 | 9796.6 | 170.4 | 1.7 | 10,191.6 | 91.6 | 0.9 | 10,146.8 | 37.3 | 0.4 | 9927.1 | 92.9 | 0.9 |
| a Assessment of SN-38 and SN-38G concentrations (ng/mL) in non-hydrolyzed and acid hydrolyzed processed samples prepared in triplicated with each sample analyzed by a separate single HPLC run. Log-transformed ratios of the AUC for each product compared to the AUC for the internal 10H-CPT standard were derived, and plotted against the log-transformed product concentration, which was fit to a linear regression. Shown are the means (Obs) ± standard deviation (SD), with the percent coefficient of variation (% CV) derived for each triplicate value. |

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| **Table S2. SN-38 extraction efficiency*a.*** |
|  | Serum | Liver | Feces |
| Expected (ng/mL) | % Diff | % CV | % Diff | % CV | % Diff | % CV |
| 50 | 3.2 | 0.10 | 17.2 | 0.12 | 30.1 | 0.36 |
| 750 | 5.0 | 0.04 | 3.9 | 0.14 | 10.4 | 0.34 |
| 7500 | 8.2 | 0.06 | 8.8 | 0.05 | 13.9 | 0.85 |
| *a*Each tissue sample was spiked with different amounts of SN-38, diluted in water (serum) or homogenized in water (liver and feces), then extracted with precipitating media. Triplicate HPLC runs were used to determine the percent difference between the expected and measured concentrations (% Diff), with its associated % coefficient of variance (% CV). |

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| **Table S3. Area under the curve (AUC) analysis of various products in the serum of nude mice given irinotecan or IMMU-132.** |
|  | **AUC (µg/mL⋅h)** |
|  | **Irinotecan-treated** |  | **IMMU-132-treated** |
|  | **Irinotecan** | **SN-38** | **SN-38G** |  | **SN-38 [TOTAL]** | **SN-38 (Free)** | **SN-38G (Free)** |
| Study 1 (Capan-1) | 21.04 | 2.52 | 2.84 |  | 145.34 | 5.04 | ND |
| Study 3 (NCI-N87) | 24.51 | 3.21 | 3.00 |  | 171.72 | 3.88 | 1.39 |
| ND, not determined (<3 samples) |

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| **Table S4.** ***In vitro* glucuronidation assay*a.*** |
|  | Sample | SN-38 | SN-38G | % conversion |
| Non-hydrolyzed samples | SN-38 | 157.4 | 139.5 | 47.0 |
| IMMU-132 | 16.1 | 7.1 | 30.6 |
|  |
| Acid-hydrolyzed samples | IMMU-132 | 452.6 | 4.5 | 1.0 |
| ***a***All concentrations expressed in nM and are based on a volume of 150 µL (i.e., 50 µL sample + 100 µL serum). Expected concentration = 425.3 nM (assuming SN-38 = 392 Da; SN-38G = 550 Da). Control samples for both the non-hydrolyzed and acid hydrolyzed samples that did not include the UGT1A1 supersomes found no SN-38G (not shown). |

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| **Table S5. Analysis of products in the large intestine of animals given irinotecan or IMMU-132 (Study 2).** Values represent the mean ± SD (N = 3) and are expressed in µg*a.* |
| Administered agent | SN-38 | SN-38G | Irinotecan |
| 1 h | 6 h | 1 h | 6 h | 1 h | 6 h |
| Irinotecan | 0.723 ± 0.184 | 20.74 ± 3.72 | Not detected | 0.036.1 ± 0.002 | 22.45 ± 3.13 | 136.9 ± 40.76 |
| IMMU-132 | 0.195 ± 0.052 | 1.135 ± 0.302(0.663 ± 0.157) | 0.042 ± 0.012 | 0.036 ± 0.007(0.064 ± 0.017) | NA | NA |
| *a* Values in parentheses after the 6-h data for the IMMU-132 animals are the concentrations found in the 24-h feces samples NA, not applicable. |