Molecular Imaging of Death Receptor 5 Occupancy and Saturation Kinetics In Vivo by Humanized Monoclonal Antibody CS-1008


Abstract

Purpose: CS-1008 (tigatuzumab; phase I/II), an antihuman death receptor 5 (DR5) agonist, induces apoptosis and has cytotoxic activity against human cancer cell lines. This study reports on the preclinical validation of 111In-labeled anti-DR5 humanized antibody CS-1008 as a diagnostic tool to study the DR5 occupancy in patients with cancer and establish dose ranges for receptor saturation kinetics in vivo.

Experimental Design: CS-1008 was radiolabeled and characterized for DR5 binding and labeling efficiency on TRAIL-sensitive DR5-positive colorectal cancer cells (COLO 205 and WiDr). Pharmacokinetic and biodistribution studies were conducted in BALB/c nu/nu mice bearing COLO 205, WiDr, or DR5-negative CT26 colon tumors. Planar gamma camera imaging and computed tomography (CT) images were obtained to study receptor occupancy in vivo.

Results: Scatchard analysis showed high and specific binding affinity (Kd 1.05 ± 0.12 nmol/L) of 111In-labeled CS-1008. 111In-labeled CS-1008 was specifically taken up in mice bearing COLO 205 and WiDr tumors with prolonged tumor retention (26.25 ± 2.85%ID/g vs. 12.20 ± 2.24 at 168 hours post injection; n = 5, SD), and uptake correlated both with DR5 expression on tumor cells and antitumor activity. DR5 saturation was shown in vivo via both biodistribution studies and planar gamma camera imaging/CT imaging of 111In-labeled CS-1008. Saturation of DR5 corresponded to maximal in vivo antitumor efficacy.

Conclusions: Imaging of DR5 receptor occupancy in vivo correlates with tumor concentration and in vivo efficacy, and is a novel molecular imaging technique that can be used to determine receptor occupancy and effective dose levels of DR5 agonist antibodies in the clinic. Clin Cancer Res; 19(21); 5984–93. ©2013 AACR.
Molecular Imaging of DR5 with Tigatuzumab

Translational Relevance

CS-1008 (tigatuzumab) is an antihuman death receptor (DR5) agonist that induces apoptosis and has cytotoxic activity against human cancer cell lines. Tumor concentration of CS-1008 in vivo and the impact of dose on tumor saturation of DR5 remain key questions in the determination of the optimal dosing schedule for therapeutic benefit. This study reports on the exploration of targeting of anti-DR5 humanized antibody CS-1008 to DR5-expressing tumors in vivo, and quantitative analysis of antibody:DR5 binding and occupancy. The relationship of in vivo imaging of CS-1008 in tumor to therapeutic response was also established. Our results showed that in vivo saturation of DR5 was predictive of therapeutic dose–response threshold. This novel and important information is highly relevant to DR5 modulation as a therapeutic approach and informs the design of clinical trials of anti-DR5 antibodies in patients with cancer.

CS-1008 has similar in vitro and in vivo antitumor activity as mTRA-8 (2).

Phase 1 trials in humans have shown linear pharmacokinetics in doses ranging from 1 to 8 mg/kg/week with a half-life of 8 to 16 days. There were no partial responses to treatment but stable disease was noted in 7 of 17 treated patients with the duration of response ranging from 81 to 798 days. CS-1008 dose-limiting toxicity was not reached, and no evidence of immunogenicity to the antibody was detected (8).

CS-1008 induces cell death in a variety of DR5-expressing human cancer cell lines in vitro. DR5 expression is required to induce cell death but expression alone is not sufficient. The development of a bioimaging technique for assessing DR5-positive tumors in vivo could help in selecting patients that benefit from CS-1008 treatment. Tumor concentration of CS-1008 in vivo and the impact of dose on tumor saturation of DR5 remain key questions in the determination of the optimal dosing schedule for therapeutic benefit. We therefore aimed to establish and validate a molecular imaging technique for measuring DR5 occupancy and saturation in vivo, and to determine the relationship of antibody:DR5 interaction with therapeutic response.

Materials and Methods

Monoclonal antibodies and cell lines

The murine anti-DR5 monoclonal antibody mTRA-8 (IgG1) and CS-1008, a humanized anti-DR5 monoclonal antibody (IgG1), were provided by Daichi Sankyo Company Ltd. Hu3S193, a humanized anti-LewisY (LeY), was provided by the Ludwig Institute for Cancer Research (Melbourne, Australia) and was used as an IgG1 isotype control (9). COLO 205, a DR5-expressing human colorectal adenocarcinoma cell line (10); C126, a DR5-negative mouse colorectal adenocarcinoma cell line (11); and A431, a LeY-expressing human epidermoid carcinoma cell line used to assess LeY antigen binding of hu3S193 were obtained from American Type Culture Collection (ATCC). WiDr, a low-level DR5-expressing colorectal cancer cell line, was obtained from J.M. Mariadason, Ludwig Institute for Cancer Research (Melbourne, Australia). Cell lines were authenticated at the DNA Sequencing and Fragment Analysis Facility at QIMR (Queensland Institute for Medical Research, Queensland, Australia) and expression levels of target receptors (DR5 or LeY) have been tested by single-point Lindmo assays as part of each study using radiolabeled CS-1008 or hu3S193. C126 is a mouse colon cancer cell line and was tested for its mouse origin at QIMR. A431 cells were grown in DMEM/F-12 with 10% v/v FBS, 1% v/v penicillin–streptomycin (10,000 U/mL in 0.85% NaCl), and 1% v/v L-glutamine (200 mM/mL in 0.85% NaCl; Invitrogen). COLO 205, WiDr, and CT26 cells were grown in RPMI-1640 with 10% v/v FBS and 1% v/v penicillin–streptomycin (10,000 U/mL in 0.85% NaCl).

Flow cytometry

Adherent COLO 205, WiDr, and CT26 cells were trypsinized and resuspended in fresh culture medium. Cells were aliquotted into 1.5 mL eppendorf tubes at a density of 1 × 10⁶ cells per tube. Cells were centrifuged at 3,000 g for 3 minutes, and the pellets were resuspended in 100 μL of an antihuman CD262/DR5-PE antibody (1:5 concentration; Affymetrix ebioScience Inc.). A cells-only control and a negative control (1:100 Goat antihuman IgG chain specific F(ab′)2 PE; Sigma-Aldrich) were included. All samples were incubated on ice for 30 minutes. Cells were then centrifuged at 3,000 × g for 3 minutes and the supernant was discarded. Cells were washed three times with 1 mL Dulbecco PBS (LifeTechnologies). After the final wash, cells were resuspended in 100 μL of FACS-FIX (n-glucose 3.2% w/v, formaldehyde 2.5% v/v in DPBS). Samples were transferred to a Corning Costar 96-well v-bottom plate (Corning Life Sciences). Samples were analyzed on the Guava EasyCyte Mini System, GuavaExpress Pro, CytoSoft v 5.3 (Merck Millipore).

TRAIL sensitivity testing

Cells were seeded in 96-well microtiter plates at 2,500 to 5,000 cells per well. TRAIL (Life Technologies) or mTRA-8 (0–1,000 ng/μL) were added the following day and one plate was harvested for a time 0 (T = 0) measurement. Remaining cell plates were incubated for 72 hours. Cell viability was assessed using the MTS colorimetric viability assay with MTS as a substrate (Promega). Absorbance was assessed at 490 nm using a VersaMax Microplate Reader (Molecular Devices) and SoftMax Pro 5.4.1 software (Molecular Devices). Absorbance at 630 nm was also determined as background, and the value was subtracted from the 490 nm reading. Experiments were performed in triplicates and repeated for two independent runs. All data were normalized to signal at the time of compound addition (T = 0). Dose–response...
curves were analyzed using GraphPad Prism 4.03 (GraphPad Software Inc.).

**Radiolabeling and quality assurance**

Analytic grade reagents, sterile technique, and pyrogen-free plasticware were used in all labeling steps. mTRA-8, CS-1008, and hu3S193 were radiolabeled with Indium-111 (111In; MDS Nordion) via the bifunctional metal ion chelating agent CHX-A\(^{2-}\)-DTPA as previously described (12, 13).

Radiolabeling was performed on the day of injection into mice. Before injection, the percentage of unbound radio-nuclide content was determined by instant thin-layer chromatography (ITLC) as previously described (14). Serum stability was assessed by incubating 5.0 µg of radiolabeled antibody in 200 µL of healthy donor human serum at 37°C for an 11-day period. Radiochemical purity and single-point immunoreactivity assays at 0 (no incubation), 2, and 11 days of incubation were determined.

**In vitro binding of 111In-CHX-A\(^{2-}\)-DTPA-CS-1008**

**Lindmo assay.** Immunoreactivity of radiolabeled CS-1008 for COLO 205 cells was determined by nonlinear extrapolation to binding at infinite antigen excess using a “Lindmo” assay (15). Briefly, 20 ng of radiolabeled CS-1008 was added to a range of COLO 205 cell concentrations (0, 3.13, 6.25, 12.5, 25, 50, 100, and 200 \(\times 10^5\) cells/mL) and incubated for 45 minutes at room temperature with continuous mixing throughout to keep the cells in suspension. Cells were washed three times to remove unbound antibody, and pellets were measured in a gamma counter (Wizard2; PerkinElmer). Three radioactive antibody standards of radiolabeled CS-1008 at the same concentration as that initially added to the cells were measured at the same time as cell pellets. The percentage of binding of CS-1008 to COLO 205 cells was calculated by the formula (cpm cell pellet/mean cpm radioactive antibody standards) \(\times 100\). The percentage of binding was graphed against COLO 205 pellet/mean cpm radioactive antibody standards (11–12 days post injection).

**Competitive binding assay.** Unlabeled CS-1008 at concentrations ranging from 5 to 200 µg/mL was added to 200 \(\times 10^5\) COLO 205 cells and mixed. Samples were done in duplicate. Subsequently, 20 ng of radiolabeled mTRA was added. After 45-minute incubation at room temperature, the cells were washed and counted as described earlier for the Lindmo assay.

**Saturation binding and Scatchard.** 111In-labeled CS-1008 at concentrations ranging from 0.1 to 5 µg/mL in duplicate were added to 20 \(\times 10^5\) per mL COLO 205 cells and mixed. To estimate nonspecific binding, duplicate samples of 111In-labeled CS-1008 at concentrations ranging from 0.1 to 5 µg/mL were added to 20 \(\times 10^5\) per mL COLO 205 cells in the presence of 140 µg/mL of cold CS-1008 and mixed. After 45-minute incubation at room temperature, the cells were washed and total bound antibody samples and nonspecific bound antibody samples were counted. The immunoreactive fraction was taken into account in calculating the amount of free, reactive antibody [(immunoreactive fraction \(\times\) total bound antibody) – specific bound antibody], and specific binding (total bound antibody – nonspecific bound antibody) was graphed against reactive free (nmol/L). The apparent dissociation constant (\(K_d\)) and total receptor number per cell (\(B_{\text{max}}\)) at equilibrium were determined by nonlinear regression \(Y = B_{\text{max}} \times X/(K_d + X)\) using GraphPad Prism version 4.03. To show the Scatchard plot, specific bound antibody/reactive free versus specific bound (nmol/L) was plotted. To convert specific bound in cpm/mL to specific bound in nmol/L, the following formula was used: specific bound (nmol/L) = specific bound (cpm/mL) \(\times 1.000 \text{ mL/L} \times (L \times 10^{-16} \text{ g/µg}) \times (1/150,000 \text{ mol/g}) \times 10^9 \text{ nmol/mol}.

**ELISA.** To further assess the impact of radiolabeling on CS-1008 binding, ELISA of samples of CS-1008, 111In-labeled CS-1008, and a control IgG1 was performed. NUNC MaxiSorp F96 plates were coated with TRAIL R2 antigen (R&D Systems) and blocked with 3% FCS–PBS. All samples (in triplicate) were diluted in half-log series (10 µg/mL to 0.01 µg/mL). Goat antihuman IgG-AP (Sigma-Aldrich) was used as a secondary antibody. Plates were washed with 0.05% Tween20–PBS between each step. After adding substrate of pNPP (\(\phi\)-Nitrophenyl Phosphate; Sigma-Aldrich), the absorbance was measured at 405 nm after 10 minutes.

**In vivo binding of 111In-CHX-A\(^{2-}\)-DTPA-CS-1008**

**Animal model.** In vivo studies were conducted in athymic BALB/c nu/nu mice (female, 4–6 week-old) obtained from the Animal Resources Centre (Western Australia, Australia). All animal studies were approved by the Austin Hospital Animal Ethics Committee and were conducted in compliance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

To establish tumors, mice were injected subcutaneously into the left underside flank with COLO 205 cells (2–5 \(\times 10^6\) cells), WiDr cells (5 \(\times 10^6\) cells with Matrigel), or CT26 cells (1 \(\times 10^6\) cells) in a total volume of 0.1 mL PBS. Tumor volume was calculated by the formula [(length \(\times\) width\(^2\))/2], in which length was the longest axis and width was the measurement at right angles to length. In general, in vivo studies commenced when tumor volume was 100 to 200 mm\(^3\) (11–12 days post injection).

**Biodistribution study.** In the initial studies, the in vivo biodistribution of 111In-labeled CS-1008 at a dose level of 0.2 mg/kg was assessed in mice bearing COLO 205, WiDr, or CT26 tumors. On day 0, mice received a single intravenous tail vein injection of 111In-labeled CS-1008 (5.0 µg, 8.5 µCi) in a total volume of 0.1 mL 0.9% w/v of NaCl. At designated time points after injection of the radioconjugates (COLO 205: 3, 24, 72, 168, and 240 hours; WiDr: 4, 24, 72, and 168 hours; CT26: 3, 24, 72, and 168 hours), groups of 5 mice were sacrificed by overinhalation of isoflurane followed by cervical dislocation. Blood, tumor, and normal tissues [brain, heart, lung, stomach, spleen, liver, kidney, small intestine, colon, muscle, bone (femur) and skin] in triplicate were collected for analysis.
and counted for radioactivity (Wizard\textsuperscript{2} gamma counter). The tissue distribution data were calculated as the mean ± SD percentage injected dose per gram tissue [%ID/g; \{radioactivity tissue (counts per minute)/radioactivity standard (counts per minute)\} × 100/tissue weight (g)] for each radiolabeled construct per time point.

The \textit{in vivo} biodistribution of \textsuperscript{111}In-labeled CS-1008 and \textsuperscript{111}In-labeled IgG\textsubscript{1} control at a dose level of 10 mg/kg was also assessed in mice bearing COLO 205 tumors. On day 0, mice with established xenografts received a single intravenous tail injection of CS-1008 or control IgG\textsubscript{1} containing trace labeled \textsuperscript{111}In radioconjugates \textsuperscript{111}In-CS-1008 (6.2 \mu g, 17.8 \mu Ci), \textsuperscript{111}In-IgG\textsubscript{1} (5.0 \mu g, 8.8 \mu Ci)] at 0.2 mg/kg plus unlabeled antibody in a total volume of 0.1 mL. Blood, tumor, and normal tissues were collected at designated time points from euthanized animals (n = 5/time point). Mice injected with radiolabeled control IgG\textsubscript{1} were euthanized at 3, 7, 12, 16, 18, and 336 hours post injection (n = 4/time point) and blood, tumor, and tissues were collected.

\textbf{Gamma camera imaging and computerized tomography imaging.} Planar gamma camera images of \textsuperscript{111}In-labeled CS-1008 (0.2 mg/kg) and \textsuperscript{111}In-labeled IgG\textsubscript{1} control antibody (0.2 mg/kg) distributions in COLO 205 tumor-bearing mice, and computerized tomography (CT) images, were obtained for 2 mice per antibody preparation on days 0, 2, 3, 7, and 10 post injection. Imaging procedures involved anesthesia of mice by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) mixture. Mice were placed supine on a Philips SkyLight gamma camera with energy windows set for \textsuperscript{111}In (20\% windows at 171 and 245 keV). Images were acquired in a 128 × 128 or 256 × 256 matrix and a standard containing a known amount of \textsuperscript{111}In activity was included in the field of view. Twenty-minute images were collected. CT scanning, to generate cross-sectional imaging of anatomic structures, was also undertaken (Philips Gemini 2 slice PET-CT scanner; slice thickness, 0.097 mm).

Imaging analysis involved whole body, liver, and tumor regions of interest (ROI) being defined for each time point. Counts of whole body and liver were compared between groups as an internal control to guarantee that equal amounts of radioactivity were administered in each dose-level group. Counts of the tumors were converted to %ID/g using a standard of known activity that was scanned at the same time. Tumor volumes (mL) were determined on the basis of markup of tumors in cross-sectional CT images. To convert tumor volumes (mL) to tumor mass (g), animals were sacrificed after CT imaging, and tumors were removed and weighed (n = 12). The conversion factor was determined as the slope of the linear regression curve.

\textbf{Receptor occupancy study.} A further series of biodistribution and planar gamma camera imaging studies explored the saturation of the DR5 receptor \textit{in vivo}. Details of the experimental procedures and calculation of receptor occupancy are provided in Supplementary Data.

\textbf{Pharmacokinetics.} Mice (n = 5) were injected with \textsuperscript{111}In-labeled CS-1008 (0.2 mg/kg) at three different protein dose levels. Unlabeled CS-1008 was added to \textsuperscript{111}In-labeled CS-1008 (0.2 mg/kg) to obtain total protein doses of 0.2, 1, and 10 mg/kg. At 0.5, 1, 3, 5, 7, 24, 48, 72, 120, 168, 240, and 336 hours post injection, blood samples (10 \mu L) were collected and counted in a Wizard\textsuperscript{2} gamma counter.

A two-compartment IV bolus model with macroparameters, no lag time, and first-order elimination (WNL Model 8) was fitted to serum data for each animal using unweighted nonlinear, least squares with WinNonLin version 5.2 (Pharsight Corp.).

\textbf{Therapeutic studies.} Mice bearing WiDr tumors (100–200 mm\textsuperscript{3}) were treated with CS-1008, control IgG\textsubscript{1}, or vehicle (on day 0, 7, and 14) at 0.1, 0.3, 1.0, 3.0, 10.0, or 30.0 mg/kg dose levels (n = 5/dose level), and monitored for tumor growth using methods previously reported (9). The therapeutic effects of CS-1008 in COLO 205 tumor-bearing mice have been previously described (2).

\textbf{Statistical analysis.} Data were analyzed using one-way ANOVA when comparing multiple groups. Data were analyzed using an unpaired two-tailed Student t test when comparing two groups (treated to control). Differences were considered significant at a P value of less than 0.05. All analyses were conducted using Graphpad Prism version 4.03.

\textbf{Results}

\textbf{Flow cytometry.} COLO 205 and WiDr cells were positive for DR5 expression, with a median fluorescence for COLO 205 cells of 44.5 and WiDr cells of 29.9. CT26 cells were negative (similar to controls) with a median fluorescence of 4.6.

\textbf{TRAIL sensitivity.} COLO 205 cells showed a 50% proliferation inhibition at 3.9 ng/\mu L of mTRA-8 and 14 ng/\mu L of TRAIL, and WiDr cells at 54 ng/\mu L of TRAIL and 208 ng/\mu L of mTRA-8. CT26 cells showed minimal responsiveness to TRAIL or mTRA-8.

\textbf{Antibody radiolabeling.} Radiochemical purity assessment of radiolabeled mTRA-8, CS-1008, and control IgG\textsubscript{1} by ITLC confirmed more than 98\% bound \textsuperscript{111}In-CHX-A’DTPA. Figure 1 shows the immunoreactivity-binding curve for radiolabeled CS-1008 (Fig. 1A). The immunoreactivity of \textsuperscript{111}In-radiolabeled CS-1008 was 13.3\%. Because of the low immunoreactivity, a competition binding assay was performed to demonstrate specific binding of CS-1008 (Fig. 1B). At 10 ng/mL of CS-1008, 75\% of \textsuperscript{111}In-labeled mTRA binding could be blocked with CS-1008, indicating that CS-1008 and mTRA are competing for the same binding sites. Saturation binding analysis of \textsuperscript{111}In-labeled CS-1008 showed specific binding with high-affinity binding sites on COLO 205 cells (Fig. 1C). The apparent K\textsubscript{s} value for \textsuperscript{111}In-labeled CS-1008 was 1.05 ± 0.12 × 10\textsuperscript{-9} mol/L. The number of binding sites per cell was 6,578 ± 284 binding sites per cell. The stability of the radiolabeled \textsuperscript{111}In-CS-1008 in normal human serum at

\textbf{Molecular Imaging of DR5 with Tigatuzumab}
37°C was high, with retention of immunoreactivity, radiocolidal purity, and construct integrity for up to 11 days.

ELISA
The labeling of CS-1008 with 111In showed minimal impact on binding to DR5 (TRAIL R2; Fig. 1D).

Biodistribution
In an initial biodistribution study, tumor uptake and biodistribution of 111In-labeled CS-1008 at a dose of 0.2 mg/kg was studied in mice bearing human COLO 205 tumors and mice bearing murine CT26 tumors (Table 1 and Supplementary Table S1). CS-1008 does not cross-react to the rodent death receptor and therefore a murine colon cancer cell line was chosen as negative control. The %ID/g of 111In-labeled CS-1008 (0.2 mg/kg) in COLO 205 tumors peaked at 168 hours post injection (%ID/g, 26.25 ± 2.85).

The tumor:blood ratio for 111In-labeled CS-1008 increased over time and peaked at 2.92 ± 0.53 at 240 hours post injection. The tumor uptake of 111In-labeled CS-1008 in

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aData presented as %ID/g tissue ± SD (n = 5).
CT26 DR5-negative tumor–bearing mice was significantly less than that seen in COLO 205 DR5-positive tumor–bearing mice at 72 and 168 hours post injection. Maximum uptake of $^{111}$In-labeled CS-1008 in CT26 was observed at 72 hours post injection (%ID/g, 11.79/C6 1.68; Fig. 2A).

The specific uptake in vivo was confirmed by an additional biodistribution study comparing $^{111}$In-labeled CS-1008 at a dose level of 10 mg/kg with $^{111}$In-labeled IgG1 isotype control hu3S193 at a dose level of 10 mg/kg in COLO 205 tumor-bearing mice at different time points after injection. At the 10 mg/kg dose level, $^{111}$In-CS-1008 tumor uptake was lower at all time points compared with the 0.2 mg/kg dose level, and peaked at 72 hours post injection (%ID/g, 11.64/C6 1.59, n=4). The isotype control antibody showed an initial tumor uptake of 5.55/C6 0.97%ID/g at 3 hours post injection, declining progressively over time.

A further biodistribution study of $^{111}$In-labeled CS-1008 (0.2 mg/kg) in WiDr and CT26 tumor-bearing mice was performed and showed specific localization of $^{111}$In-labeled CS-1008 in WiDr tumors, but at a lower level than in COLO 205 tumors (Fig. 2C and Supplementary Table S2).

Gamma camera imaging and DR5 occupancy of $^{111}$In-CHX-A”-DTPA-CS-1008 in vivo

Whole-body imaging of $^{111}$In-labeled CS-1008 (0.2 mg/kg) in BALB/c nu/nu mice bearing DR5-positive COLO 205 or DR5-negative CT26 tumors was performed. Tumor uptake of $^{111}$In-labeled CS-1008 was evident as of day 2 post injection and continued for more than 7 days (Supplementary Fig. S1). Initial blood pool activity was seen on day 0 and decreased over time. In a further series of imaging studies, tumor uptake of $^{111}$In-labeled CS-1008 at different antibody dose levels was conducted to explore the saturation of DR5 in vivo in tumors with different levels of DR5 expression, high (COLO 205) and low (WiDr). Six groups of mice (n=4) were injected with $^{111}$In-labeled CS-1008 (5 μg, 8.5 μCi) at different dose levels of unlabeled CS-1008 (0.2, 0.5, 1, 3, 10, and 30 mg/kg). Planar gamma camera imaging and CT imaging were conducted at 48 hours post injection when the effect of CS-1008 on tumor size was minimal (Fig. 2B and D). Figure 3 shows representative planar gamma camera images and CT scans at 48 hours post injection for 0.2 mg/kg (Fig. 3A), 0.5 mg/kg (Fig. 3B), and 10 mg/kg (Fig. 3C) CS-1008 in COLO 205 tumor-bearing mice. After imaging, mice were sacrificed; tumors were weighed and counted for radioactivity. A terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (Supplementary Fig. S2) of COLO 205 tumor sections collected from mice treated with 0.2 mg/kg (Fig. 3A) and 10 mg/kg (Fig. 3C) CS-1008 in COLO 205 tumor-bearing mice showed an increase in apoptosis in tumors from animals treated with 10 mg/kg compared with animals treated with 0.2 mg/kg. Supplementary Fig. S3 shows representative images of WiDr tumor-bearing mice at 48 hours post injection for 0.2 mg/kg (Supplementary Fig. S3A), 0.5 mg/kg (Supplementary Fig. S3B), and 10 mg/kg (Supplementary Fig. S3C) CS-1008. Figure 3D shows %ID/g in the COLO 205 tumors at different doses (n=4). At 48 hours post injection at a 0.2 mg/kg dose level, tumor uptake of $^{111}$In-labeled CS-1008 was highest (28.15 ± 2.02%ID/g; n=4), declining progressively with increased doses of cold CS-1008. DR5 saturation starts at 1 mg/kg dose level and is complete at
3 mg/kg. Figure 3E shows the %ID/cm³ curve generated from region of interest (ROI) analysis of tumor images.

To correct for the difference between tumor volumes (cm³) measured by CT analysis and tumor volumes (g) weighed, a correlation curve was established and conversion tumor volumes (cm³) into tumor volumes (g) required a correlation factor of 0.69 ± 0.01. Figure 3F shows the %ID/g generated from image analysis after correcting the tumor volumes and this curve is consistent with the curve generated from the biodistribution data (Fig. 3D). The results for WiDr tumor analysis are shown in Fig. 3G.

Using a baseline value of 5.07%ID/g at 48 hours post injection of 0.2 mg/kg ¹¹¹In-labeled isotype control, a receptor occupancy curve was generated. Figure 4A shows the occupancy of COLO 205 DR5 antigen binding sites by ¹¹¹In-CS-1008 with increasing protein dose at 48 hours post injection. To compare receptor occupancy at different dose levels with in vivo tumor growth inhibition (TGI) at different dose levels, TGI values (%) measured by Yada and colleagues were plotted against the imaged CS-1008 antibody dose levels (Fig. 4B; ref. 2). The curves in Fig. 4A and B show the same saturation trend, with DR5 saturation occurring between 1 and 3 mg/kg dose levels. WiDr tumors showed a similar trend for receptor saturation between 1 and 3 mg/kg dose levels (Fig. 4C), and this receptor saturation level also predicted the dose of maximal therapeutic response (Fig. 4D). The level of TGI was less with WiDr tumors than with COLO 205 tumors, in keeping with the reduced DR5 expression and lower concentration of CS-1008 achieved in tumors in vivo.

Pharmacokinetics

No differences in serum clearance were observed between different antibody dose levels (0.2, 1 mg/kg vs. 10 mg/kg) with mean values (n = 15; SD) for α half-life (tᵌ/₂α) of 5.71 ± 0.91 hours, β half-life (tᵌ/₂β) of 141.78 ± 20.69 hours, total clearance (C_L) of 0.02 ± 0.1 mL/h, and volume of distribution at steady state (Vss) of 4.47 ± 0.25 mL. The pharmacokinetic parameters for each dose level are shown in Table 2.

Discussion

We have shown that CS-1008, a humanized IgG₁ anti-DR5 antibody, demonstrates specific uptake in TRAIL-sensitive, DR5-positive tumors that is dose dependent and in proportion to DR5 expression in tumor cells. Moreover, CS-1008 has tumor inhibitory effects that are dependent on CS-1008 tumor concentration and reach maximal efficacy when antibody:DR5 binding is saturated, which can be quantitatively measured by molecular imaging of receptor occupancy in vivo with ¹¹¹In-CS-1008.
Anti-DR5 antibodies were successfully chelated and radiolabeled with $^{111}$In-CHX-A$^\text{D}$TTPA, with both $^{111}$In-labeled mTRA and CS-1008 showing high-affinity binding for DR5 expressing COLO 205 cells. Retention of binding affinity of $^{111}$In-CHX-A$^\text{D}$TTPA-CS-1008 following radiolabeling was confirmed by ELISA. The in vivo biodistribution and imaging studies showed specific DR5-positive tumor localization, with prolonged retention in COLO 205 tumors despite the low receptor number available for CS-1008 binding observed in saturation binding analysis (6,578 receptors per cell). The number of binding sites on COLO 205 cells is consistent with the number of DR5 found on other cell lines: the number of DR5 per MIA PaCa–2 cell was 8,620 ± 1,920, whereas that per 2LMP cell was 11,170 ± 4,170, without significant difference between the two cell lines ($P = 0.6141$; ref. 16). WiDr tumors, which have lower DR5 expression, also demonstrated specific uptake at lower levels than seen in COLO 205 tumors.

Endocytosed $^{111}$In-DTPA antibodies are delivered to lysosomes and hydrolyzed by lysosomal enzymes into small molecular weight metabolites that are retained within tumor lysosomes (17, 18). Consistent with a low receptor number expressed on the cell membrane, accumulation of $^{111}$In-CHX-A$^\text{D}$TTPA-CS-1008 in DR5-positive tumors was gradual with high tumor uptake by 72 hours, and maintaining or slightly increasing uptake over a 1-week period. In contrast to the COLO 205 and WiDr xenografts, no specific uptake of radiolabeled $^{111}$In-CS-1008 was observed in DR5-negative CT26 tumors. Pharmacokinetic analysis showed no differences between increasing dose levels of unlabeled CS-1008. The uptake of $^{111}$In-labeled CS-1008 in all normal tissues was consistent with blood pool activity. Other DR5 imaging antibodies reported on are $^{99m}$Tc-HYNIC-mTRA (19, 20) and $^{64}$Cu-DOTA-conatumumab (21). Comparable tumor uptake (%ID/g) was observed with $^{111}$In-labeled CS-1008, but in contrast with $^{64}$Cu-DOTA-conatumumab, no

<table>
<thead>
<tr>
<th>CS-1008 (mg/kg)</th>
<th>$C_{\text{max}}$ (µg/mL)</th>
<th>AUC$_{24}$ (µg/mL h)</th>
<th>$t_{1/2a}$ (h)</th>
<th>$t_{1/2b}$ (h)</th>
<th>CL (mL/h)</th>
<th>$V_{ss}$ (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>1.7 ± 0.1</td>
<td>124.5 ± 11.5</td>
<td>6.1 ± 0.9</td>
<td>120.1 ± 18.7</td>
<td>0.03 ± 0.01</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>7.7 ± 0.4</td>
<td>746.2 ± 101.4</td>
<td>4.7 ± 1.1</td>
<td>143.9 ± 29.4</td>
<td>0.02 ± 0.01</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>60.7 ± 2.6</td>
<td>7,784.4 ± 2,101.7</td>
<td>6.4 ± 2.1</td>
<td>161.3 ± 61.7</td>
<td>0.02 ± 0.01</td>
<td>4.8 ± 0.6</td>
</tr>
</tbody>
</table>

Abbreviations: $C_{\text{max}}$, maximum plasma–serum concentration; AUC, area under the curve; $t_{1/2a}$, serum distribution half-life; $t_{1/2b}$, serum elimination half-life; CL, clearance; $V_{ss}$, volume of distribution at steady state.
splenic uptake was seen with $^{111}$In-labeled CS-1008. Radiiodinated recombinant human TRAIL has also been explored as a biomarker for DR4 and DR5 targeting (22).

Potent antitumor activity of CS-1008 has been previously demonstrated in vitro and in vivo against the colorectal cell line COLO 205, with tumor growth inhibition observed in a dose-dependent manner up to 1 mg/kg dose level (2). We have demonstrated that receptor occupancy and saturation can be accurately measured by molecular imaging of $^{111}$In-CHX-A-DTPA-CS-1008 imaging in COLO 205 tumors, and that receptor occupancy and saturation correlate precisely with the dosage required for maximal tumor growth inhibition. We have also demonstrated that in tumors with lower levels of DR5 expression (WiDr), similar correlations can be made through molecular imaging of DR5 occupancy and saturation. Interestingly, DR5 expression levels in tumor (assessed by peak uptake of $^{111}$In-CHX-A-DTPA-CS-1008 in tumor) also correlated with the maximal tumor growth inhibition achieved, with COLO 205 tumors showing higher uptake and tumor inhibition than WiDr tumors.

Calculation of antigen-binding site occupancy from the imaging data showed that DR5 binding is saturable at protein doses above 1 mg/kg. Assuming a cell diameter of 10 μm for COLO 205 and WiDr cells, measured CT tumor volumes and an immunoreactive fraction of 10%, approximately three antibodies per receptor at 1 mg/kg were enough to saturate the tumor and elicit maximum therapeutic effect in vivo. This finding indicates the importance of dose selection on achieving optimal concentration of CS-1008 in tumor, and the ability of molecular imaging approaches to quantitate receptor occupancy in vivo.

There are several factors that might influence the success of imaging DR5 occupancy in patients and might cause patient variability in therapy outcome when using $^{111}$In-labeled anti-DR5 antibodies in the clinic. First, when calculating %ID/g tumor in the mouse models, 1% of the total labeled anti-DR5 antibodies in the clinic. First, when calculating $^{111}$In-CS-1008 in tumor, and the ability of molecular imaging approaches to quantitate receptor occupancy in vivo.

There are several factors that might influence the success of imaging DR5 occupancy in patients and might cause patient variability in therapy outcome when using $^{111}$In-labeled anti-DR5 antibodies in the clinic. First, when calculating %ID/g tumor in the mouse models, 1% of the total injected $^{111}$In-CS-1008 was typically measured in the tumors of which 60% was specific. When translating these findings into the clinic, many solid tumors in patients only show values around 0.01% of the injected dose when using radiolabeled antibodies. Using $^{111}$In-labeled CS-1008 might help to select the patients that show sufficient tumor uptake of $^{111}$In-CS-1008 at tracer levels of cold CS-1008 (e.g., 0.2 mg/kg). Second, Fcγ receptors have been proposed as an Fc cross-linking interface in vivo promoting DR5-mediated agonistic cell killing by anti-DR5 antibodies (23). In patients, these Fcγ receptors might be available in the tumor via infiltrating immune cells, but the population and the amount might vary among patients. Third, the data presented here show DR5 occupancy and therapeutic response to CS-1008 in COLO 205 and WiDr tumor models; however, in cells with similar or higher DR5 expression levels, but with slower induction of the apoptotic pathway, receptor turnover, receptor internalization, and antibody pharmacokinetics might play a much larger role. Although the results here have shown proof-of-principle that $^{111}$In-CHX-A-DTPA-CS-1008 can aid in patient selection and estimate optimal therapeutic dosing, further studies are warranted comparing resistant versus sensitive tumor models, and exploring the role of apoptosis pathway induction on effectiveness of treatment (24). Such studies are currently being performed by our laboratory.

In conclusion, these results suggest that molecular imaging of DR5 occupancy and saturation can play a major role for clinical development, optimal dosing, and patient selection for trials of DR5-targeting antibodies.

Disclosure of Potential Conflicts of Interest

R.A. Beckman is founder and chief scientific officer at Onco-Mind LLC. No potential conflicts of interest were disclosed by the other authors.

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References


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