**Supplementary Materials**

**Supplementary Figures**

Figure S1 – Immunogenicity of P53-SADA-BsAb

Experiment schematic (top) and results (bottom). Mice (n=5) were immunized with P53-SADA-BsAb or IgG-scFv-BsAb and bled 4 weeks later. Mice received a follow up dose of BsAb and were bled again 4 weeks later. Anti-BsAb titers were measured by ELISA and normalized to a monoclonal anti-BsAb standard. Statistical significances were calculated using a Mann Whitney test. \*\*P = 0.0079 for IgG-scFv-BsAb compared to P53-SADA-BsAb.

Figure S2 – Toxicology analysis of DOTA[177Lu] treated nude mice

(A) White blood cell (WBC, top left), Red blood cell (RBC, top right), and platelet (PLT, bottom left) counts from mouse blood. All mice were bled 14 days after the first dose of DOTA[177Lu]. Each symbol refers to a single mouse (n=10). The black dotted line refers to mean values from age-matched mice irradiated with 300 cGy of total body irradiation (TBI) on day 0, and the grey bar represents the one standard deviation above and below this mean. FLT3L levels in plasma of treated mice (bottom right). All mice were bled 21 days after the first dose of DOTA[177Lu]. Each symbol refers to a single mouse (n=10). (B) Weight change in treated mice. Weights were monitored at least once per week and normalized to each individual mouse’s pre-treatment weight. Each solid line represents one treatment group (n=10). The dotted black line represents 15% increases or decreases in weight. Average weights were calculated until at least one mouse had to be euthanized. Data are shown as means ± standard deviation. (C) Summary of ovarian and bladder toxicity (left), with representative H&E slides of ovaries from treated nude mice (right). Normal ovary (top, littermate control), grade 3 atrophied ovary (left, P53-SADA-BsAb) and grade 4 atrophied ovary (right, IgG-scFv-BsAb). Mice were sacrificed between day 110 and day 230 after treatment start.

Figure S3 – Toxicology analysis of DOTA[177Lu] treated BRG mice

(A) White blood cell (WBC, left), Red blood cell (RBC, center), and platelet (PLT, right) counts from mouse blood. All mice were bled 14 days after the first dose of DOTA[177Lu]. Each symbol refers to a single mouse (n=5). (B) Weight change in treated mice. Weights were monitored at least once per week and normalized to each individual mouse’s pre-treatment weight. Each solid line represents one treatment group (n=5). The dotted black line represents 15% increases or decreases in weight. Average weights were calculated until at least one mouse had to be euthanized. Data are shown as means ± standard deviation. (C) Graphical representation of bladder pathologies observed in treated mice (left), with representative H&E slides of bladders from treated BRG (right). Each bar represents one treatment group (n=5). Y-axis values represent the percentage of analyzed mice displaying the toxicity. Red represents grade 4 toxicity (bottom right), orange represents grade 3 (bottom left), green represents grade 2 (top right), and blue represents no pathologies (top, left). Mice were sacrificed at day 120 after treatment start.

Figure S4 – Toxicology analysis of Proteus[225Ac] treated BRG mice.

(A) White blood cell (WBC, left), Red blood cell (RBC, center), and platelet (PLT, right) counts from mouse blood. All mice were bled 14 days after the first dose of DOTA[177Lu]. Each symbol refers to a single mouse (n=5). (B) Weight change in treated mice. Weights were monitored at least once per week and normalized to each individual mouse’s pre-treatment weight. Each solid line represents one treatment group (n=5). The dotted black line represents 15% increases or decreases in weight. Average weights were calculated until at least one mouse had to be euthanized. Data are shown as means ± standard deviation. (C) Graphical representation of kidney pathologies observed in treated mice (left) with representative slides of kidneys from treated mice (right). All pathologies were measured as the number of observations per 10-consecutive fields, beginning with the field containing the most pathologies. Each group (x-axis) represents one treatment group or age-matched littermate control, and each individual scatter plot represents a different stain for kidney damage. Blue represents tubular proteinosis (bottom right), red represents epithelial cell apoptosis (bottom left), green represents Cleaved Caspase 3 (CC-3) positive cells (top left), and purple represents Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells (top right). Mice were sacrificed between day 100 and 120 after treatment start.

**Supplementary Tables**

Table S1 – Structural properties of candidate SADA domains

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Protein Name | Gene Name | Sequence | PDB ID | Size of monomer (kDa) | Surface area between dimers (Å2) | H bonds between dimers (#) | Surface area between monomers (Å2) | H bonds between monomers (#) | Total surface area (Å2) |
| Tumor protein 53 | TP53 | 326-357 | 2J0Z | 3.8 | 242 | 3 | 478 | 20 | 1199 |
| Tumor protein 63 | TP63 | 358-401 | 4A9Z | 7.3 | 1188 | 33 | 646 | 32 | 2480 |
| Tumor protein 63 | TP73 | 355-398 | 2WQI | 6.1 | 1066 | 32 | 617 | 24 | 2301 |
| Heterogeneous nuclear ribonucleoproteins C1/C2 | HNRNPC | 180-207 | 1TXP | 3.3 | 630 | 3 | 172 | 4 | 973 |
| Synaptosomal-associated protein 23 | SNAP23 | 28-81 | 1NHL | 6.2 | 957 | 16 | 465 | 9 | 1887 |
| Protein CBFA2T1 | RUNX1T1 | 462-521 | 4JOL | 7.5 | 1207 | 18 | 514 | 15 | 2235 |

Summary of candidate SADA domains. The sequence refers to the specific amino acids used, counting from the N-terminal amino acid. PDB ID refers to a referenced crystal structure. The molecular size of monomer displays the theoretical molecular weight for each SADA domain. The surface areas and the number of hydrogen bonds were calculated using Discovery Studio.

Table S2 – Biochemical properties of candidate SADA-BsAb

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| SADA-BsAb | SADA domain | Estimated monomer size (kDa) | Yield | High molecular weight aggregates (%) | Tetramer purity (%) | Low molecular weight products (%) | Stability @ 37°C |
| hu3F8-scFv\_huC825-scFv\_P53 | P53 | 53.8 | >5mg/L | 10.1 | 89.4 | 0.5 | >3 weeks |
| hu3F8-scFv\_huC825-scFv\_P63 | P63 | 57.3 | >5mg/L | 6.8 | 92.8 | 0.5 | >3 weeks |
| hu3F8-scFv\_huC825-scFv\_P73 | P73 | 56.1 | >5mg/L | 10.8 | 88.4 | 0.9 | >3 weeks |
| hu3F8-scFv\_huC825-scFv\_HNRNPC | HNRNPC | 53.3 | >5mg/L | 3.8 | 92.8 | 3.3 | - |
| hu3F8-scFv\_huC825-scFv\_SNAP23 | SNAP23 | 56.2 | - | - | - | - | - |
| hu3F8-scFv\_huC825-scFv\_ RUNX1T1 | RUNX1T1 | 57.5 | - | - | - | - | - |

Summary of candidate SADA-BsAb proteins. Total monomer size was calculated assuming 25 kDa for each scFv. Yield was calculated from at least 2 transfections using expi293 cells. Purity was determined by SEC-HPLC. High and low molecular weight impurities were defied as peaks before or after the main peak, respectively. Stability was determined by incubation at 37 °C with weekly quantitation by SEC-HPLC.

Table S3 – Pharmacokinetics of SADA-BsAb

|  |  |
| --- | --- |
| Protein | P53-SADA-BsAb |
| Terminal Half-life (hr) | 8.9±1.4 |
| Volume of distribution (ml) | 1.2±0.3 |
| Mean Residence Time (hr) | 10.4±2.8 |
| AUC (ml/hr) | 10.9±2.1 |

Summary of the pharmacokinetic properties of P53-SADA-BsAb. NSG mice (n=10) were serially bleed from 0.5 to 168 hours after intravenous BsAb administration (100 mg). Pharmacokinetic analysis was carried out by non-compartmental analysis of the serum concentration-time data using WinNonlin software program (Pharsight Corp.).

Table S4 – SADA PRIT Dosimetry

|  |  |  |
| --- | --- | --- |
|  | Dose (cGy/MBq) | Ratio (Tumor:Organ) |
| Organs | P53-SADA-BsAb | P53-SADA-BsAb |
| Blood | 2.9 | 109.0 |
| Brain | 0.1 | 2575.4 |
| Lower Large Intestine Wall | 2.6 | 123.6 |
| Small Intestine | 1.4 | 235.6 |
| Stomach Wall | 0.6 | 564.9 |
| Upper Large Intestine Wall | 2.6 | 123.6 |
| Heart Wall | 1.0 | 313.8 |
| Kidneys | 12.7 | 25.1 |
| Liver | 10.0 | 31.9 |
| Lungs | 2.3 | 137.7 |
| Muscle | 0.5 | 581.7 |
| Bone | 1.8 | 180.9 |
| Spleen | 32.8 | 9.7 |
| Tumor | 319.6 | - |

Dosimetry estimates calculated from mouse biodistribution studies, and their corresponding tumor-to-non-tumor ratios. Tumor bearing mice (n=3-5 per time point) were dosed with each BsAb (1.25 nmol) and DOTA[177Lu] (18.5 MBq), 48 hours apart. Mice were sacrificed either 2, 24, 48, or 120 hours after payload delivery.

Table S5 – PET/CT Dosimetry

|  |  |  |  |
| --- | --- | --- | --- |
| Treatment | IgG-scFv-BsAb  No CA (2-step) | IgG-scFv-BsAb + CA (3-step) | P53-SADA-BsAb  (2-step) |
| Blood | 16.4 | 2.8 | 0.1 |
| Tumor | 58.8 | 39.3 | 31.0 |
| Heart | 6.4 | 1.2 | 0.2 |
| lungs | 7.7 | 1.8 | 0.5 |
| liver | 6.5 | 2.1 | 0.8 |
| spleen | 8.2 | 2.3 | 0.8 |
| stomach | 1.2 | 0.2 | 0.1 |
| small intestine | 1.8 | 1.0 | 0.3 |
| large intestine | 3.4 | 1.2 | 0.7 |
| kidneys | 5.5 | 2.0 | 2.5 |
| muscle | 1.7 | 0.3 | 0.1 |
| bone | 3.5 | 0.0 | 0.6 |
| tail | 4.0 | 1.1 | 0.7 |

Summary of tissue biodistribution of DOTA[86Y] after PET/CT scan. Tumor bearing mice were dosed with each BsAb (1.25 nmol) and DOTA[86Y] (3.7 MBq), 48 hours apart, and sacrificed immediately after imaging. Values are normalized to percentage of injected dose per gram of tissue (%ID/g). 2-step IgG-scFv-BsAb treated mice did not receive clearing agent (CA). 3-step IgG-scFv-BsAb treated mice received 25 g of CA.

Table S6 – Summary of Mouse Serum Chemistry, Complete Blood Counts, and Histopathology: Nude mice with DOTA[177Lu] payload

|  |  |  |
| --- | --- | --- |
| Treatment | IgG-scFv-BsAb  DOTA[177Lu] 3x-3x  n=9 | P53-SADA-BsAb  DOTA[177Lu] 3x-3x  n=9 |
| Serum chemistry | Normal | Normal |
| Complete blood counts | Normal | Normal |
| Alimentary and Hepatobiliary Systems | Normal | Normal |
| Skeletal and Cardiovascular System | Normal | Normal |
| Endocrine System - Adrenals\* | Cortical hyperplasia and hypertrophy  day 230 n=3/3 | Normal |
| Endocrine System - Other | Normal | Normal |
| Immune System | Normal | Normal |
| Ocular and Nervous Systems | Normal | Normal |
| Reproductive System - Ovaries\* | Ovarian atrophy  day 111 n=2/3 day 155 n=2/3  day 230 n=3/3 | Ovarian atrophy day 155 n=1/3 |
| Reproductive System - Other | Normal | Normal |
| Respiratory System | Normal | Normal |
| Urinary System - Bladder\* | Urothelial hyperplasia with neutrophilic and lymphocytic infiltrate  day 111 n=1/3 | Urothelial hyperplasia, eosinophilic infiltrate, and fibroplasia day 230 n=1/3 |
| Urinary System - Other | Normal | Normal |
| Other | Normal | Normal |

Summary of nude mouse toxicology analysis. Interpretation was performed by board-certified veterinary pathologists. Normal was defined as being not significantly different from untreated age-matched littermate control mice, or within known normal ranges for this strain of mice at this age. Histopathologic abnormalities were determined by microscopic analysis of H&E slides. Mice were submitted for assessment 111, 155 and 230 days after treatment began.

Table S7 – Summary of Mouse Serum Chemistry, Complete Blood Counts, and Histopathology: BRG mice with DOTA[177Lu] or Proteus[225Ac]

|  |  |  |
| --- | --- | --- |
| Treatment | P53-SADA-BsAb  DOTA[177Lu] 3x-3x  n=5 | P53-SADA-BsAb  Proteus[225Ac] 1x-1x  n=3 |
| Serum chemistry | Normal | Normal |
| Complete blood counts | Normal | Normal |
| Alimentary and Hepatobiliary Systems | Normal | Normal |
| Skeletal and Cardiovascular Systems | Normal | Normal |
| Endocrine System | Normal | Normal |
| Immune System | Normal | Normal |
| Ocular and Nervous Systems | Normal | Normal |
| Reproductive System | Normal | Normal |
| Respiratory System | Normal | Normal |
| Urinary System – Other | Normal | Normal |
| Urinary System - Bladder\* | Urothelial degeneration, necrosis, hyperplasia, ulceration, with fibrosis  day 120 n=5/5 | Normal |
| Urinary System - Kidneys (H&E) | Normal | Normal |
| Urinary System - Kidneys (TUNEL + CC3 IHC) | Normal | Normal |
| Other | Normal | Normal |
|  |  |  |  |  |

Summary of DKO mouse toxicology analysis. Interpretation was performed by board-certified veterinary pathologists. Normal was defined as being not significantly different from untreated age-matched littermate control mice, or within known normal ranges for this strain of mice at this age. Histopathologic abnormalities were determined by microscopic analysis of H&E slides. Mice were submitted for assessment 120 days after treatment began. CC-3: Cleaved caspase-3 immunohistochemistry.

**Supplementary Videos**

Video S1 – Representative PET/CT of P53-SADA-BsAb + DOTA[86Y]

Rotating PET/CT overlay video of a representative mouse bearing a subcutaneous tumor on its right shoulder, treated with P53-SADA-BsAb and DOTA[86Y].

Video S2 – Representative PET/CT of IgG-scFv-BsAb + clearing agent + DOTA[86Y]

Rotating PET/CT overlay video of a representative mouse bearing a subcutaneous tumor on its right shoulder, treated with IgG-scFv-BsAb, clearing agent, and DOTA[86Y].

Video S3 – Representative PET/CT of IgG-scFv-BsAb + DOTA[86Y]

Rotating PET/CT overlay video of a representative mouse bearing a subcutaneous tumor on its right shoulder, treated with IgG-scFv-BsAb and DOTA[86Y].

**Supplementary Methods**

*Radiometal labeling*

For DOTA[86Y], S-2-(4-Aminobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA, Macrocyclics, Cat# B-200, CAS 181065-46-3) was mixed with 86Y nitrate (Radiological Chemistry and Imaging Laboratory at Washington University in St. Louis) 60 minutes at 80 °C. Labeled DOTA was separated from free radiometal by passing through a sepak column (Waters). For DOTA[177Lu], DOTA was incubated with 177LuCl3 (Perkin Elmer) at a ratio of 37 MBq to 1.1 mmol of DOTA in ammonium acetate (pH 5.6) for 60 minutes at 85 °C. For Proteus[225Ac], 225Ac nitrate (Oak Ridge National Laboratory) was mixed with Proteus for 30 min at 60°C. After incubation the sample was purified using a Sephadex C-25 column (GE) pre-equilibrated with 6 mL of normal sterile isotonic saline solution (NSS).

*Pharmacokinetic analysis*

NSG mice were injected with 100 g of P53-SADA-BsAb and bled serially over 7 days (30 minutes – 168 hours). Blood was processed as plasma and frozen until all samples were acquired. Plasma concentrations of BsAb were determined by ELISA. Briefly, for each plate, half of the wells were coated with ganglioside GD2 overnight at 4°C (EMD Millipore, Cat# 345743, 1ug/ml in 90% ethanol, 20ul/well), and half were left blank. Plates were washed with PBS and blocked with PBS supplemented with 0.5% bovine serum albumin (Sigma, Cat# A7906) for one hour at room temperature. Plasma samples were added at 1:100 and 1:200 dilutions (> 48 hours) or 1:2000 and 1:4000 dilutions (0.5-24 hours) in duplicate across both coated and uncoated wells and incubated at 37°C for 2.5 hours. P53-SADA-BsAb was used as a standard curve (100 ng/ml to 0.41 ng/ml, 3-fold dilutions). Samples were detected using a mouse anti-HIS specific secondary antibody (Biorad, clone AD1.1.10, Cat# MCA1396) for one hour at room temperature. Samples were then incubated with a rat anti-mouse detection antibody conjugated to horse-radish peroxidase (Jackson ImmunoResearch, Cat# 415-035-166) for one hour at 4°C. The color reaction was developed with o-phenylenediamine (Sigma, Cat# P8287-100TAB, 150 ul/well) and stopped with 5N sulfuric acid (30 ul/well). Plates were read at 490 nm using a Biotek H1 plate reader (Synergy) with the Gen5 software (version v2.09). Protein concentrations were calculated using a standard curve fitted to a linear regression. Pharmacokinetic analysis was carried out by non-compartmental analysis of the serum concentration-time data using WinNonlin software program (Pharsight Corp.).

Serum clearance measurements were also determined using 131I-labeled SADA-BsAb. SADA-BsAb were labeled with 131I (IBA Molecular or MSKCC) using precoated IODOGEN tubes (Pierce) as previously described for the radioiodination of the IgG-scFv-BsAb8. Purity of the 131I-SADA-BsAb was validated by SEC-HPLC. Each mouse (nude, tumor free) was injected with 740 kBq of 131I-SADA-BsAb and bled serially (0.5 to 48 hours). Blood samples were radio-assayed on a gamma counter (PerkinElmer, Wallac Wizard 3 automatic gamma counter) and plotted using GraphPad Prism 8.

*Immunogenicity Analysis*

C57BL/6J mice were injected with P53-SADA-BsAb or IgG-scFv-BsAb (0.5 nmol) on days 0 and 28, intravenously and intraperitoneally, respectively. Mice were bled retro-orbitally on days 27 and 55. Blood was processed as plasma and frozen at -80°C until all samples were acquired. Plasma concentrations of each BsAb were determined by ELISA. Briefly, for each plate, half of the wells were coated with P53-SADA-BsAb or IgG-scFv-BsAb (10 g/ml in PBS, 50 l/well) overnight at 4°C, and the other half were left blank). After this, plates were washed with PBS and blocked with PBS supplemented with 0.5% bovine serum albumin (Sigma, A7906) for one hour at room temperature. Plasma samples were added at 1:100 and 1:200 dilutions in duplicate across both coated and uncoated wells and incubated at 37°C for 2.5 hours. A standard curve was generated using either mouse-anti-HIS antibody (P53-SADA-BsAb) or an anti-human IgG-hinge (IgG-scFv-BsAb, Southern Biotech, Clone 4E3, Cat# 9052-01) monoclonal antibodies. Next, samples were detected with a goat anti-mouse antibody detection antibody conjugated to horse-radish peroxidase (Jackson ImmunoResearch, Cat# 115-005-003). The color reaction was developed with o-phenylenediamine (Sigma, Cat# P8287-100TAB, 150 ul/well) and stopped with 5 N sulfuric acid (30 l/well). Plates were read at 490 nm using a Biotek H1 plate reader (Synergy) with the Gen5 software (version v2.09). Protein concentrations were estimated using a standard curve fitted to a linear regression. Data was plotted using GraphPad Prism 8.

*Anatomic and Clinical Pathology for Toxicology Assessment*

Mice were sacrificed by carbon dioxide asphyxiation, and immediately dissected and fixed in 10% neutral buffered formalin. Age-matched littermates were used as reference in all studies. Tissues were processed in ethanol and xylene and embedded in paraffin in a Leica ASP6025 tissue processor. Paraffin blocks were sectioned at 5 microns, stained with hematoxylin and eosin (H&E), and histopathologic examination was performed by two board-certified veterinary pathologists. (SM, AOM). The following tissues were processed and evaluated: heart, lungs, thymus, kidneys, liver, gallbladder, stomach, duodenum, jejunum, ileum, cecum, colon, mesenteric lymph node, salivary glands, submandibular lymph node, uterus, cervix, vagina, urinary bladder, spleen, pancreas, adrenals, ovaries, oviducts, trachea, esophagus, thyroid, parathyroid, skin (trunk, perigenital, head), mammary glands, bones (femur, tibia, sternum, vertebrae, skull), bone marrow (femur, tibia, sternum, vertebrae), stifle joint, skeletal muscles (hind limb, spine), nerves (hind limb, spine), spinal cord, oral cavity, teeth, nasal cavity, eyes, harderian gland, pituitary, brain, ears. For serum chemistry, blood was collected into tubes containing a serum separator and centrifuged. Serum samples were analyzed on an AU 680 chemistry analyzer (Beckman Coulter Inc, Pasadena, CA) and the concentration of the following analytes was determined: alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, creatine kinase, gamma-glutamyl transpeptidase, albumin, total protein, globulin, total bilirubin, blood urea nitrogen, creatinine, cholesterol, triglycerides, glucose, calcium, phosphorus, chloride, potassium, and sodium. Na/K ratio, albumin/globulin ratio were calculated. For hematology, blood was collected into tubes containing EDTA and automated Complete blood counts (CBC) were performed on a Procyte Dx (Idexx laboratories Inc., Westbrook, ME) with manual differential performed by blood smear examination for validation. For further kidney analysis, sections of kidney were also stained with the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method as previously described56 and IHC against cleaved caspase 3 (Cell Signaling Technology Inc., Cat# 9661) were performed on a Leica Bond RX automated stainer using Bond reagents (Leica Biosystems, Buffalo Grove, IL). Following heat-induced epitope retrieval in a citrate buffer, the primary antibody was applied at a 1:250 concentration and was followed by a polymer detection system (Novocastra Bond Polymer Refine Detection, Leica Biosystems, Cat# DS9800). The chromogen was 3,3 diaminobenzidine tetrachloride (DAB), and sections were counterstained with hematoxylin. The total number of TUNEL positive and CC-3 immunoreactive cells were counted in ten, 400x fields on an Olympus BX45 microscope with a UPlanFL 40x/0.75 objective (Olympus Corp., Tokyo, Japan).