A human monoclonal antibody specific to placental alkaline phosphatase, a marker of ovarian cancer

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Introduction

Most chemotherapeutic agents used for cancer therapy do not selectively localize to solid tumors or metastatic masses, which leads to suboptimal pharmacological activity and potential damage to normal organs.¹ To overcome this limitation, monoclonal antibodies (mAbs) are increasingly being considered as “delivery vehicles” for bioactive payloads, such as cytotoxic drugs,²,³ radionuclides⁴ or cytokines.⁵

Ovarian cancer accounts for approximately 3% of all cancers in women and is the fifth leading cause of cancer-related death among women in the United States. Due to the lack of early symptoms and of effective ovarian cancer screening tests, it has the highest mortality of all cancers of the female reproductive system (National Cancer Institute). The biology of ovarian carcinoma differs from that of hematogenously metastasizing tumors because ovarian cancer cells primarily disseminate within the peritoneal cavity and are only superficially invasive. However, since the rapidly proliferating tumors compress visceral organs and are only temporarily chemo-sensitive, ovarian carcinoma is a deadly disease, with a cure rate of only 30%.⁶ Catumaxomab (Removab®, Fresenius/Trion), a bispecific antibody that simultaneously recognizes the tumor-associated EpCam antigen and CD3, received marketing authorization in 2009 for the treatment of ovarian cancer patients.⁷ Furthermore, antibody-drug conjugates specific to MUC16 (DMUC-5754A, Genentech) and to FOLR1 (IMGN853, ImmunoGen) are currently in clinical development for the treatment of ovarian cancer (clinicaltrials.gov). Placental alkaline phosphatase consists of two isoenzymes: placental alkaline phosphatase (PLAP) and the 97% identical germ cell alkaline phosphatase (GCAP), both originating from syncytiotrophoblast cells.⁸ Numerous associations have been reported between the expression of PLAP and GCAP and malignancy, particularly in testicular seminoma,⁹,¹² cancer of the ovary¹³ and the uterus.¹⁴,¹⁵ In normal tissues, PLAP is only detectable in term placenta and endometrium.⁸,¹⁶,¹⁷ By contrast, PLAP is strongly expressed in ovarian cancer, especially in ovarian adenocarcinoma, serous cystadenocarcinoma, undifferentiated carcinoma and dysgerminoma.⁸

The groups of Agamemnon Epenetos and of Torgny Stigbrand have previously demonstrated that radiolabeled preparations of murine antibodies specific to PLAP in IgG1 format¹⁸ were able to selectively localize to xenografted human cancer tumors in immunocompromised mice, following intravenous administration. Because antibodies of rodent origin are frequently immunogenic in humans, generation of human mAbs specific to PLAP for use as modular building blocks in product development and protein engineering applications is desirable. For industrial pharmaceutical applications, fully human mAbs are typically isolated either by immunization of transgenic mice that bear the human immunoglobulin locus¹⁹,²⁰ or by antibody
phage technology.24 Our group has constructed synthetic human antibody phage display libraries containing billions of antibody clones, which have yielded useful binding specificities and product candidates in clinical trials.5,22-24 The phage display library used in this article (“PHILO-Diamond”) contains >50 billion antibody clones (unpublished results) and features antibodies in scFv format,25 all constructed on the basis of the DP47 VH germline gene.26 This heavy chain variable domain is the most frequently used VH gene in human immunoglobulins27 and binds directly to Protein A, thus facilitating antibody purification of antibody fragments.28

Here, we describe the isolation and characterization of human antibodies specific to human PLAP. As commercial preparations of human PLAP are not pure, containing both unrelated proteins and inactive forms of PLAP, we optimized the purification process of the antigen, which facilitated subsequent phage selections and affinity-maturation procedures. The best antibody isolated so far, termed B10, bound to human PLAP with a dissociation constant $K_d$ of 10 nM and strongly reacted with the cognate antigen in freshly frozen tissues and fixed cancer cell lines, as well as in FACS experiments.

**PLAP Purification and Characterization**

Figure 1A and B show a SDS-PAGE and gel-filtration analysis of a commercial preparation of PLAP derived from human placenta (Sigma Aldrich). To produce a more homogenous antigen preparation for phage selections, a preparative gel-filtration procedure was performed, which led to the product described in Figure 1C and D. The purified PLAP was enzymatically active, as assessed by CDP-Star® chemiluminescent assay for alkaline phosphatases (Fig. 1E). From 10 mg of starting material, approximately 1.8 mg of purified product were obtained. This amount was sufficient for antibody selection and for affinity measurements.

**Isolation, Affinity Maturation, and Characterization of Anti-PLAP Antibodies**

The PHILO-Diamond antibody phage display library was panned using biotinylated PLAP using an affinity capture method on avidin-coated or streptavidin-coated wells. ELISA screening of bacterial supernatants and sequencing led to the identification of clone B5. To further improve the binding properties of this antibody, an affinity maturation procedure (featuring the combinatorial mutagenesis of CDR1 loops of VH and VL domains) was performed, using a methodology previously described by our group.29 This process yielded clones B10 and D9. The most relevant amino acid positions of the CDR1 and CDR3 loops of these antibodies are reported in Table 1. A characterization of the SDS-PAGE, gel-filtration, ELISA and Biacore performance of the parental B5 antibody and of the affinity matured B10 and D9 clones is shown in Figure 2. The B10 and D9 antibodies exhibited $K_d$ values of 10 and 30 nM, respectively, and a lower propensity to form diabodies or higher order structures compared with the parental antibody.

The ability of the B10 and D9 antibodies to recognize the cognate antigen in tissues was confirmed by fluorescence microscopy on freshly-frozen sections of placenta.

**Reformatting into Small Immunoprotein Format and Further Characterization**

The B10 and D9 antibodies were reformatted into recombinant small immunoprotein (SIP) format as described by Borsi et al.5 and tested in placenta tissue, as well as in cancer cell lines of gynecological origin, using immunofluorescence methodologies and FACS.

Fluorescence microscopy analysis on freshly frozen sections of placenta, a human tissue characterized by abundant PLAP expression, exhibited a strong and homogenous staining pattern for both B10 and D9 in SIP format, which was comparable to the commercial anti-PLAP 8B6 murine antibody. The anti-hen egg lysozyme KSF antibody30 and a phosphate-buffered saline (PBS) solution that were used as negative controls did not stain placenta, confirming the selectivity of PLAP recognition by B10 and D9 (Fig. 3).

An intense staining was observed for the ovarian cancer cell lines A2780, TOV112D, TOV21G, SKOV3, OVCAR3, IGROV1, for A431 cells (derived from an epidermal carcinoma of the vulva) and for HeLa cells (epithelial cervical carcinoma). As expected, we could not detect a staining for WEHI164 (murine fibrosarcoma) and F9 (murine testicular teratoma), which were tumors of rodent origin chosen as negative controls (Fig. 4).

The selectivity of our antibodies was tested in ELISA and in Biacore against related alkaline phosphatases (ALPI and ALPPL2), as well as other unrelated proteins to probe binding specificity (Fig. S1). A strong binding was detected for PLAP, but not for commercial preparations of ALPI and ALPPL2.

A431 tumor sections were stained with B10 and D9 in SIP format, as well as PBS or SIP (KSF) as negative control. The study revealed a preferential staining with the anti-PLAP antibodies (Fig. 5A). In a quantitative biodistribution experiments, nude mice bearing subcutaneously grafted A431 tumors were injected with 10 µg of a radioiodinated SIP(B10) preparation. Twenty-four hours after injection, a preferential tumor accumulation was observed, with tumor:organ ratios ranging between 2 and 7.5 (Fig. 5B).

To our knowledge, B10 and D9 represent the first fully human mAbs specific to PLAP, a validated marker of ovarian cancer. While our laboratory typically relies on antibody fragments in SIP format for pharmacodelivery applications,5 other groups may prefer to use the antibodies in full IgG format. Starting from the amino acid sequence of the antibodies reported in Table 1 and in Table S1, the antibody reformattting into the corresponding fully human IgG1 format can easily performed, using established procedures for expression in mammalian cells.31,32 We anticipate that B10 and D9 may serve as modular building blocks for the development of ovarian cancer targeting agents. The availability of the cognate antigen in large amounts and in a pure form facilitates the analytical characterization of anti-PLAP products, including affinity measurements and immunoreactivity determinations.
Materials and Methods

Characterization and purification of the antigen

Human PLAP isolated from human placenta was purchased from Sigma Aldrich (P1391) and dissolved in Heps buffer (25 mM HEPES, 100 mM NaCl pH 7.4) at a concentration of 5 mg/ml. It was then filtered through a 0.22 µm PES membrane (TPP) and centrifuged (7 min 2000 g at 4 °C) in a Vivaspin® 20 MWCO 300,000 (Sigma Aldrich) device to remove large-size contaminants. Subsequently, PLAP was purified by size exclusion chromatography (ÄKTA FPLC GE Healthcare) on a Superdex200 10/300 GL column (GE Healthcare) in Hepes buffer applying a flow of 0.5 ml/min. Collected fractions were analyzed by SDS-PAGE electrophoresis on a 4–12% Bis-Tris gel (NuPAGE®) in MOPS buffer and checked for enzymatic activity.

Activity assay of PLAP

Enzymatic activity of the purified and unpurified PLAP was assessed using the chemiluminescent substrate CDP-Star® (Roche). Serial dilutions of the protein (from 1200 nM to 18 nM) were incubated for 30 min at RT in an alkaline buffer (100 mM DEA, 1 mM MgCl₂, 0.06 mM ZnCl₂, 4% DMSO pH 9.8) in the presence of 250 µM CDP-Star® substrate. The

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**Figure 1.** Characterization of PLAP from human placenta. (A, C) SDS–PAGE analysis of PLAP under nonreducing (left) and reducing (right) conditions before (A) and after (C) gel filtration chromatography purification. (B, D) Size-exclusion chromatography profiles of unpurified (B) and purified (D) PLAP. (E) Activity assay of purified PLAP.
Table 1. Sequences of the scFv fragments specific to the human placental alkaline phosphatase

| scFv | VH chain | VL chain |
|------|----------|----------|
|      | 31–33×  | 95–100×  | 31–32×  | 91–96×  |
| B5   | SYA      | TPWLPK   | SYY     | NPTTLR |
| B10  | LYH      | TPWLPK   | WKR     | NPTTLR |
| D9   | MYS      | TPWLPK   | LKY     | NPTTLR |

Relevant amino acid positions of antibody clones isolated from the designed synthetic libraries. Positions that are mutated in the primary antibody library are underlined. Residues in B10 and D9, mutated during the affinity maturation procedure, are in boldface. Single amino acid codes are used according to standard IUPAC nomenclature. ×Aminoacid positions are numbered according to Tomlinson et al.26

Figure 2. Anti-PLAP antibodies. (A, C, E) Size-exclusion chromatography profiles of the purified B5, B10 and D9 scFv fragments, respectively. The retention volume (ml) of the major peak corresponds to the monomeric form of the scFv fragments. (B, D, F) Biacore analysis of the binding of B5, B10 and D9 scFv fragments to the human placental alkaline phosphatase (PLAP). Purified monomeric preparations of the scFv fragments were injected at different concentrations and the kinetic constants were calculated with the BIAevaluation 3.1 software. (G) SDS–PAGE analysis of the purified B5, B10 and D9 scFv fragments under nonreducing (left) and reducing (right) conditions. (H) ELISA assay of the purified B5, B10 and D9 scFv fragments against PLAP.
chemiluminescence emission was measured every minute over 3 h at a wavelength of 466 nm. Negative controls were tested using protein without substrate, substrate without protein and heat inactivated protein (30 min at 95 °C) with substrate.

**Antibodies selection from PHILO-DIAMOND scFvs library by phage display**

Purified PLAP was biotinylated with EZ-Link™ Sulfo-NHS-SS-Biotin (Pierce) according to manufacturer’s instructions and immobilized (1 µM in PBS) for 15 min on a streptavidin coated 8-wells strip (StreptaWell®, Roche) (first and third round of panning, respectively) or on an Maxisorp 8-wells strip (Nunc) pre-coated (overnight at 4 °C) with 0.1 mg/mL Avidin (Apollo Scientific) (second round of panning) and blocked for 2h at room temperature (RT) with PBS 2% milk. The phage particles (diluted in PBS 2% milk) were added to the coated wells for 30 min on a shaker at RT and then for 90 min at RT without shaking. The wells were then rinsed 10 times (20 times for second and third round, respectively) with PBS 0.1% Tween-20 and 10 times with PBS (20 times for second and third round, respectively). Bound phages were eluted by incubation with 100 mM triethylamine for 5 min at RT. The eluted phages were used to infect exponentially growing *Escherichia coli* TG-1 bacteria. The next steps of selection and enrichment were performed according to standard antibody phage display protocols.33

**Construction of the affinity maturation library**

The scFv fragment B5, selected against PLAP from the PhiloDiamond phage-display library, was used as template for the construction of an affinity-maturation library using a procedure recently published by our group.29,34 Sequence variability in the CDR1 region of both VH (positions 31–33) and VL (positions 31, 31b, and 32) was introduced by PCR using partially degenerated primers DP47rCDR1fw and DPL16rCDR1rev. VH-VL combinations were assembled by PCR using the gel-purified CDR1-randomized VH and VL segments as templates and the primers LMB3long and Fdsseqlong. The assembled VH–VL fragments were doubly digested with NcoI/NotI and cloned into NcoI/NotI-digested pHEN1 phagemid vector.35 The resulting ligation product was electroporated into electrocompetent *E.coli* TG-1 as previously described,33 giving rise to a library containing 2.5 × 10^9 individual antibody clones. All oligonucleotides used in this work were purchased from Operon Biotechnologies and listed in Table S2.

**Supernatants screening by ELISA and surface plasmon resonance**

Induced supernatant of individual clones were screened by ELISA as described by Viti et al33 using high-bind StreptaWell plates (Roche) coated with biotinylated antigen. Bound antibody was detected using the mouse anti-myc tag antibody 9E10,36 followed by an anti-mouse immunoglobulin horseradish peroxidase (HRP) conjugate (Sigma Aldrich). The assay was detected by a colorimetric reaction using BM-Blue POD soluble substrate (Roche). Supernatant of clones that were positive in ELISA were further screened by surface plasmon resonance real-time interaction analysis on a CM5 chip coated with PLAP using a Biacore3000 instrument (Biacore Life Sciences).

**Sequencing of scFv genes**

Selected scFvs were sequenced by GATC Biotech using a standard Sanger sequencing. The template DNA for sequencing was generated by PCR reactions, using primers LMB3long (annealing upstream the scFv gene) and Fdsseqlong (annealing 100 bp downstream the scFv gene) (Eurofins MWG Operon).

**Characterization of scFv antibodies**

ScFv antibody fragments were expressed in E.coli TG-1 strain after an overnight induction with IPTG and purified from

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Figure 3. Immunofluorescence analysis performed on human placenta tissue. PLAP was stained using anti-PLAP antibodies B10 or D9 in SIP format (green). A positive control was performed using the commercial anti-PLAP 8B6 antibody (green). An anti-hen egg lysozyme antibody (KSF) in SIP format was used as negative control (green). Nuclei were stained with DAPI (blue). Scale bar = 50 µm.
|       | B10 | D9 | KSF |
|-------|-----|----|-----|
| A431  | ![Image](Image1) | ![Image](Image2) | ![Image](Image3) | ![Image](Image4) |
| A2780 | ![Image](Image5) | ![Image](Image6) | ![Image](Image7) | ![Image](Image8) |
| HEla  | ![Image](Image9) | ![Image](Image10) | ![Image](Image11) | ![Image](Image12) |
| IGROV1| ![Image](Image13) | ![Image](Image14) | ![Image](Image15) | ![Image](Image16) |
| OVCAR3| ![Image](Image17) | ![Image](Image18) | ![Image](Image19) | ![Image](Image20) |
| SKOV3 | ![Image](Image21) | ![Image](Image22) | ![Image](Image23) | ![Image](Image24) |
| TOV112D| ![Image](Image25) | ![Image](Image26) | ![Image](Image27) | ![Image](Image28) |
| TOV21G| ![Image](Image29) | ![Image](Image30) | ![Image](Image31) | ![Image](Image32) |
| WEHI164| ![Image](Image33) | ![Image](Image34) | ![Image](Image35) | ![Image](Image36) |
| F9    | ![Image](Image37) | ![Image](Image38) | ![Image](Image39) | ![Image](Image40) |

Figure 4. For figure legend, see page 92.
the supernatant by affinity chromatography using Protein A Sepharose (Sino Biological). Purified antibodies were analyzed by size-exclusion chromatography on a Superdex 75 10/300 GL column (ÄKTA FPLC GE Healthcare). Peaks representing monomeric fractions were collected and used for ELISA or for affinity measurements by Biacore on a CM5 chip (Biacore Lifescience, GE Healthcare) coated with PLAP.

Cloning, expression, and purification of antibodies in the SIP format

The single chain fragment variables (scFvs) of B10 and D9 were converted into the SIP format by cloning the variable fragments (VH and VL) with a CH4 domain into pcDNA3.1 (Invitrogen) using a previously described strategy. The plasmids were transfected into CHO-S cells (Invitrogen) using transient gene expression (TGE) technique. After 6 d, the SIP antibodies were purified from culture medium by affinity chromatography using Protein A Sepharose (Sino Biological Inc).

Biotinylation of SIP antibodies

SIP antibodies (B10 and D9) were biotinylated through their cysteine residues using biotin-maleimide. The disulfide bond between the cysteine residues at the N-terminus of the SIP was reduced by incubation with 30 times molar excess of tris(2-carboxyethyl)phosphine (TCEP) (ABCR) at 4 °C overnight. The day after a conjugation step was performed incubating the reduced SIP with a 20 times molar excess of biotin-maleimide (Sigma-Aldrich) on an horizontal shaker for 3h at RT. A size exclusion chromatography using a PD10 column (GE Healthcare) was performed to purify the biotin-conjugated SIP.

Immunofluorescence on frozen placenta

Placental tissues were collected from patients who had undergone caesarean section at the Klinik für Geburtshilfe, Universitätsspital, Zürich; an informed written consent module to read and sign before surgery was given to patients, including detailed explanations of tissue sample collection and sensitive data management. Tissues were immediately embedded in NEG-50 freezing medium (Thermo Scientific) and stored at -80 °C until sectioning. Tissue sections (10 µm) were fixed for 10 min with ice-cold acetone, rehydrated with PBS and blocked for 1 h with fetal calf serum (FCS). Immunofluorescence studies were performed incubating the slides with biotinylated SIP antibodies (250 nM in PBS 1% BSA) for 1 h at RT. The staining of primary antibodies was detected using Streptavidin AlexaFluor488 conjugate (10 µg/ml in PBS 1% BSA) (Invitrogen) for 30 min at RT. Nuclei were stained with DAPI (Invitrogen). Each step was followed by 3 washes with PBS. Slides were mounted with Fluorescent Mounting medium (Dako) and images were acquired with a Zeiss Axioskop 2 MOT Plus (Carl Zeiss AG). Image analysis was performed using ImageJ (NIH) and AxioVision 4.7 image analysis software (Carl Zeiss AG).

A commercial mouse monoclonal anti-PLAP antibody (8B6 clone) (Santa Cruz Biotechnologies) was used as positive control (10 µg/ml in PBS 1% BSA for 1 h at RT). The primary antibody was detected with goat anti-mouse IgG AlexaFluor488 (Invitrogen) (10 µg/ml in PBS 1% BSA) for 30 min at RT.

Immunofluorescence on cell lines

10^5 cells/well were plated on chamber slides (Nalgene Nunc International) and grown overnight at 37 °C 5% CO₂. Cells were fixed with a PBS -4% paraformaldehyde (PFA) (Sigma-Aldrich) solution for 10 min at RT, blocked for 30 min at 37 °C with PBS -3% bovine serum albumin (BSA) (Sigma-Aldrich) and then incubated with primary antibodies in SIP format (250 nM in PBS 1% BSA) for 1 h at RT.

The signal of primary antibodies was detected performing two sequential incubations for 45 min at RT with rabbit anti-human IgE produced in rabbit (10 µg/ml in PBS 1% BSA) (Dako) and with goat anti-rabbit AlexaFluor488 conjugate (10 µg/ml in PBS 1% BSA) (Invitrogen). Nuclei were stained with DAPI. Each step was followed by three washes with PBS. Slides were mounted with Fluorescent Mounting medium (Dako) and images were acquired with a Zeiss Axioskop 2 MOT Plus. Image analysis was performed using ImageJ (NIH) and AxioVision 4.7 image analysis software (Carl Zeiss AG). Negative controls were performed using SIP KSF (anti-hen egg lysozyme) as primary antibody or without primary antibody.

Flow cytometry analysis of SIP on living tumor cells

Cells were grown in a 75 cm² flask (TPP AG) until they reach roughly 80% confluence. Cells were detached by adding Accutase® solution (Sigma-Aldrich), counted, resuspended in 3% BSA in PBS and distributed in a 96 well U-bottom plate to achieve a final concentration of 5 × 10^5 cells per well. Cells were centrifuged at 250 g, washed with PBS and resuspended FACS Buffer (PBS 0.5%, BSA 5 mM, EDTA pH 7.4). The signal of primary antibodies was detected performing two sequential incubations for 45 min at RT with rabbit anti-human IgE (10 µg/ml in PBS 1% BSA) (Dako) and with goat anti-rabbit AlexaFluor488 conjugate (10 µg/ml in PBS 1% BSA) (Invitrogen). A propidium iodide (Sigma, 1:1000) staining was performed to evaluate cell viability. Cells were resuspended in FACS Buffer and 10000 events were analyzed on a FACS Canto II (BD Bioscience).
Results were analyzed by FlowJo 8.7 software. Whenever not specified, each step was followed by at least two washes with PBS. Negative controls were performed using SIP KSF (anti-hen egg lysozyme) as primary antibody, without primary antibody and without staining at all.

Selectivity screening by ELISA and surface plasmon resonance

50 µl of 200 nM ALPI (Sino Biological, 13225-H08H), ALPPL2 (Abnova, H00000251-P01) and PLAP (Sigma-Aldrich, purified as described above) were immobilized on Maxisorp 96-well plate (Nunc) at 4 °C overnight. After blocking with 3% BSA in PBS, the proteins were detected with biotinylated SIP (B10, D9 and KSF) and streptavidin-HRP. After a colorimetric reaction using BM-Blue POD soluble substrate (Roche), the plates were read at 450 nm. On SPR (Biacore 3000), a CM5 Chip was coated with ALPI (14 000 RU), ALPPL2 (1500 RU) and PLAP (7000 RU) and the SIPs were run over all flow cells at different concentrations (125–1000 nM) with 10 µL/min for 3 min.

Quantitative biodistribution studies

Four BALB/c nude mice (Charles River) were injected subcutaneously with 5 × 10⁶ A431 cells diluted in Hank’s balanced salt solution (HBSS) (Invitrogen). After 9 d, the time necessary for the tumors to reach a size between 250 and 700 mm³, mice were injected (caudal vein) with 10 µg of radioiodinated SIP (B10) (13 µCi per mouse). The radiolabeling of SIP (B10) with iodine-125 (¹²⁵I) (Perkin Elmer) was performed as previously described³¹ immediately prior use. Mice were sacrificed after 24 h and the organs were excised and weighed. Radioactivity was measured on a Packard Cobra gamma counter. Radioactivity content of representative organs was expressed as percentage of the injected dose per gram of tissue (% ID/g).

All animal experiments were performed on the basis of project license (42/2012) administered by the Veterinaeramt des Kanton Zuerichs and approved by all participating institutions.

Immunofluorescence on tumor section

A BALB/c nude mice was injected subcutaneously with 5 × 10⁶ A431 cells diluted in HBSS (Invitrogen). After 9 d, the mouse developed an A431 xenograft tumor. The mouse was sacrificed and the tumor was excised, embedded in NEG-50 freezing medium (Thermo Scientific) and stored at -80 °C until sectioning. An immunofluorescence analysis on tumor sections (10 µm) using biotin-conjugated primary antibodies in SIP format was performed according to the protocol described above for human placenta.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/mabs/article/27230

Figure 5. Testing recognition of PLAP by SIPs on solid A431 tumors. (A) Staining of PLAP with SIP (B10 D9 and KSF) (green) and nuclei were stained by DAPI (blue). (B) Data of the quantitative biodistribution. Four mice bearing an A431 tumor were injected with SIP (B10) labeled with I125 and sacrificed after 24 h. Organs were excised, radioactivity measured and expressed as percentage injected dose per gram (%ID/g). Scale bar = 50 µm.
