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Humanized anti-Trop-2 IgG-SN-38 conjugate for effective treatment of diverse epithelial cancers: Preclinical studies in human cancer xenograft models and monkeys

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Abstract

Purpose: Evaluate the efficacy of an SN-38-anti-Trop-2 antibody-drug conjugate (ADC) against several human solid tumor types, and to assess its tolerability in mice and monkeys, the latter with tissue cross-reactivity to hRS7 similar to humans.

Experimental Design: Two SN-38 derivatives, CL2-SN-38 and CL2A-SN-38, were conjugated to the anti-Trop-2 humanized antibody, hRS7. The immunoconjugates were characterized *in vitro* for stability, binding, and cytotoxicity. Efficacy was tested in five different human solid tumor-xenograft models that expressed Trop-2 antigen. Toxicity was assessed in mice and in Cynomolgus monkeys.

Results: The hRS7 conjugates of the two SN-38 derivatives were equivalent in drug substitution (~6), cell-binding (Kd~1.2 nM), cytotoxicity (IC_{50}~2.2 nM), and serum stability *in vitro* ($t_{½}$~20 h). Exposure of cells to the ADC demonstrated signaling pathways leading to PARP cleavage, but differences *versus* free SN-38 in p53 and p21 up-regulation were noted. Significant anti-tumor effects were produced by hRS7-SN-38 at non-toxic doses in mice bearing Calu-3 ($P \leq 0.05$), Capan-1 ($P < 0.018$), BxPC-3 ($P < 0.005$), and COLO 205 tumors ($P < 0.033$) when compared to non-targeting control ADCs. Mice tolerated a dose of 2 × 12 mg/kg (SN-38 equivalents) with only short-lived elevations in ALT and AST liver enzyme levels. Cynomolgus monkeys infused with 2 × 0.96 mg/kg exhibited only transient decreases in blood counts, although, importantly, the values did not fall below normal ranges.

Conclusions: The anti-Trop-2 hRS7-CL2A-SN-38 ADC provides significant and specific anti-tumor effects against a range of human solid tumor types. It is well tolerated in monkeys, with tissue Trop-2 expression similar to humans, at clinically relevant doses, and warrants clinical investigation.

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Keywords

Antibody-drug conjugate; hRS7; SN-38; Trop-2; EGP-1; irinotecan

INTRODUCTION

Human trophoblast cell-surface antigen (Trop-2), also known as GA733–1 (gastric antigen 733–1), EGP-1 (epithelial glycoprotein-1), and TACSTD2 (tumor-associated calcium signal transducer), is expressed in a variety of human carcinomas and has prognostic significance in some, being associated with more aggressive disease (1–14). Studies of the functional role of Trop-2 in a mouse pancreatic cancer cell line transfected with murine Trop-2 revealed increased proliferation in low serum conditions, migration, and anchorage-independent growth *in vitro*, and enhanced growth rate with evidence of increased Ki-67 expression *in vivo* and a higher likelihood to metastasize (15).

Trop-2 antigen's distribution in many epithelial cancers makes it an attractive therapeutic target, but its normal tissue expression profile casts some doubt on Trop-2 as a suitable target (11, 14). Stein et al. (4) characterized an antibody, designated RS7–3G11 ("RS7"), that bound to EGP-1 (16, 17), which was present in a number of solid tumors, but the antigen was also expressed in some normal tissues, usually in a lower intensity, or in restricted regions. Targeting and therapeutic efficacies were documented in a number of human tumor xenografts using radiolabeled RS7(18–20), but this internalizing antibody did not show therapeutic activity in unconjugated form (18). However, *in vitro* it has demonstrated antibody-dependent cellular cytotoxicity (ADCC) activity against Trop-2 positive carcinomas (21).

We reported the preparation of antibody-drug conjugates (ADC) using an anti-CEACAM5 (CD66e) IgG coupled to several derivatives of SN-38, a topoisomerase-I inhibitor that is the active component of irinotecan, or CPT-11 (22, 23). The derivatives varied in their *in vitro* serum stability properties, and *in vivo* studies found one form (designated CL2) to be more effective in preventing or arresting the growth of human colonic and pancreatic cancer xenografts than other linkages with more or less stability. Importantly, these effects occurred at non-toxic doses, with initial testing failing to determine a dose-limiting toxicity (23). These results were encouraging, but also surprising, since the CEACAM5 antibody does not internalize, a property thought to be critical to the success of an ADC. We speculated that the therapeutic activity of the anti-CEACAM5-SN-38 conjugate might be related to the slow release of SN-38 within the tumor after the antibody localized. Since irinotecan performs best when cells are exposed during the S-phase of their growth cycle, a sustained release is expected to improve responses (24–26). Indeed, SN-38 coupled to non-targeting, plasma extending agents, such as polyethylene glycol (PEG) or micelles, has shown improved efficacy over irinotecan or SN-38 alone (27–33), lending additional support to this mechanism.

Given the RS7 antibody's broad reactivity with epithelial cancers and its internalization ability, we hypothesized that an RS7-SN-38 conjugate could benefit not only from the sustained release of the drug, but also from direct intracellular delivery. Therefore, we

prepared and tested the efficacy of SN-38 conjugates using a humanized version of the murine RS7 antibody (hRS7). A slight modification was made to the SN-38 derivative that was reported previously (22, 23), which improved the quality of the conjugate without altering its *in vitro* stability or its efficacy *in vivo*, and thus this new derivative (designated CL2A) is currently the preferred agent for SN-38 coupling to antibodies. Herein, we show the efficacy of the hRS7-SN38 conjugate in several epithelial cancer cell lines implanted in nude mice at non-toxic dosages, with other studies revealing that substantially higher doses could be tolerated. More importantly, toxicity studies in monkeys that also express Trop-2 in similar tissues as humans showed that hRS7-SN38 was tolerated at appreciably higher amounts than the therapeutically effective dose in mice, providing evidence that this conjugate is a promising agent for treating patients with a wide range of epithelial cancers.

MATERIALS & METHODS

Cell lines, antibodies, and chemotherapeutics

All human cancer cell lines used in this study were purchased from the American Type Culture Collection (Manassas, VA). These include Calu-3 (non-small cell lung carcinoma), SK-MES-1 (squamous cell lung carcinoma), COLO 205 (colonic adenocarcinoma), Capan-1 and BxPC-3 (pancreatic adenocarcinomas), and PC-3 (prostatic adenocarcinomas). Humanized RS7 IgG and control humanized anti-CD20 (hA20 IgG, veltuzumab) and anti-CD22 (hLL2 IgG, epratuzumab) antibodies were prepared at Immunomedics, Inc. Irinotecan (20 mg/mL) was obtained from Hospira, Inc. (Lake Forest, IL).

SN-38 immunoconjugates and *in vitro* aspects

Synthesis of CL2-SN-38 has been described previously (22). Its conjugation to hRS7 IgG and serum stability were performed as described (22, 23). Preparations of CL2A-SN-38 (M.W. 1480) and its hRS7 conjugate, and stability, binding and cytotoxicity studies, were conducted as described previously (22), and are presented in the Supplemental Data. Cell lysates were prepared and immunoblotting for $p21^{Waf1/Cip}$, p53, and PARP (poly-ADP-ribose polymerase) was done as described in Supplemental Data. Concentrations, timing, and primary antibodies are shown in the figure legends.

In vivo therapeutic studies

For all animal studies, the doses of SN-38 immunoconjugates and irinotecan are shown in SN-38 equivalents. Based on a mean SN-38/IgG substitution ratio of six, a dose of 500 μg ADC to a 20-gram mouse (25 mg/kg) contains 0.4 mg/kg of SN-38. Irinotecan doses are likewise shown as SN-38 equivalents (i.e., 40 mg irinotecan/kg is equivalent to 24 mg/kg of SN-38).

NCr female athymic nude (*nu*/*nu*) mice, 4–8 weeks old, and male Swiss-Webster mice, 10 weeks old, were purchased from Taconic Farms (Germantown, NY). All animal studies were approved by the Center for Molecular Medicine and Immunology's Institutional Animal Care and Use Committee (IACUC). Tolerability studies were performed in Cynomolgus monkeys (*Macaca fascicularis*; 2.5–4 kg male and female) by SNBL USA, Ltd. (Everett, WA) after approval by SNBL USA's IACUC.

Animals were implanted subcutaneously with different human cancer cell lines as described in the Supplemental Information. Tumor volume (TV) was determined by measurements in two dimensions using calipers, with volumes defined as: $L \times w^2/2$, where L is the longest dimension of the tumor and w the shortest. Tumors ranged in size between 0.10 to 0.47 cm^3 when therapy began. Treatment regimens, dosages, and number of animals in each experiment are described in the Results. The lyophilized hRS7-CL2A-SN-38 and control ADC were reconstituted and diluted as required in sterile saline. All reagents were administered intraperitoneally (0.1 mL), except irinotecan, which was administered intravenously. The dosing regimen was influenced by our prior investigations, where the ADC was given every 4 days or twice weekly for varying lengths of time (22, 23). This dosing frequency reflected a consideration of the conjugate's serum half-life *in vitro*, in order to allow a more continuous exposure to the ADC.

Statistics

Growth curves are shown as percent change in initial tumor volume over time. Statistical analysis of tumor growth was based on area under the curve (AUC). Profiles of individual tumor growth were obtained through linear-curve modeling. An *f*-test was employed to determine equality of variance between groups prior to statistical analysis of growth curves. A two-tailed *t*-test was used to assess statistical significance between the various treatment groups and controls, except for the saline control, where a one-tailed *t*-test was used (significance at $P \leq 0.05$). Statistical comparisons of AUC were performed only up to the time that the first animal within a group was euthanized due to progression.

Pharmacokinetics and biodistribution

^{111}In-radiolabeled hRS7-CL2A-SN-38 and hRS7 IgG were injected into nude mice bearing s.c. SK-MES-1 tumors (~0.3 cm^3). One group was injected intravenously with 20 μCi (250-μg protein) of ^{111}In-hRS7-CL2A-SN-38, while another group received 20 μCi (250-μg protein) of ^{111}In-hRS7 IgG. At various time-points mice (5 per time-point) were anesthetized, bled *via* intra-cardiac puncture, and then euthanized. Tumors and various tissues were removed, weighed, and counted by gamma scintillation to determine the percentage injected dose per gram tissue (% ID/g). A third group was injected with 250 μg of unlabeled hRS7-CL2A-SN-38 three days before the administration of ^{111}In-hRS7-CL2A-SN-38 and likewise necropsied. A two-tailed *t*-test was used to compare hRS7-CL2A-SN-38 and hRS7 IgG uptake after determining equality of variance using the *f*-test. Pharmacokinetic analysis on blood clearance was performed using WinNonLin software (Parsight Corp., Mountain View, CA).

Tolerability in Swiss-Webster mice and Cynomolgus monkeys

Details of these tolerability studies are given in the Supplemental Information. Briefly, mice were sorted into four groups each to receive 2-mL i.p. injections of either a sodium acetate buffer control or three different doses of hRS7-CL2A-SN-38 (4, 8, or 12 mg/kg of SN-38) on days 0 and 3 followed by blood and serum collection, as described in Results. Cynomolgus monkeys (3 male and 3 female; 2.5–4.0 kg) were administered two different doses of hRS7-CL2A-SN-38. Dosages, times, and number of monkeys bled for evaluation of possible hematologic toxicities and serum chemistries are described in the Results.

RESULTS

Stability and potency of hRS7-CL2A-SN38

Two different linkages were used to conjugate SN-38 to hRS7 IgG. The first is termed CL2-SN-38 and has been described previously (22, 23). A minor change was made to the synthesis of our CL2 linker in that the phenylalanine moiety was removed. (Fig. 1A). This change simplified the synthesis, but did not affect the conjugation outcome (e.g., both CL2-SN-38 and CL2A-SN-38 incorporated ~6 SN-38 per IgG molecule). Side-by-side comparisons found no significant differences in serum stability, antigen binding, or *in vitro* cytotoxicity.

To confirm that the change in the SN-38 linker from CL2 to CL2A did not impact *in vivo* potency, hRS7-CL2A- and hRS7-CL2-SN-38 were compared in mice bearing COLO 205 or Capan-1 tumors (Fig. 1B and 1C, respectively), using 0.4 mg or 0.2 mg/kg SN-38 twice weekly x 4 weeks, respectively, and with starting tumors of 0.25 cm^3 size in both studies. Both the hRS7-CL2A- and CL2-SN-38 conjugates significantly inhibited tumor growth compared to untreated (AUC_{14days} $P<0.002$ *vs.* saline in COLO 205 model; AUC_{21days} $P<0.001$ *vs.* saline in Capan-1 model), and a non-targeting anti-CD20 control ADC, hA20-CL2A-SN-38 (AUC_{14days} $P<0.003$ in COLO-205 model; AUC_{35days}: $P<0.002$ in Capan-1 model). At the end of the study (day 140) in the Capan-1 model, 50% of the mice treated with hRS7-CL2A-SN-38 and 40% of the hRS7-CL2-SN-38 mice were tumor-free, while only 20% of the hA20-ADC-treated animals had no visible sign of disease. Importantly, there were no differences in efficacy between the two specific conjugates in both the tumor models.

Mechanism of action

In vitro cytotoxicity studies demonstrated that hRS7-CL2A-SN-38 had IC_{50} values in the nM range against several different solid tumor lines (Table 1). The IC_{50} with free SN-38 was lower than the conjugate in all cell lines. While there was no correlation between Trop-2 expression and sensitivity to hRS7-CL2A-SN-38, the IC_{50} ratio of the ADC *vs.* free SN-38 was lower in the higher Trop-2-expressing cells, most likely reflecting the enhanced ability to internalize the drug when more antigen is present.

SN-38 is known to activate several signaling pathways in cells, leading to apoptosis (*34–37*). Our initial studies examined the expression of two proteins involved in early signaling events ($p21^{Waf1/Cip1}$ and p53) and one late apoptotic event (cleavage of poly-ADP-ribose polymerase (PARP)) *in vitro* (Fig. 2). In BxPC-3 (Fig. 2A), SN-38 led to a 20-fold increase in $p21^{Waf1/Cip1}$ expression, while hRS7-CL2A-SN-38 resulted in only a 10-fold increase, a finding consistent with the higher activity with free SN-38 in this cell line (Table 1). However, hRS7-CL2A-SN-38 increased $p21^{Waf1/Cip1}$ expression in Calu-3 more than 2-fold over free SN-38 (Fig. 2B).

A greater disparity between hRS7-CL2A-SN-38- and free SN-38-mediated signaling events was observed in p53 expression. In both BxPC-3 and Calu-3, up-regulation of p53 with free SN-38 was not evident until 48 h, while hRS7-CL2A-SN-38 up-regulated p53 within 24 h. Additionally, p53 expression in cells exposed to the ADC was higher in both cell lines

compared to SN-38. Interestingly, while hRS7 IgG had no appreciable effect on $p21^{Waf1/Cip1}$ expression, it did induce the up-regulation of p53 in both BxPC-3 and Calu-3, but only after a 48-h exposure. In terms of later apoptotic events, cleavage of PARP was evident in both cell lines when incubated with either SN-38 or the conjugate (Fig. 2C). The presence of the cleaved PARP was higher at 24 h in BxPC-3, which correlates with high expression of p21 and its lower IC_{50}. The higher degree of cleavage with free SN-38 over the ADC was consistent with the cytotoxicity findings.

Efficacy of hRS7-SN-38

Since Trop-2 is widely expressed in several human carcinomas, studies were performed in several different human cancer models, which started with an evaluation of the hRS7-CL2-SN-38 linkage, but later, conjugates with the CL2A-linkage were used. Calu-3-bearing nude mice given 0.04 mg SN-38/kg of the hRS7-CL2-SN-38 every 4 days x 4 had a significantly improved response compared to animals administered the equivalent amount of hLL2-CL2-SN-38 (TV=0.14 ± 0.22 cm^3 *vs.* 0.80 ± 0.91 cm^3, respectively; AUC_{42days} $P<0.026$) (Fig. 3A). A dose-response was observed when the dose was increased to 0.4 mg/kg SN-38. At this higher dose level, all mice given the specific hRS7 conjugate were ‘cured’ within 28 days, and remained tumor-free until the end of the study on day 147, while tumors re-grew in animals treated with the irrelevant ADC (specific *vs.* irrelevant AUC_{98days}: $P=0.05$). In mice receiving the mixture of hRS7 IgG and SN-38, tumors progressed >4.5-fold by day 56 (TV=1.10 ± 0.88 cm^3; AUC_{56days} $P<0.006$ *versus* hRS7-CL2-SN-38).

Efficacy also was examined in human colonic (COLO 205) and pancreatic (Capan-1) tumor xenografts. In COLO 205 tumor-bearing animals, (Fig. 3B), hRS7-CL2-SN-38 (0.4 mg/kg, q4dx8) prevented tumor growth over the 28-day treatment period with significantly smaller tumors compared to control anti-CD20 ADC (hA20-CL2-SN-38), or hRS7 IgG (TV=0.16 ± 0.09 cm^3, 1.19 ± 0.59 cm^3, and 1.77 ± 0.93 cm^3, respectively; AUC_{28days} $P<0.016$). The MTD of irinotecan (24 mg SN-38/kg, q2dx5) was as effective as hRS7-CL2-SN-38, since mouse serum can more efficiently convert irinotecan to SN-38 (38–41) than human serum, but the SN-38 dose in irinotecan (2400 μg cumulative) was 37.5-fold greater than with the conjugate (64 μg total). Animals bearing Capan-1 showed no significant response to irinotecan alone when given at an SN-38-dose equivalent to the hRS7-CL2-SN-38 conjugate (e.g., on day 35, average tumor size was 0.04 ± 0.05 cm^3 in animals given 0.4 mg SN-38/kg hRS7-SN-38 *vs.* 1.78 ± 0.62 cm^3 in irinotecan-treated animals given 0.4 mg/kg SN-38; AUC_{day35} $P<0.001$) (Fig. 3C). When the irinotecan dose was increased 10-fold to 4 mg/kg SN-38, the response improved, but still was not as significant as the conjugate at the 0.4 mg/kg SN-38 dose level (TV=0.17 ± 0.18 cm^3 *vs.* 1.69 ± 0.47 cm^3, AUC_{day49} $P<0.001$). An equal dose of non-targeting hA20-CL2-SN-38 also had a significant anti-tumor effect as compared to irinotecan-treated animals, but the specific hRS7 conjugate was significantly better than the irrelevant ADC (TV=0.17 ± 0.18 cm^3 *vs.* 0.80 ± 0.68 cm^3, AUC_{day49} $P<0.018$).

Studies with the hRS7-CL2A-SN-38 ADC were then extended to two other models of human epithelial cancers. In mice bearing BxPC-3 human pancreatic tumors (Fig. 3D), hRS7-CL2A-SN-38 again significantly inhibited tumor growth in comparison to control

mice treated with saline or an equivalent amount of non-targeting hA20-CL2A-SN-38 (TV=0.24 ± 0.11 cm^3 *vs.* 1.17 ± 0.45 cm^3 and 1.05 ± 0.73 cm^3, respectively; AUC_{day21} $P<0.001$), or irinotecan given at a 10-fold higher SN-38 equivalent dose (TV=0.27 ± 0.18 cm^3 *vs.* 0.90 ± 0.62 cm^3, respectively; AUC_{day25} $P<0.004$). Interestingly, in mice bearing SK-MES-1 human squamous cell lung tumors treated with 0.4 mg/kg of the ADC (Fig. 3E), tumor growth inhibition was superior to saline or unconjugated hRS7 IgG (TV=0.36 ± 0.25 cm^3 *vs.* 1.02 ± 0.70 cm^3 and 1.30 ± 1.08 cm^3, respectively; $AUC_{28\ days}$, $P<0.043$), but non-targeting hA20-CL2A-SN-38 or the MTD of irinotecan provided the same anti-tumor effects as the specific hRS7-SN-38 conjugate.

In all murine studies, the hRS7-SN-38 ADC was well tolerated in terms of body weight loss (not shown).

Biodistribution of hRS7-CL2A-SN-38

The biodistributions of hRS7-CL2A-SN-38 or unconjugated hRS7 IgG were compared in mice bearing SK-MES-1 human squamous cell lung carcinoma xenografts (Suppl. Table S1), using the respective ^{111}In-labeled substrates. A pharmacokinetic analysis was performed to determine the clearance of hRS7-CL2A-SN-38 relative to unconjugated hRS7 (Fig. 4A). The ADC cleared faster than the equivalent amount of unconjugated hRS7, with the ADC exhibiting ~40% shorter half-life and mean residence time. Nonetheless, this had a minimal impact on tumor uptake (Fig. 4B). While there were significant differences at the 24- and 48-h time-points, by 72 h (peak uptake) the amounts of both agents in the tumor were similar. Among the normal tissues, hepatic (Fig. 4C) and splenic (Fig 4D) differences were the most striking. At 24 h post-injection, there was >2-fold more hRS7-CL2A-SN-38 in the liver than hRS7 IgG. Conversely, in the spleen there was three-fold more parental hRS7 IgG present at peak uptake (48-h time-point) than hRS7-CL2A-SN-38. Uptake and clearance in the rest of the tissues generally reflected differences in the blood concentration.

Since twice-weekly doses were given for therapy, tumor uptake in a group of animals that first received a pre-dose of 0.2 mg/kg (250 μg protein) of the hRS7 ADC 3 days before the injection of the ^{111}In-labeled antibody was examined. Tumor uptake of ^{111}In-hRS7-CL2A-SN-38 in pre-dosed mice was substantially reduced at every time-point in comparison to animals that did not receive the pre-dose (e.g., at 72 h, pre-dosed tumor uptake was 12.5 ± 3.8% ID/g *vs.* 25.4 ± 8.1% ID/g in animals not given the pre-dose; $P = 0.0123$; Fig. 4E). Pre-dosing had no appreciable impact on blood clearance or tissue uptake (Suppl. Table S2). These studies suggest that in some tumor models, tumor accretion of the specific antibody can be reduced by the preceding dose(s), which likely explains why the specificity of a therapeutic response could be diminished with increasing ADC doses and why further dose escalation is not indicated.

Tolerability of hRS7-CL2A-SN-38 in Swiss-Webster mice and Cynomolgus monkeys

Swiss-Webster mice tolerated two doses over three days, each of 4, 8, and 12 mg SN-38/kg of the hRS7-CL2A-SN-38, with minimal transient weight loss (Suppl. Figure S2). No hematopoietic toxicity occurred and serum chemistries only revealed elevated aspartate transaminase (AST) and alanine transaminase (ALT) (Figure 5). Seven days after treatment,

AST rose above normal levels (>298 U/L) in all three treatment groups (Fig. 5A), with the largest proportion of mice being in the 2 × 8 mg/kg group. However, by 15 days post-treatment, most animals were within the normal range. ALT levels were also above the normal range (>77 U/L) within seven days of treatment (Fig. 5B) and with evidence of normalization by Day 15. Livers from all these mice did not show histologic evidence of tissue damage (not shown). In terms of renal function, only glucose and chloride levels were somewhat elevated in the treated groups. At 2 × 8 mg/kg, 5 of 7 mice had slightly elevated glucose levels (range of 273 to 320 mg/dL, upper end of normal 263 mg/dL) that returned to normal by 15 days post-injection. Likewise, chloride levels were slightly elevated, ranging from 116 to 127 mmol/L (upper end of normal range 115 mmol/L) in the two highest dosage groups (57% in the 2 × 8 mg/kg group and 100% of the mice in the 2 × 12 mg/kg group), and remained elevated out to 15 days post-injection. This also could be indicative of gastrointestinal toxicity, since most chloride is obtained through absorption by the gut; however, at termination, there was no histologic evidence of tissue damage in any organ system examined (not shown).

Since mice do not express Trop-2, a more suitable model was required to determine the potential of the hRS7 conjugate for clinical use. Immunohistology studies revealed binding in multiple tissues in both humans and Cynomolgus monkeys (breast, eye, gastrointestinal tract, kidney, lung, ovary, fallopian tube, pancreas, parathyroid, prostate, salivary gland, skin, thymus, thyroid, tonsil, ureter, urinary bladder, and uterus) (not shown). Based on this cross-reactivity, a tolerability study was performed in monkeys.

The group receiving 2 × 0.96 mg SN-38/kg of hRS7-CL2A-SN-38 had no significant clinical events following the infusion and through the termination of the study. Weight loss did not exceed 7.3% and returned to acclimation weights by day 15. Transient decreases were noted in most of the blood count data (neutrophil and platelet data shown in Fig. 5C and 5D), but values did not fall below normal ranges. No abnormal values were found in the serum chemistries. Histopathology of the animals necropsied on day 11 (eight days after last injection) showed microscopic changes in hematopoietic organs (thymus, mandibular and mesenteric lymph nodes, spleen, and bone marrow), gastrointestinal organs (stomach, duodenum, jejunum, ileum, cecum, colon, and rectum), female reproductive organs (ovary, uterus, and vagina), and at the injection site. These changes ranged from minimal to moderate and were fully reversed at the end of the recovery period (day 32) in all tissues, except in the thymus and gastrointestinal tract, which were trending towards full recovery at this later time-point.

At the 2 × 1.92 mg SN-38/kg dose level of the conjugate, there was one death arising from gastrointestinal complications and bone marrow suppression, and other animals within this group showed similar, but more severe adverse events than the 2 × 0.96 mg/kg group. These data indicate that dose-limiting toxicities were identical to that of irinotecan; namely, intestinal and hematologic. Thus, the MTD for hRS7-CL2A-SN-38 lies between 2 × 0.96 to 1.92 mg SN-38/kg, which represents a human equivalent dose of 2 × 0.3 to 0.6 mg/kg SN-38.

DISCUSSION

Trop-2 is a protein expressed on many epithelial tumors, including lung, breast, colorectal, pancreas, prostate, and ovarian cancers, making it a potentially important target for delivering cytotoxic agents (7–9, 11, 13). The RS7 antibody internalizes when bound to Trop-2 (18), which enables direct intracellular delivery of cytotoxics.

Conjugation of chemotherapeutic drugs to antibodies has been explored for over 30 years (42, 43). Because a substantial portion of an ADC is not processed by the tumor, but by normal tissues, there is a risk that these agents will be too toxic to normal organ systems before reaching the therapeutic level in tumors. As with any therapeutic, the therapeutic window is a key factor determining the potential of an ADC, and thus rather than examining "ultratoxic" drugs, we chose SN-38 as the drug component of the Trop-2-targeted ADC.

SN-38 is a potent topoisomerase-I inhibitor, with IC_{50}-values in the nanomolar range in several cell lines. It is the active form of the prodrug, irinotecan, that is used for the treatment of colorectal cancer, and which also has activity in lung, breast, and brain cancers. We reasoned that a directly targeted SN-38, in the form of an ADC, would be a significantly improved therapeutic over CPT-11, by overcoming the latter's low and patient-variable bioconversion to active SN-38 (26).

The Phe-Lys peptide inserted in the original CL2 derivative allowed for possible cleavage via cathepsin B (44). In an effort to simplify the synthetic process, in CL2A, phenylalanine was eliminated, and thus the cathepsin B cleavage site was removed. Interestingly, this product had a better-defined chromatographic profile compared to the broad profile obtained with CL2 (not shown), but more importantly, this change had no impact on the conjugate's binding, stability, or potency in side-by-side testing. These data suggest that SN-38 in CL2 was released from the conjugate primarily by the cleavage at the pH-sensitive benzyl carbonate bond to SN-38's lactone ring and not the cathepsin B cleavage site (Figure 1A).

In vitro cytotoxicity of hRS7 ADC against a range of solid tumor cell lines consistently had IC_{50} values in the nM range. However, cells exposed to free SN-38 demonstrated a lower IC_{50} value compared to the ADC. This disparity between free and conjugated SN-38 was also reported for ENZ-2208 (28, 29) and NK012 (27). ENZ-2208 utilizes a branched PEG to link about 3.5 to 4 molecules of SN-38 per PEG, while NK012 is a micelle nanoparticle containing 20% SN-38 by weight. With our ADC, this disparity (i.e., ratio of potency with free vs. conjugated SN-38) decreased as the Trop-2 expression levels increased in the tumor cells, suggesting an advantage to targeted delivery of the drug. In terms of *in vitro* serum stability, both the CL2- and CL2A-SN-38 forms of hRS7-SN-38 yielded a $t_{½}$ of ~20 h, which is in contrast to the short $t_{½}$ of 12.3 minutes reported for ENZ-2208 (29), but similar to the 57% release of SN-38 from NK012 under physiological conditions after 24 hours (27).

Treatment of tumor-bearing mice with hRS7-SN-38 (either with CL2-SN-38 or CL2A-SN-38) significantly inhibited tumor growth in five different tumor models. In four of them, tumor regressions were observed, and in the case of Calu-3, all mice receiving the highest dose of hRS7-SN-38 were tumor-free at the conclusion of study. Unlike in humans,

irinotecan is very efficiently converted to SN-38 by a plasma esterase in mice, with a greater than 50% conversion rate (38), and yielding higher efficacy in mice than in humans (39–41). When irinotecan was administered at 10-fold higher or equivalent SN-38 levels, hRS7-SN-38 was significantly better in controlling tumor growth. Only when irinotecan was administered at its MTD of 24 mg/kg q2dx5 (37.5-fold more SN-38) did it equal the effectiveness of hRS7-SN-38. In patients, we would expect this advantage to favor hRS7-CL2A-SN-38 even more, since the bioconversion of irinotecan would be substantially lower.

We also showed in some antigen-expressing cell lines, such as SK-MES-1, that using an antigen-binding ADC does not guarantee better therapeutic responses than a non-binding, irrelevant conjugate. This is not an unusual or unexpected finding. Indeed, the non-binding SN-38 conjugates mentioned above enhance therapeutic activity when compared to irinotecan, and so an irrelevant IgG-SN-38 conjugate is expected to have some activity. This is related to the fact that tumors have immature, leaky vessels that allow the passage of macromolecules better than normal tissues (45). With our conjugate, 50% of the SN-38 will be released in ~13 h when the pH is lowered to a level mimicking lysosomal levels (e.g., pH 5.3 at 37^{o} C; data not shown), whereas at the neutral pH of serum, the release rate is reduced nearly 2-fold. If an irrelevant conjugate enters an acidic tumor microenvironment, it is expected to release some SN-38 locally. Other factors, such as tumor physiology and innate sensitivities to the drug, will also play a role in defining this “baseline” activity. However, a specific conjugate with a longer residence time should have enhanced potency over this baseline response as long as there is ample antigen to capture the specific antibody. Biodistribution studies in the SK-MES-1 model also showed that if tumor antigen becomes saturated as a consequence of successive dosing, tumor uptake of the specific conjugate is reduced, which yields therapeutic results similar to that found with an irrelevant conjugate.

While it is challenging to make direct comparisons between our ADC and the published reports of other SN-38 delivery agents, some general observations can be made. In our therapy studies, the highest individual dose was 0.4 mg/kg of SN-38. In the Calu-3 model, only four injections were given for a total cumulative dose of 1.6 mg/kg SN-38 or 32 μg SN-38 in a 20 g mouse. Multiple studies with ENZ-2208 were done using its MTD of 10 mg/kg x 5 (28, 31), and preclinical studies with NK012 involved its MTD of 30 mg/kg x 3 (27). Thus, significant anti-tumor effects were obtained with hRS7-SN-38 at 30-fold and 55-fold less SN-38 equivalents than the reported doses in ENZ-2208 and NK012, respectively. Even with 10-fold less hRS7 ADC (0.04 mg/kg), significant anti-tumor effects were observed, whereas lower doses of ENZ-2208 were not presented, and when the NK012 dose was lowered 4-fold to 7.5 mg/kg, efficacy was lost (27). Normal mice showed no acute toxicity with a cumulative dose over 1 week of 24 mg/kg SN-38 (1500 mg/kg of the conjugate), indicating that the MTD was higher. Thus, tumor-bearing animals were effectively treated with 7.5- to 15-fold lower amounts of SN-38 equivalents.

As a topoisomerase-I inhibitor, SN-38 induces significant damage to a cell’s DNA, with up-regulation of p53 and p21$^{WAF1/Cip1}$ resulting in caspase activation and cleavage of PARP (34–37). When we exposed BxPC-3 and Calu-3 cells to our ADC, both p53 and p21$^{WAF1/Cip1}$ were up-regulated above basal levels. Additionally, PARP cleavage was also

evident in both cell lines, confirming an apoptotic event in these cells. Of interest was the higher up-regulation of $p21^{WAF1/Cip1}$ in BxPC-3 and Calu-3 relative to p53 by both free SN-38 and our hRS7-SN-38. This may be indicative of the mutational status of p53 in these two cell lines (46–48) and the use of a p53-independent pathway for $p21^{WAF1/Cip1}$-mediated apoptosis (49).

An interesting observation was the early up-regulation of p53 in both BxPC-3 and Calu-3 at 24 h mediated by the hRS7-ADC relative to free SN-38. Even the naked hRS7 IgG could up-regulate p53 in these cell lines, though only after a 48-h exposure. Trop-2 over-expression and cross-linking by antibodies has been linked to several MAPK-related signaling events (11), as well as intracellular calcium release (5). While binding of hRS7 was not sufficient to induce apoptosis in BxPC-3 and Calu-3, as evidenced by the lack of PARP cleavage, it may be enough to prime a cell, such that the inclusion of SN-38 conjugated to hRS7 may lead to a greater effect on tumor growth inhibition. Studies are currently underway to understand which pathways are involved with hRS7-delivery of SN-38 and how they may differ from free SN-38, and what effect p53 status may play in this signaling.

Biodistribution studies revealed the hRS7-CL2A-SN-38 had similar tumor uptake as the parental hRS7 IgG, but cleared substantially faster with 2-fold higher hepatic uptake, which may be due to the hydrophobicity of SN-38. With the ADC being cleared through the liver, hepatic and gastrointestinal toxicities were expected to be dose-limiting. While mice had evidence of increased hepatic transaminases, gastrointestinal toxicity was mild at best, with only transient loss in weight and no abnormalities noted upon histopathologic examination. Interestingly, no hematological toxicity was noted. However, monkeys showed an identical toxicity profile as expected for irinotecan, with gastrointestinal and hematological toxicity being dose-limiting.

Since Trop-2 is not expressed in mice, it was critically important to perform toxicity studies in monkeys that have a similar tissue expression of Trop-2 as humans. Monkeys tolerated 0.96 mg/kg/dose (~12 mg/m^2) with mild and reversible toxicity, which extrapolates to a human dose of ~0.3 mg/kg/dose (~11 mg/m^2). In a Phase I clinical trial of NK012, patients with solid tumors tolerated 28 mg/m^2 of SN-38 every 3 weeks with Grade 4 neutropenia as dose-limiting toxicity (DLT) (30). Likewise, Phase I clinical trials with ENZ-2208 revealed dose-limiting febrile neutropenia, with a recommendation to administer 10 mg/m^2 every three weeks or 16 mg/m^2 if patients were administered G-CSF (48, 49). Since monkeys tolerated a cumulative human equivalent dose of 22 mg/m^2, it is possible that even though hRS7 binds to a number of normal tissues, the MTD for a single treatment of the hRS7 ADC could be similar to that of the other non-targeting SN-38 agents. Indeed, the specificity of the anti-Trop-2 antibody did not appear to play a role in defining the DLT, since the toxicity profile was similar to that of irinotecan. More importantly, if anti-tumor activity can be achieved in humans as in mice that responded with human equivalent dose of just at 0.03 mg SN-38 equivalents/kg/dose, then significant anti-tumor responses could be realized clinically.

In conclusion, toxicology studies in monkeys, combined with *in vivo* human cancer xenograft models in mice have indicated that this ADC targeting Trop-2 is an effective therapeutic in several tumors of different epithelial origin, supporting future clinical testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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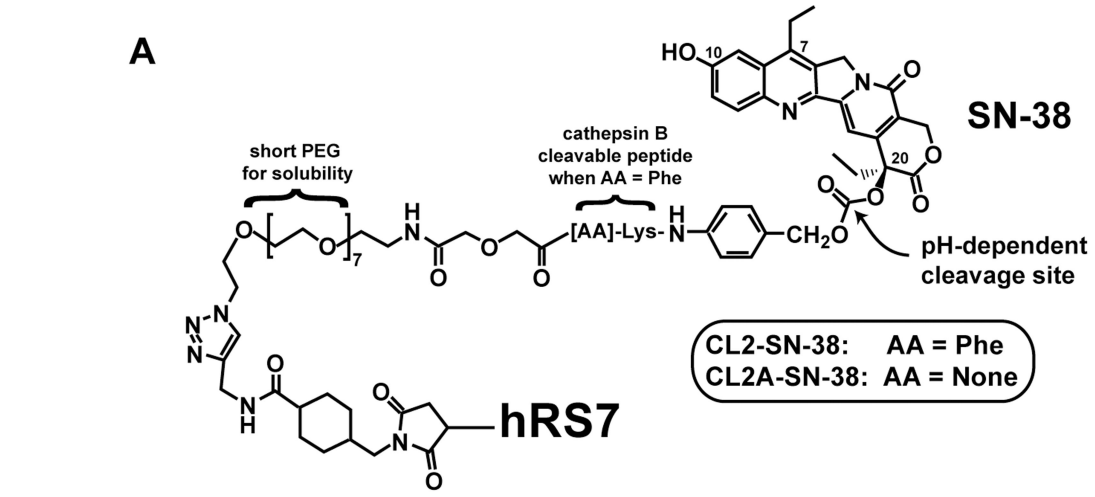
STATEMENT OF TRANSLATIONAL RELEVANCE

Successful irinotecan treatment of patients with solid tumors has been limited due in large part to the low conversion rate of the CPT-11 prodrug into the active SN-38 metabolite. Others have examined non-targeted forms of SN-38 as a means to bypass the need for this conversion and to deliver SN-38 passively to tumors. We conjugated SN-38 covalently to a humanized anti-Trop-2 antibody, hRS7. This antibody-drug conjugate has specific anti-tumor effects in a range of s.c. human cancer xenograft models, including non-small cell lung carcinoma, pancreatic, colorectal, and squamous cell lung carcinomas, all at non-toxic doses (e.g., ≤ 3.2 mg/kg cumulative SN-38 equivalent dose). Trop-2 is widely expressed in many epithelial cancers, but also some normal tissues, and therefore a dose escalation study in Cynomolgus monkeys was performed to assess the clinical safety of this conjugate. Monkeys tolerated 24 mg SN-38 equivalents/kg with only minor, reversible, toxicities. Given its tumor-targeting and safety profile, hRS7-SN-38 may provide an improvement in the management of solid tumors responsive to irinotecan.



Agent	SN-38/IgG Substitution Ratio	Free SN-38 (%)	Human Serum Half-Life (h)	Cell Binding: Kd (nM) (95% C.I.)	Cytotoxicity: IC_{50} (nM) (95% C.I.)
hRS7-CL2-SN-38	6.2	0.7	22.1	1.19 (0.89 to 1.49)	4.12 (2.88 to 5.89)
hRS7-CL2A-SN-38	6.1	0.6	20.3	1.09 (0.97 to 1.21)	4.24 (2.99 to 6.01)

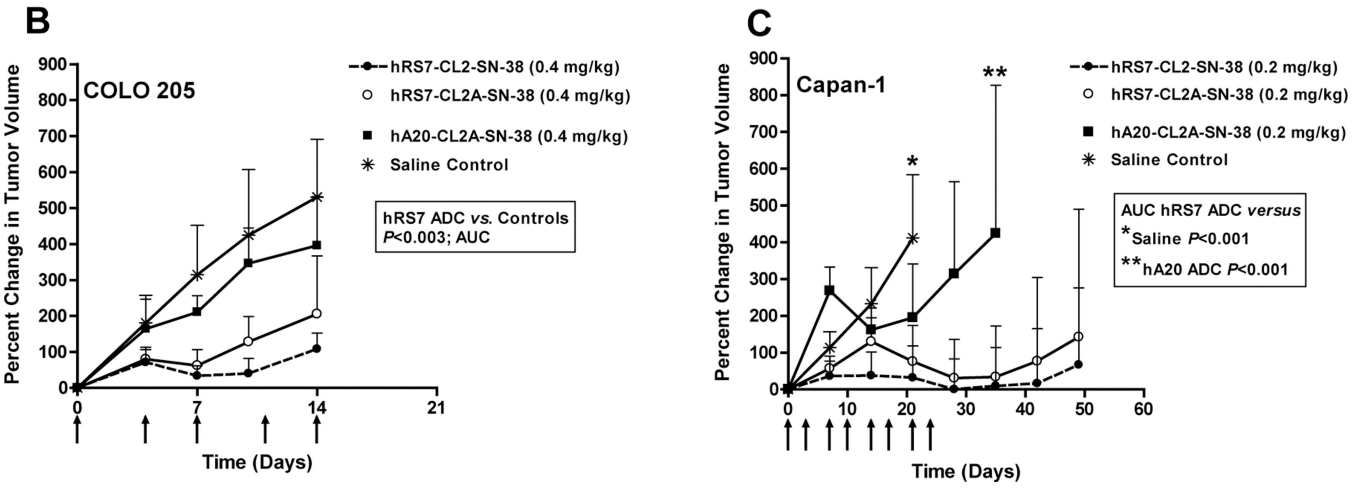
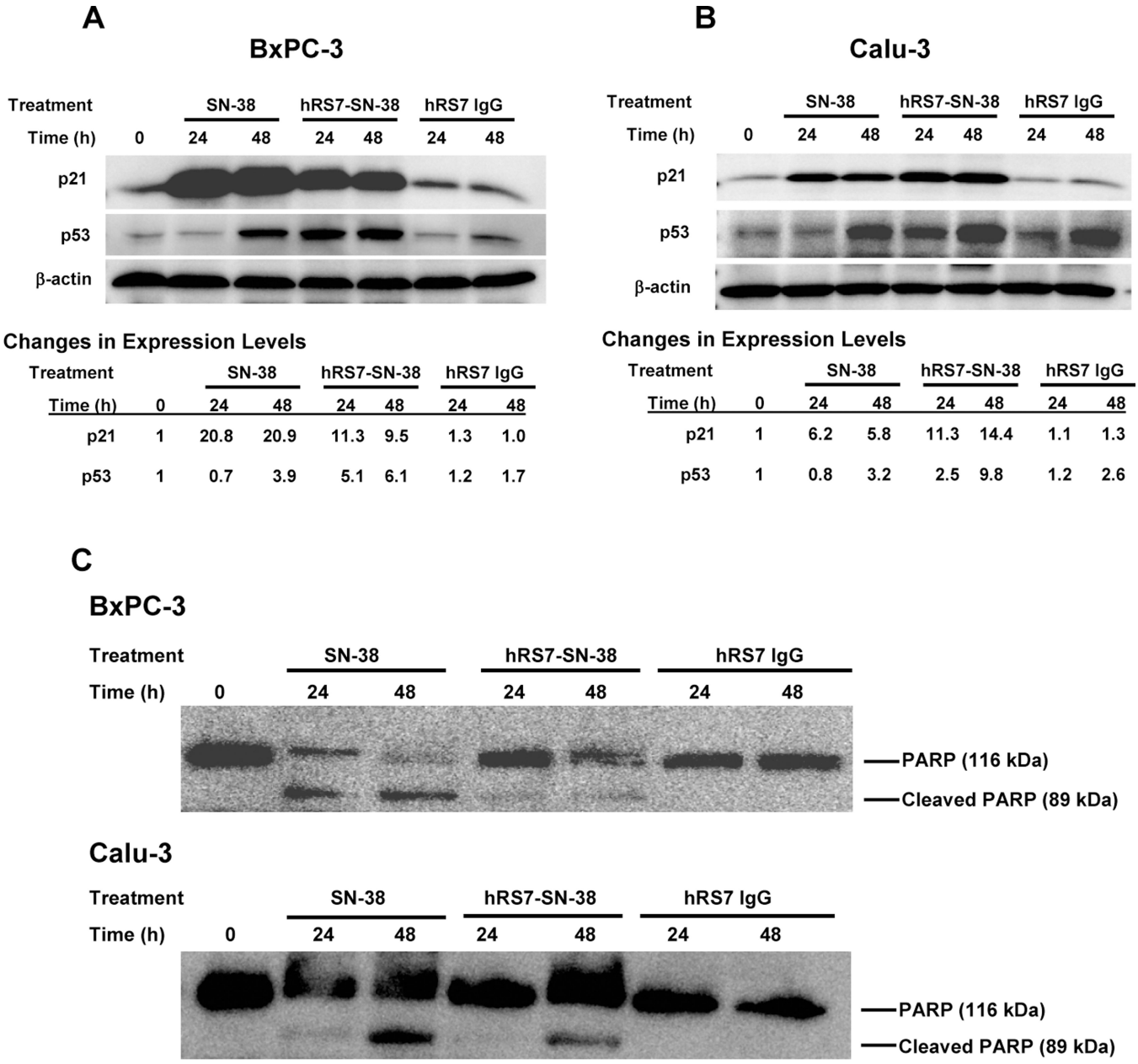


Figure 1. Structure and characterization of SN-38 conjugates: hRS7-CL2-SN-38 and hRS7-CL2A-SN-38.

(A) The chemical structure of the linkage of SN-38 (shown in red) to the hRS7 antibody (blue) via the CL-linker. A single amino acid deletion shows the difference between the CL2-SN-38 and CL2A-SN-38 forms of the hRS7 anti-Trop-2 ADC. Comparisons in chemistry and potency are shown in the table. Comparability of hRS7-CL2-SN-38 *versus* hRS7-CL2A-SN-38 in mice bearing COLO 205 (*B*) and Capan-1 tumors (*C*). Animals were treated twice weekly for four weeks as indicated by the arrows. COLO 205 mice (N=6) were treated with 0.4 mg/kg ADC and tumors measured twice a week. Capan-1 mice (N=10) were treated with 0.2 mg/kg ADC and tumors measured weekly.



Changes in Expression Levels

Treatment		SN-38		hRS7-SN-38		hRS7 IgG	
Time (h)	0	24	48	24	48	24	48
p21	1	20.8	20.9	11.3	9.5	1.3	1.0
p53	1	0.7	3.9	5.1	6.1	1.2	1.7

Changes in Expression Levels

Treatment		SN-38		hRS7-SN-38		hRS7 IgG	
Time (h)	0	24	48	24	48	24	48
p21	1	6.2	5.8	11.3	14.4	1.1	1.3
p53	1	0.8	3.2	2.5	9.8	1.2	2.6

Figure 2. Western blot analysis for early and late signaling events mediated by hRS7-CL2A-SN-38.

Cells were plated overnight in 6-well plates before the addition of hRS7-CL2A-SN-38 (1 μM SN-38 equivalents), free SN-38 (1 μM), or the protein equivalent of hRS7 IgG (25 μg/mL). At the indicated times, cells were lysed and 20 μg protein from these lysates subjected to SDS-PAGE (4–20%) followed by blotting with antibodies to p21, p53, β-Actin, or PARP. Time 0 lanes are from untreated cells grown in the plates for 48 h in growth media alone and represent basal expression levels. Changes in expression are normalized to β-actin loading controls and are relative to levels in the untreated cells.

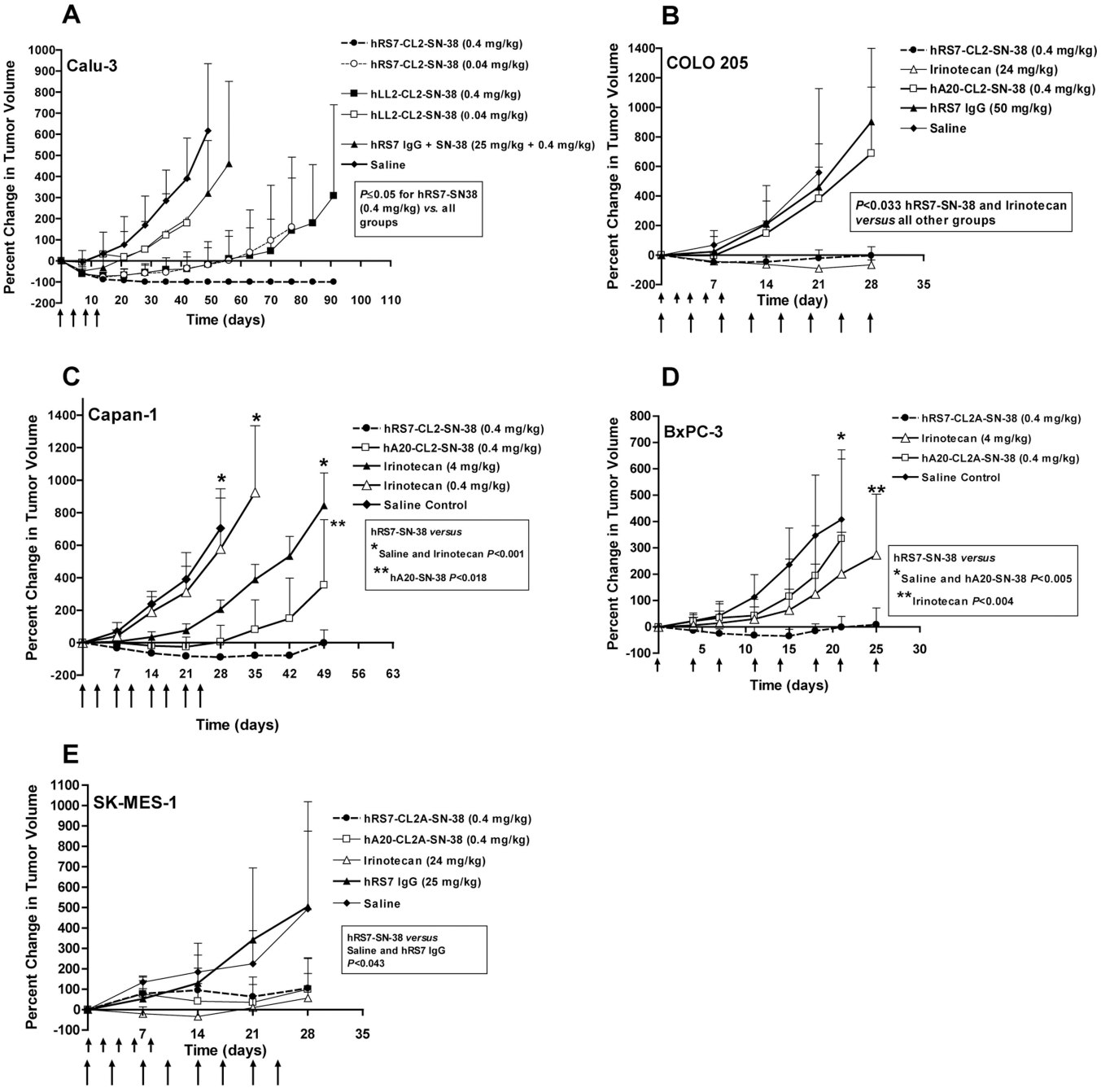


Figure 3. Therapeutic efficacy of hRS7-SN-38 ADC in several solid tumor-xenograft disease models.

Efficacy of hRS7-CL2-SN-38 and hRS7-CL2A-SN-38 ADC treatment was studied in mice bearing human non-small cell lung, colorectal, pancreatic, and squamous cell lung tumor xenografts. All the ADCs and controls were administered in the amounts indicated (expressed as amount of SN-38 per dose; long arrows = conjugate injections, short arrows = irinotecan injections). *(A)* Mice bearing Calu-3 tumors (N = 5–7) were injected with hRS7-CL2-SN-38 every four days for a total of four injections (q4dx4). *(B)* COLO 205 tumor-bearing mice (N=5) were injected eight times (q4dx8) with the ADC or every two

days for a total of five injections (q2dx5) with the MTD of irinotecan. Capan-1 (*C*; N=10) or BxPC-3 tumor-bearing mice (*D*; N = 10) were treated twice weekly for 4 weeks with the agents indicated. (*E*) In addition to ADC given twice weekly for 4 week, SK-MES-1 tumor-bearing (N=8) mice received the MTD of CPT-11 (q2dx5).

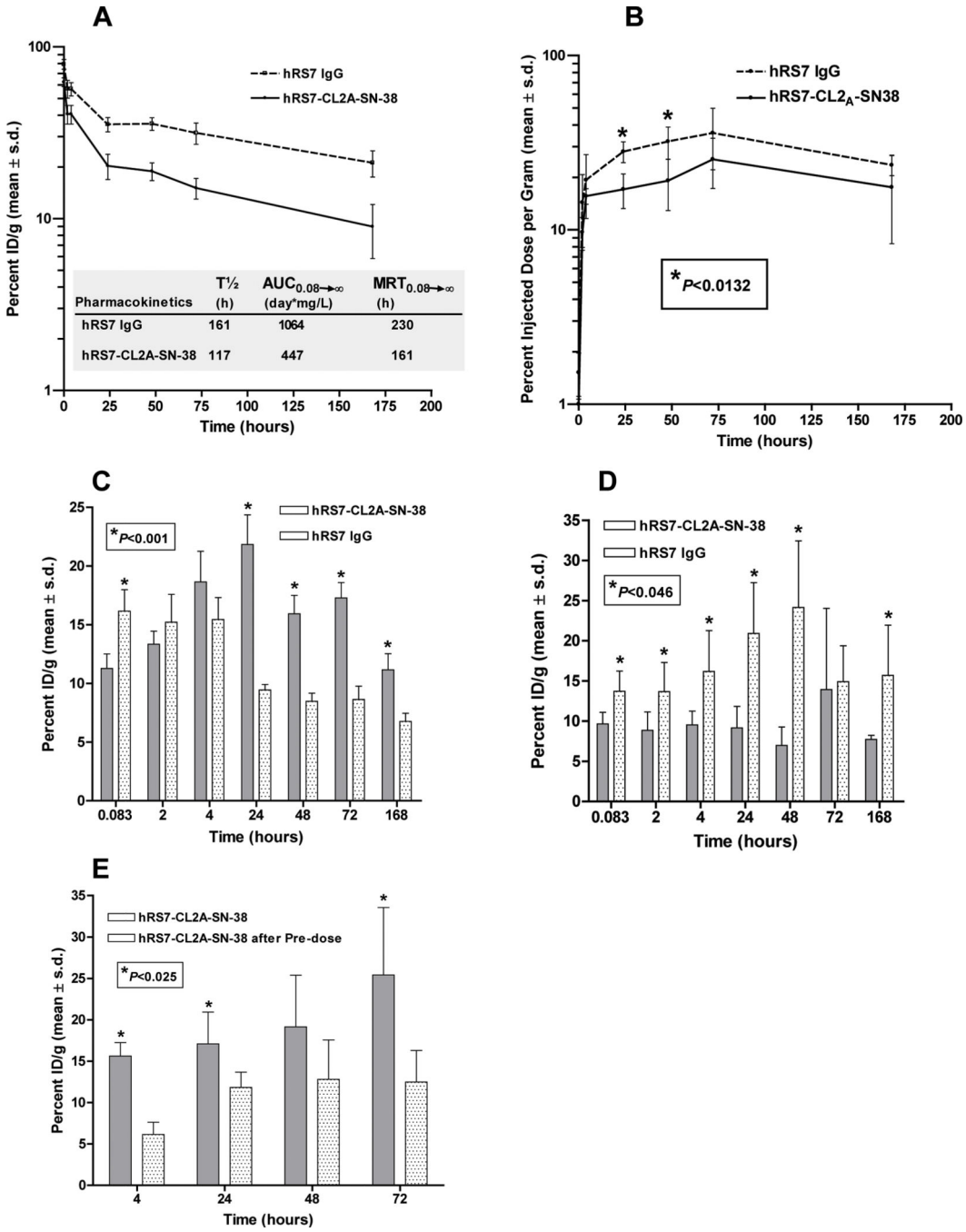


Figure 4. Biodistribution and pharmacokinetics of hRS7-CL2A-SN-38 in SK-MES-1 tumor-bearing mice.

(A-D) Mice were injected with either ^{111}In-DTPA-hRS7-CL2A-SN-38 or ^{111}In-DTPA-hRS7 IgG (N = 5/observation). Data are expressed as % ID/g. *(A)* Blood clearance and pharmacokinetics parameters, *(B)* tumor, *(C)* liver, and *(D)* spleen are shown. *(E)* Pre-dosing with 250 μg of hRS7-CL2A-SN-38 three days before receiving ^{111}In-DTPA-hRS7-CL2A-SN-38 effect on tumor uptake.

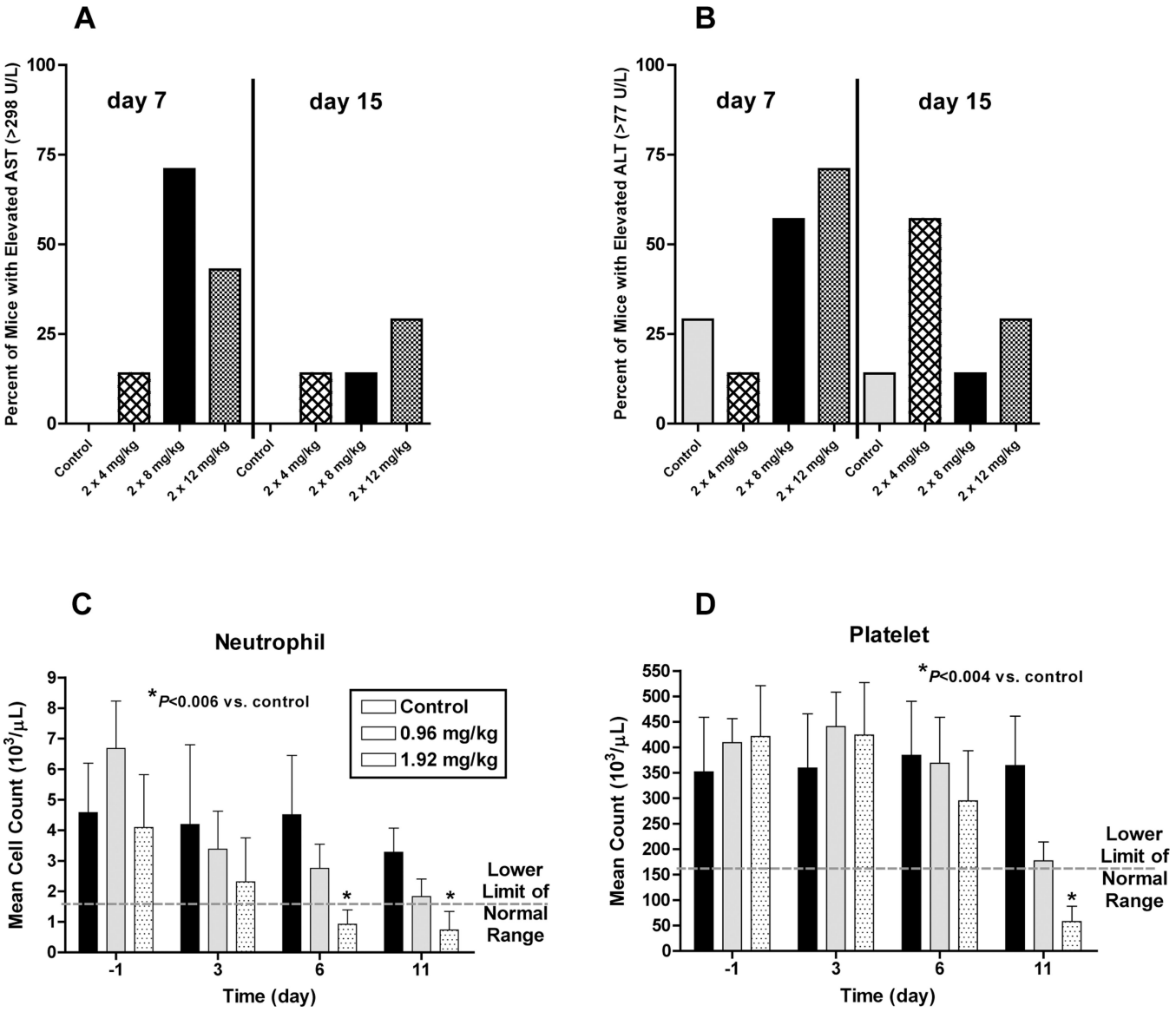


Figure 5. Tolerability of hRS7-CL2A-SN-38 in Swiss-Webster mice and Cynomolgus monkeys.

(A-B) Fifty-six Swiss-Webster mice were administered two i.p. doses of buffer or the hRS7-CL2A-SN-38 three days apart (4, 8, or 12 mg/kg of SN-38 per dose; 250, 500, or 750 mg conjugate protein/kg per dose). Seven and 15 days after the last injection, seven mice from each group were euthanized, with blood counts and serum chemistries performed. Graph show the percent of animals in each group that had elevated levels of AST *(A)* and ALT *(B)*. Six monkeys per group were injected twice 3 days apart with buffer (control) or hRS7-CL2A-SN-38 at 0.96 mg/kg or 1.92 mg/kg of SN-38 equivalents per dose (60 and 120 mg/kg conjugate protein). All animals were bled on day −1, 3, and 6. Four monkeys were bled on day 11 in the 0.96 mg/kg group, 3 in the 1.92 mg/kg group. Changes in neutrophil *(C)* and platelet counts *(D)*, respectively in Cynomolgus monkeys.

Table 1.

Expression of Trop-2 and *in vitro* cytotoxicity of SN-38 and hRS7-SN-38 in several solid tumor lines

	Trop-2 Expression *via* FACS		Cytotoxicity Results				
Cell Line	**Median Fluorescence (Background)**	**Percent Positive**	**SN-38 IC_{50} (nM)**	**95% C.I. IC_{50} (nM)**	**hRS7-SN-38* IC_{50} (nM)**	**95% C.I. IC_{50} (nM)**	**ADC/Free SN-38 Ratio**
Calu-3	282.2 (4.7)	99.6%	7.19	5.77 – 8.95	9.97	8.12 – 12.25	1.39
COLO 205	141.5 (4.5)	99.5%	1.02	0.66 – 1.57	1.95	1.26 – 3.01	1.91
Capan-1	100.0 (5.0)	94.2%	3.50	2.17 – 5.65	6.99	5.02 – 9.72	2.00
PC-3	46.2 (5.5)	73.6%	1.86	1.16 – 2.99	4.24	2.99 – 6.01	2.28
SK-MES-1	44.0 (3.5)	91.2%	8.61	6.30 – 11.76	23.14	17.98 – 29.78	2.69
BxPC-3	26.4 (3.1)	98.3%	1.44	1.04 – 2.00	4.03	3.25 – 4.98	2.80

*IC_{50}-value is shown as SN-38 equivalents of hRS7-SN-38