A Recombinant Immunotoxin Derived from a Humanized Epithelial Cell Adhesion Molecule-specific Single-Chain Antibody Fragment Has Potent and Selective Antitumor Activity

Claudio Di Paolo, Jörg Willuida, Susanne Kubetzko, Ikar Lauffer, Dominique Tschudi, Robert Waibel, Andreas Plückthun, Rolf A. Stahel, and Uwe Zangemeister-Witte

Clinic and Policlinic for Oncology, University Hospital, CH-8044 Zürich [C. D. P., I. L., R. A. S., U. Z.-W.]; Clinic of Otorhinolaryngology, Head and Neck Surgery, University Hospital, CH-8091 Zürich [D. T.]; Department of Biochemistry, University of Zürich, CH-8057 Zürich [J. W., S. K., A. P.]; and Center for Radiopharmaceutical Science, Paul Scherrer Institute, CH-5232 Villigen [R. W.], Switzerland

ABSTRACT

Purpose: Epithelial cell adhesion molecule (Ep-CAM) is a tumor-associated antigen overexpressed in many solid tumors but shows limited expression in normal epithelial tissues. To exploit this favorable expression pattern for targeted cancer therapy, an Ep-CAM-specific recombinant immunotoxin was developed and its antitumor activity investigated.

Experimental Design: The immunotoxin 4D5MOCB-ETA was developed by genetically fusing a truncated form of Pseudomonas aeruginosa exotoxin A (ETA) (ETA<sub>Δ52-60KDEL</sub>) to the highly stable humanized single-chain antibody fragment (scFv) 4D5MOCB. Cytotoxicity of 4D5MOCB-ETA was measured in cell growth and leucine incorporation assays in vitro. Tumor localization and antitumor activity were assessed in athymic mice bearing established human tumor xenografts.

Results: Fusion of the toxin moiety to the scFv did not affect its thermal stability nor its antigen-binding affinity. In vitro, 4D5MOCB-ETA potently and specifically inhibited protein synthesis and reduced the viability of Ep-CAM-positive carcinoma cells of diverse histological origins with IC<sub>50</sub><sup>8</sup> ranging from 0.005 to 0.2 pm. Upon systemic administration in mice, 4D5MOCB-ETA showed similar organ distribution as the scFv 4D5MOCB and preferentially localized to Ep-CAM-positive tumor xenografts with a tumor:blood ratio of 5.4. The potent antitumor activity of 4D5MOCB-ETA was demonstrated by its ability to strongly inhibit the growth and induce regression of relatively large tumor xenografts derived from lung, colon, or squamous cell carcinomas.

Conclusions: We describe for the first time the development of a fully recombinant Ep-CAM-specific immunotoxin and demonstrate its potent activity against solid tumors of various histological origins. 4D5MOCB-ETA is currently being evaluated in a Phase I study in patients with refractory squamous cell carcinoma of the head and neck.

INTRODUCTION

Despite favorable initial responses, most advanced solid tumors develop resistance to standard treatments and relapse as incurable metastatic diseases (1). Because increasing the dose of conventional anticancer agents results in unacceptable side effects, the design of novel therapies based on the use of tumor-selective targeting ligands and effector domains using different mechanisms of action is of great importance. Antibodies targeting tumor-associated antigens and equipped with intrinsic cytotoxic or immunostimulatory effector functions have shown promising antitumor activity in preclinical and clinical studies (2–4).

Ep-CAM<sup>4</sup> is a 40-kDa transmembrane protein overexpressed in many solid tumors, including carcinomas of the lung, breast, ovary, colorectum, and squamous cell carcinoma of the head and neck (5). The limited expression of Ep-CAM in normal epithelial tissues (5, 6) makes this antigen an attractive target for cellular and antibody-based immunotherapy (7–9). Recently, a transgenic mouse model mimicking the Ep-CAM expression pattern in humans additionally validated the suitability of this target for immunotherapy by showing no localization of the monoclonal antibody MOC31 in Ep-CAM-positive normal tissues (10).

The role of Ep-CAM in carcinogenesis and malignant progression is still unclear, but there is increasing evidence that

Received 9/24/02; revised 2/22/03; accepted 2/24/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Cancer League of the Kanton Zürich Grant 23.2197.

2 Present address: Schering AG, Experimental Oncology, D-13342 Berlin, Germany.

3 To whom requests for reprints should be addressed, at University Hospital of Zürich, Clinic and Policlinic for Oncology, Häldeliweg 4, CH-8044 Zürich, Switzerland. Phone: 411-6342877; Fax: 411-6342872; E-mail: uwe.zangemeister@usz.ch.

4 The abbreviations used are: Ep-CAM, epithelial cell adhesion molecule; ETA, Pseudomonas aeruginosa exotoxin A; scFv, single-chain antibody fragment; dsFv, disulfide-stabilized single-chain antibody fragment; ER, endoplasmic reticulum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MFI, mean fluorescence intensity; EGF, epithelial growth factor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TNF, tumor necrosis factor; IPTG, isopropyl-1-thio-β-D-galactopyranoside; IDA, iminodiacetic acid; %ID/g, % of injected dose/g.
it modulates cell-cell interactions (11) and that its expression correlates with the rate of cell proliferation (12). In addition, a promoting role of Ep-CAM in tissue invasion and metastasis has been suggested (13), and a strong correlation between Ep-CAM expression and tumor progression has been found in patients with squamous cell carcinoma of the head and neck.\(^5\) Ep-CAM-specific antibodies have been used in imaging studies to detect primary tumors and localize distant metastases in patients with small cell lung cancer (14) and non-small cell lung cancer (15). Moreover, they have been shown to trigger antitumor immune responses (16) and to deliver cytotoxic effector molecules to tumors in preclinical models (17, 18) and in patients (19).

An immunotoxin is a chimeric protein in which a toxin moiety is chemically or genetically linked to a monoclonal or recombinant antibody. Antibodies with specificities for various tumor-associated antigens have been investigated as carriers of toxins, and the majority of those that target solid tumors have used the effector function of truncated ETA, which lacks the cell-binding domain I (aa 1–252; Ref. 20). ETA irreversibly inhibits protein synthesis by ADP-ribosylation of elongation factor 2 and therefore has to gain access to its intracellular target in the cytoplasm (20, 21). Thus, the most promising antigens for immunotoxin therapy are those that are efficiently internalized into tumor cells upon antibody binding by receptor-mediated endocytosis (22–25).

Several immunotoxins, either as chemically linked first generation or recombinant second generation formats, have been tested in animal models and in patients with advanced solid tumors (20, 26). Although early clinical data using immunotoxin therapy for refractory tumors are promising, the induction of neutralizing antibodies and dose-limiting side effects associated with vascular leak syndrome or liver toxicity still remain obstacles to effective therapy (27–29). To overcome these limitations, more promising tumor-associated antigens have to be validated as targets for new immunotoxin generations that are equipped with rationally engineered effector functions (2, 30, 31).

In a previous study, we reported the ability of the chemically conjugated immunotoxin MOC31-ETA to eradicate small tumor xenografts in mice as well as its failure to delay the growth of larger tumors (18). We concluded that because of its relatively large size (\(M_f\) of approximately 200,000), the immunotoxin was unable to homogeneously distribute within the tumor mass and thus could only affect an insufficiently small proportion of clonogenic tumor cells. Support for this hypothesis is provided by others who reported an inverse correlation between immunotoxin size and efficacy (32). The tumor targeting and tissue distribution properties of immunotoxins can be substantially improved by using small scFv as targeting ligands (22, 23, 25, 33–37). We have recently described the enhanced tumor localization of scFv 4D5MOCB, which was derived by grafting the hypervariable loops of monoclonal antibody MOC31 onto the humanized framework of the anti-HER-2/neu scFv 4D5 and by additionally changing eight critical core residues to obtain a high molecule stability (38). In this study, we describe the development of a fully recombinant Ep-CAM-specific single-chain immunotoxin based on 4D5MOCB and report its favorable tumor localization and potent antitumor activity against carcinomas of diverse histological origins in vitro and in vivo.

MATERIALS AND METHODS

**Tumor Cell Lines.** The colorectal carcinoma cell lines HT29 (HTB-38), COLO320 (CL-220), the breast adenocarcinoma cell line MCF7 (HTB-22), and the non-Hodgkin’s lymphoma cell line RL (CRL-2261) were obtained from the American Type Culture Collection (Manassas, VA). The squamous cell carcinoma cell line of the tongue CAL27 was kindly provided by Dr. Samuel D. Bernal (UCLA School of Medicine, Los Angeles, CA). The small cell lung carcinoma cell line SW2 was raised in our laboratory. Except for CAL27, which was maintained in DMEM (Life Technologies, Inc., Grand Island, NY), cell lines were grown in RPMI 1640 (Life Technologies, Inc.). Both media were supplemented with 10% fetal bovine serum (HyClone, Europe Ltd.), 2\(\text{mM}\) L-glutamine, 50 IU/ml penicillin, and 50 \(\mu\)g/ml streptomycin. Cell cultures were maintained at 37\(^\circ\)C in a humidified atmosphere containing 5% \(\text{CO}_2\).

**Construction of the 4D5MOCB-ETA Expression Vector.** The sequence encoding a truncated form of ETA (\(\text{ETA}_{252-608}\)) was amplified by PCR from plasmid pSW200 (25) and cloned as an 1164-bp EcoRI-HindIII fragment downstream of the Ep-CAM-binding 4D5MOCB scFv sequence present in the plg6-based (39) 4D5MOCB scFv expression vector (38). The primers (Tox1, CTCGGAATTCGGTGGCGCGCGGAGTGTCGCCAAAGCCTACCCCAGGCTTGTCCTCGGTGTTTTA; Tox2, GTCAAGCTTCTACAGTTCGTCTTTATGGTGATGTTGTTGAATATGCG GCCGTTTCCCCCGCTGGT) introduced an EcoRI restriction site between scFv and toxin and a COOH-terminal hexahistidine tag followed by the ER retention signal with the sequence KDEL, a stop codon and a HindIII restriction site. To improve purity and yield during immobilized ion-metal affinity chromatography, a second hexahistidine tag was added at the NH\(_2\) terminus between the periplasmic signal sequence and the 4D5MOCB coding region. To this end, two pairs of oligonucleotides (\(\text{XbaI}, 5’-\text{CTAGATACGAGGCCGAAAAATGAAAAAGACAGCTATCGGCGATGGCCATGCCTGGTCTGGTCTCCGTTACCGT}\) and \(\text{XbaI}, 5’-\text{GCCCAGTCTACGAGCCGATCGTGGTCTCCTGGTCTGGTCTCCGTTACCGT}\) were heated to 80\(^\circ\)C, allowed to anneal by gradually cooling to room temperature, and then ligated between the \(\text{XbaI}\) and EcoRV sites of plg6-4D5MOCB-ETA. The sequence was experimentally confirmed.

**Expression and Purification of 4D5MOCB-ETA.** For periplasmic expression of 4D5MOCB-ETA, the vector plg6 was used, which places the gene under \(\text{lac}\) promoter control in SBS536, an Escherichia coli strain devoid of the periplasmic proteases HhoA and HhoB (40). Five ml of 2Y T medium containing ampicillin (100 \(\mu\)g/ml) were inoculated with a single bacterial colony containing the 4D5MOCB-ETA expression plasmid and grown overnight at 25\(^\circ\)C. The bacteria were diluted in 1 liter of 2Y T medium supplemented with 0.5% glucose and ampicillin (100 \(\mu\)g/ml) to reach an \(A_{550}\) nm between 0.1 and 0.2

---

\(^5\) D. Tschudi, unpublished observation.
and transferred to 3-liter baffled shake flasks. The culture was further grown at 25°C to an A550nm of 0.5, and immunotoxin production was induced for 4 h by adding IPTG (Sigma) to a final concentration of 1 mM. The harvested pellet derived from a bacterial culture with a final A550nm of 6 was stored at −80°C. For purification, the pellet obtained from a 1-liter culture was resuspended in 25 mL of lysis buffer, containing 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2 mM MgSO4, and supplemented with EDTA-free protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) and DNase I. The bacterial suspension was lysed by two cycles in a French Press (SLS Instruments, Urbana, IL), centrifuged at 48,000 × g in a SS-34 rotor for 30 min at 4°C and subsequently filter-sterilized (0.22 μm). The immunotoxin present in the cleared supernatant was purified by chromatography using a BioCad-System (Applied Biosystems, Foster City, CA) with a Ni2+-IDA column and a POROS HQ/M-anion-exchange column coupled in-line as described previously (41). Before the lysate was loaded, the Ni2+-IDA column was equilibrated with 20 mM Tris (pH 7.5), 300 mM NaCl. After loading, the column was washed three times with different salt solutions, all buffered with 20 mM Tris (pH 7.5) in the order 300, 510, and 90 mM NaCl. Subsequently, the column was washed with 20 mM Tris (pH 7.5), 10 mM imidazole, and 90 mM NaCl before the bound immunotoxin was eluted with the same solution containing 200 mM imidazole (pH 7.5). The eluate was directly loaded onto the POROS HQ/M-anion-exchange column, and the bound immunotoxin was eluted with a salt gradient of 90–1000 mM NaCl, buffered with 20 mM Tris (pH 7.5). The fractions containing 4DSMOCB-ETA were collected and concentrated using a 10-kDa cutoff filter by centrifugation at 4°C (Ultrafree-MC low protein binding; Millipore). The quality of purified 4DSMOCB-ETA was analyzed by a 10% SDS-polyacrylamide gel and Western blotting using a horseradish peroxidase-conjugated antitetrahistidine antibody (Qiagen, Hilden, Germany) diluted 1:5000 according to the manufacturer’s recommendations.

**Analytical Gel Filtration and Determination of Thermal Stability.** Ten μg of purified 4DSMOCB-ETA were diluted in 50 μL of PBS (pH 7.4) containing 0.005% Tween 20 and subsequently incubated at 37°C. Samples were analyzed at different time points (after 0, 2, 4, 8, 10, and 20 h) by gel filtration using the Smart system (Pharmacia, Uppsala, Sweden) with a Superose-12 PC3.2/30 column. The column was calibrated in the same buffer with three protein standards: alcohol dehydrogenase (Mw 150,000); BSA (Mw 66,000); and carbonic anhydrase (Mw 29,000). The same analytical setting was used to assess the thermal stability of the 99mTc-labeled immunotoxin after a 20-h incubation at 37°C in human serum. The amount of immunotoxin monomers was determined by γ-scintillation counting of the eluted fractions.

**Radiolabeling and Determination of Antigen-binding Affinity.** 4DSMOCB-ETA was radioactively labeled by stable site-specific coordination of 99mTc-tricarbonyl trihydrate to the hexahistidine tags present in the protein sequence (42). This spontaneous reaction was induced by mixing 30 μL of immunotoxin solution (1 mg/mL) with one-third volume of 1 mL 2-(N-morpholino)ethanesulfonic acid (pH 6.8) and one-third volume of freshly synthesized 99mTc-tricarbonyl compound. The mixture was incubated for 1 h at 37°C, and the reaction was stopped by desalting over a BioSpin-6 column (Bio-Rad, Hercules, CA) equilibrated with PBS containing 0.005% Tween 20, according to the manufacturer’s recommendation. The percentage of immunoreactive immunotoxin was assessed as described by Lindmo et al. (43). The binding affinity of the 99mTc-labeled immunotoxin was determined on SW2 cells in a RIA, essentially as described for the scFv 4DSMOCB (38).

**Cell Growth Assay.** Inhibition of cell growth upon treatment with 4DSMOCB-ETA was determined in standard MTT assays based on the reduction of tetrazolium salt to formazan by the enzymes from viable cells (44). Briefly, 5000 tumor cells were seeded in 96-well ELISA microplates in a total volume of 50 μL of culture medium/well. Immunotoxin concentrations ranging from 0.0001 to 100 μM were added in a total volume of 100 μL/well, and cells were incubated for 72 h under standard cell culture conditions. Ten μL of a 10 mg/mL MTT (Fluka) solution were added to each well, and the plates were incubated for an additional 90 min at 37°C. Cell lysis and formazan solubilization were achieved by addition of 100 μL of lysis buffer containing 20% SDS in 50% dimethylformamide ([pH 4.7]) adjusted with a solution consisting of 80% acetate, 20% 1 M HCl], and the released formazan crystals were allowed to dissolve overnight at 37°C. Absorption was quantified at 590 nm using a SPECTRAMax 340 microplate reader (Molecular Devices, Sunnyvale, CA).

To demonstrate that the cytotoxicity of 4DSMOCB-ETA was attributable to inhibition of protein synthesis in cells, [3H]leucine incorporation assays were performed as described previously (18). Briefly, 2 × 104 cells/well in leucine-free cell culture medium were seeded into 96-well plates and incubated with increasing concentrations of 4DSMOCB-ETA diluted in leucine-free medium to a final volume of 200 μL. Cells incubated in leucine-free medium without immunotoxin were used as control. Upon a 24-h incubation at 37°C under standard cell culture conditions, cells were pulsed with 10 μM of medium containing 1 μCi of [4,5-3H]leucine (specific radioactivity 5 TBq/nmol)/well for 6 h and harvested onto glass fiber filters using a Harvester 96 (Tomtec, Hamden, CT). The radioactivity incorporated into cells was quantified in a Trilux1450 liquid scintillation MicroBeta counter (Perkin-Elmer Life Sciences, Wellesley, MA) and expressed as percentages relative to untreated controls.

**Flow Cytometry.** Cell surface expression of Ep-CAM was quantified by flow cytometry using the mouse IgG2a, KSI/4 (BD Pharmingen, San Diego, CA). As secondary antibody, a FITC-conjugated goat antimouse F(ab′)2, IgG (H+L; Zymed Laboratories, San Francisco, CA) was used. All staining steps were performed in a staining buffer consisting of PBS supplemented with 1% (w/v) BSA and 0.04% (w/v) sodium azide. Cells (5 × 105) were harvested, washed twice with ice-cold staining buffer, and incubated on ice for 45 min in a total volume of 100 μL of staining buffer containing 1 μg of the first antibody. Cells were washed and additionally incubated with 400 ng of FITC-labeled antibody in a final volume of 100 μL. After 30 min on ice, cells were washed and resuspended in 300 μL of staining buffer for analysis. Fluorescence intensity was measured at 430 nm using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) and quantified using the CellQuestPro software (Becton Dickinson, San Jose, CA).
Mice. Six-to-eight-week-old female CD-1 (ICR nu/nu) mice (Charles River Laboratories, Sulzfeld, Germany) were used. They were kept under specific pathogen-free conditions according to the guidelines of the Veterinary Office of the Kanton Zurich. Tumors were raised at the lateral flank by s.c. injection of 10² cells and randomized to constitute groups with an average tumor size of 160 mm³. Ten-week-old female C57BL/6 mice (Janvier, Saint Isle, France) were used to determine immunotoxin-caused toxicity in immunocompetent animals, which are more sensitive to the ETA-mediated T-cell stimulation that results in the production of TNF by Kupffer cells and perforin by cytotoxic T cells.

Biodistribution Study of 4D5MOCB-ETA. To investigate the distribution of 4D5MOCB-ETA in mice, 6 μg of ⁹⁹ᵐTc-labeled 4D5MOCB-ETA (specific radioactivity of 98.9 TBq/mmol) were diluted in a total volume of 150 μl of PBS and were injected i.v. into mice bearing established SW2 and Colo320 tumor xenografts at the contralateral flanks. Mice were sacrificed at different time points (10 min, 30 min, 1 h, 4 h, 16 h, 24 h, and 48 h) after treatment, and organs were removed to measure the accumulated radioactivity using a gamma counter. The amount of radioactivity/gram organ was given as percentage of the total injected dose, which was arbitrarily set to 100%.

In Vivo Toxicity of 4D5MOCB-ETA. Toxicity of the immunotoxin was investigated in C57BL/6 mice by measuring ALT/AST activity in plasma upon repeated injections of escalating doses of 4D5MOCB-ETA given every other day for three cycles (5- and 10-μg dose) or for two cycles (20-μg dose). Whole blood samples were taken to assess the degree of myelosuppression based on alterations of cellular components. In addition, tissue specimens from the livers and spleens of immunotoxintreated mice were analyzed for histopathological changes upon hematoxylin and eosin staining.

Antitumor Activity of 4D5MOCB-ETA. Mice bearing tumor xenografts derived from the Ep-CAM-positive cell lines CAL27, HT29, and SW2 and the Ep-CAM-negative cell line COLO320 were treated i.v. every second day with either 5 μg of 4D5MOCB-ETA for a total of nine applications (total dose 45 μg) or with 10 μg every second day for a total of three applications (total dose 30 μg) in a volume of 100 μl of PBS. Tumor xenografts from untreated mice were used as control. Tumor size was calculated by measurement of the shortest and longest perpendicular diameter using digital calipers according to the formula: (short diameter)² × (long diameter) × 0.5.

RESULTS

Construction and Purification of 4D5MOCB-ETA. We have recently reported the development and promising tumor-targeting properties of the Ep-CAM-specific scFv 4D5MOCB (38). In this study, this scFv antibody was fused to a truncated ETA (ETA₂₂₆₋₆₀₈) by a 20 amino acid long peptide linker (Fig. 1A). The COOH-terminal original ER retention sequence REDLK of wild-type ETA (aa 609 – 613) was replaced by the mammalian counterpart KDEL, which increases the cytotoxic potency of the toxin in tumor cells (45). Furthermore, we added a second hexahistidine sequence at the NH₂ terminus of 4D5MOCB to increase the efficiency of purification by Ni²⁺-IDA affinity chromatography (Fig. 1A). The final construct encoded a protein of 648 amino acids with a theoretical isoelectric point of 5.9. Fig. 1B shows a computer model of the mature 4D5MOCB-ETA immunotoxin molecule.

During IPTG induction, >90% of the total immunotoxin detected by Western blot was found in the periplasmic soluble fraction of E. coli and was released upon cell fractionation. The final protein yield was 0.5 mg of a 95% pure immunotoxin preparation/liter bacterial culture in standard shake flasks. The product migrated at the expected size of ~70 kDa on SDS-PAGE (Fig. 1C), and the theoretical M₀ of 69,737 was verified by mass spectrometry. The absence of proteolytic degradation was confirmed by Western blot analysis (Fig. 1D).

Immunoreactivity and Stability of 4D5MOCB-ETA. Thermal stability and resistance to protease degradation of an immunotoxin is of paramount importance for its tumor targeting properties and thus for therapeutic efficacy. To investigate the stability of 4D5MOCB-ETA, the fusion protein was incubated in PBS for different time periods at 37°C, and the rate of degradation was analyzed by gel filtration essentially as described previously (38). As shown in Fig. 2, upon a 4-h incubation at 37°C, 91% of the immunotoxin molecules still eluted as monomers at the retention volume of 1.4 ml, corresponding to a M₀ of ~66,800. The amount of 4D5MOCB-ETA only slowly decreased with time, and ~47% of the initial protein still eluted in monomeric form after 20 h at 37°C. Similar results were obtained upon incubation of ⁹⁹ᵐTc-labeled 4D5MOCB-ETA in human serum, additionally corroborating the suitability of the immunotoxin for in vivo application.

To assess the effect of the additional NH₂-terminal hexahistidine tag on the antigen-binding affinity, we determined the amount of immunoreactive immunotoxin in a binding assay as described previously (43). Upon a 1-h incubation at 37°C, the ⁹⁹ᵐTc-tricarbonyl quantitatively bound to the histidine tags of the immunotoxin. As determined in cell-binding assays, 80–90% of the immunotoxin retained its binding activity for Ep-CAM after the labeling procedure. The Kᵦ of the immunotoxin to Ep-CAM expressed on SW2 cells was determined to be 4 nM, which was essentially the same as observed for the scFv 4D5MOCB assessed in a similar test system (38). The low level of immunotoxin degradation could be completely prevented by the addition of protease inhibitors even after a 48-h incubation at 37°C in PBS (data not shown). Thus, the immunotoxin 4D5MOCB-ETA retained all of the favorable biophysical properties of the parental scFv.

Ep-CAM Expression on Tumor Cell Lines. Ep-CAM is overexpressed in many solid tumors of diverse histological origins (5). As shown in Table 1, the highest level of Ep-CAM was expressed on HT29 cells (MFI 696.1), followed by MCF7 (MFI 419.5), CAL27 (MFI 415.3), and SW2 cells (MFI 372.4). The cell lines RL and COLO320 cells did not express Ep-CAM and were used as antigen-negative controls.

Cytotoxicity of 4D5MOCB-ETA against Tumor Cells in Vitro. To determine the ability of 4D5MOCB-ETA to specifically inhibit the growth of Ep-CAM-positive tumor cells, MTT assays were performed. The immunotoxin was specifically cytotoxic against Ep-CAM-positive cell lines and did not affect the growth of the Ep-CAM-negative cells RL and COLO320 in the broad range of concentrations tested (Fig. 3). SW2, CAL27, and MCF7 cells were found to be equally sensitive to the cytotoxic
effect of 4D5MOCB-ETA, and their proliferation was inhibited with an IC\textsubscript{50} of only 0.005 pm. Despite the highest level of Ep-CAM expression (Table 1), HT29 cells were found to be the least sensitive (IC\textsubscript{50} of 0.2 pm). In the range of concentrations tested, the cytotoxicity of the immunotoxin was completely blocked by an excess of scFv 4D5MOCB (data not shown).

As determined in [\textsuperscript{3}H]leucine incorporation assays (data not shown), treatment of SW2 cells with 4D5MOCB-ETA inhibited protein synthesis with an IC\textsubscript{50} of 0.01 pm, and this effect showed the same dose-response relationship as measured in the cell viability assays described above. Protein synthesis was not inhibited in the antigen-negative control cell line RL.
Tumor Localization of 4D5MOCB-ETA in Mice. To spare normal tissues from cytotoxic damage and use the full cytotoxic potential of 4D5MOCB-ETA demonstrated in vitro for targeted cancer therapy, the selective and preferential localization of the immunotoxin to Ep-CAM-positive tumors is a prerequisite. We assessed the tumor localization properties of 4D5MOCB-ETA in a biodistribution experiment in mice bearing established Ep-CAM-positive SW2 and Ep-CAM-negative COLO320 xenografts at the contralateral flanks. As shown in Table 2, the maximum dose of radiolabeled 4D5MOCB-ETA detected in SW2 tumors was 2.93% ID/g after 4 h, which then gradually decreased to 1.95% ID/g after 24 h. After 48 h, radioactivity was still 1.13% ID/g tumor tissue. In COLO320 control tumors, 4D5MOCB-ETA localized with a maximum dose of 1.65% ID/g after 30 min, which then rapidly declined to 1.06% ID/g after 4 h and showed only background levels after 48 h. As expected from its larger size, 4D5MOCB-ETA showed a slower blood clearance than the parental scFv 4D5MOCB (data shown in Table 2 for comparison). After 24 h, the total dose of 4D5MOCB-ETA in the blood was 0.42% ID/g, which was 1.5-fold more than measured for the scFv (0.28% ID/g). Moreover, localization of the immunotoxin in SW2 tumors was also delayed compared with the scFv, and the distribution of 4D5MOCB-ETA revealed a tumor:blood ratio of 5.38 after 48 h, which was comparable with the ratio obtained with the scFv after 24 h.

Tumor Localization of 4D5MOCB-ETA in Mice. To spare normal tissues from cytotoxic damage and use the full cytotoxic potential of 4D5MOCB-ETA demonstrated in vitro for targeted cancer therapy, the selective and preferential localization of the immunotoxin to Ep-CAM-positive tumors is a prerequisite. We assessed the tumor localization properties of 4D5MOCB-ETA in a biodistribution experiment in mice bearing established Ep-CAM-positive SW2 and Ep-CAM-negative COLO320 xenografts at the contralateral flanks. As shown in Table 2, the maximum dose of radiolabeled 4D5MOCB-ETA detected in SW2 tumors was 2.93% ID/g after 4 h, which then gradually decreased to 1.95% ID/g after 24 h. After 48 h, radioactivity was still 1.13% ID/g tumor tissue. In COLO320 control tumors, 4D5MOCB-ETA localized with a maximum dose of 1.65% ID/g after 30 min, which then rapidly declined to 1.06% ID/g after 4 h and showed only background levels after 48 h. As expected from its larger size, 4D5MOCB-ETA showed a slower blood clearance than the parental scFv 4D5MOCB (data shown in Table 2 for comparison). After 24 h, the total dose of 4D5MOCB-ETA in the blood was 0.42% ID/g, which was 1.5-fold more than measured for the scFv (0.28% ID/g). Moreover, localization of the immunotoxin in SW2 tumors was also delayed compared with the scFv, and the distribution of 4D5MOCB-ETA revealed a tumor:blood ratio of 5.38 after 48 h, which was comparable with the ratio obtained with the scFv after 24 h.

At each time point, 4D5MOCB-ETA preferentially accumulated in Ep-CAM-positive SW2 tumors compared with COLO320 control tumor with a SW2:COLO320 ratio varying between 1.28 and 2.95. This indicates that 4D5MOCB-ETA was retained in Ep-CAM-positive tumors by specific antibody-antigen interactions and cellular uptake and suggests that its...
Table 2  Biodistribution of [99mTc]-labeled 4D5MOCB-ETA in mice bearing SW2 and COLO320 tumor xenografts

| Tissue                     | 10 min (n = 3) | 30 min (n = 3) | 1 h (n = 3) | 4 h (n = 3) | 16 h (n = 3) | 24 h (n = 3) | 48 h (n = 3) | 4D5MOCB scFv \( \%\) of Tissue %ID/g
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>27.21 ± 4.26</td>
<td>17.46 ± 3.28</td>
<td>10.08 ± 2.89</td>
<td>2.23 ± 0.32</td>
<td>0.57 ± 0.14</td>
<td>0.42 ± 0.04</td>
<td>0.21 ± 0.02</td>
<td>0.28 ± 0.06</td>
</tr>
<tr>
<td>Heart</td>
<td>10.84 ± 1.96</td>
<td>6.69 ± 3.88</td>
<td>6.20 ± 1.10</td>
<td>2.24 ± 0.18</td>
<td>1.14 ± 0.04</td>
<td>0.64 ± 0.47</td>
<td>0.52 ± 0.05</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>Lung</td>
<td>11.56 ± 1.66</td>
<td>8.36 ± 1.77</td>
<td>6.21 ± 0.71</td>
<td>2.59 ± 0.26</td>
<td>1.25 ± 0.13</td>
<td>0.97 ± 0.12</td>
<td>0.77 ± 0.22</td>
<td>1.14 ± 0.60</td>
</tr>
<tr>
<td>Spleen</td>
<td>7.35 ± 1.50</td>
<td>12.17 ± 1.70</td>
<td>11.64 ± 1.74</td>
<td>7.70 ± 5.65</td>
<td>8.81 ± 1.26</td>
<td>6.24 ± 0.68</td>
<td>4.07 ± 1.69</td>
<td>0.70 ± 0.13</td>
</tr>
<tr>
<td>Kidney</td>
<td>22.49 ± 8.28</td>
<td>32.68 ± 1.49</td>
<td>33.50 ± 2.32</td>
<td>42.54 ± 6.01</td>
<td>32.98 ± 1.14</td>
<td>22.79 ± 1.76</td>
<td>16.54 ± 0.39</td>
<td>300.0 ± 85.0</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.92 ± 0.30</td>
<td>1.23 ± 0.40</td>
<td>2.72 ± 1.33</td>
<td>0.85 ± 0.13</td>
<td>1.15 ± 0.69</td>
<td>0.63 ± 0.09</td>
<td>0.45 ± 0.09</td>
<td>0.24 ± 0.24</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.78 ± 0.52</td>
<td>2.19 ± 0.15</td>
<td>2.31 ± 0.52</td>
<td>2.30 ± 1.09</td>
<td>2.11 ± 0.22</td>
<td>0.90 ± 0.09</td>
<td>0.58 ± 0.06</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>Liver</td>
<td>15.47 ± 3.24</td>
<td>20.44 ± 0.70</td>
<td>19.97 ± 3.77</td>
<td>20.20 ± 1.26</td>
<td>16.28 ± 2.51</td>
<td>13.70 ± 1.83</td>
<td>8.44 ± 0.49</td>
<td>2.38 ± 0.52</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.63 ± 0.14</td>
<td>0.80 ± 0.15</td>
<td>0.57 ± 0.07</td>
<td>0.58 ± 0.19</td>
<td>0.37 ± 0.03</td>
<td>0.26 ± 0.05</td>
<td>0.16 ± 0.02</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Bone (femur)</td>
<td>3.91 ± 0.97</td>
<td>6.24 ± 0.96</td>
<td>3.96 ± 0.23</td>
<td>3.76 ± 0.43</td>
<td>3.05 ± 0.37</td>
<td>2.85 ± 0.49</td>
<td>1.08 ± 0.20</td>
<td>0.06 ± 0.05</td>
</tr>
<tr>
<td>COLO320 tumor</td>
<td>0.79 ± 0.10</td>
<td>1.65 ± 0.33</td>
<td>1.26 ± 0.11</td>
<td>1.19 ± 0.05</td>
<td>1.06 ± 0.20</td>
<td>0.66 ± 0.03</td>
<td>0.55 ± 0.14</td>
<td>ND</td>
</tr>
<tr>
<td>SW2 tumor</td>
<td>1.01 ± 0.22</td>
<td>2.45 ± 0.33</td>
<td>2.45 ± 0.47</td>
<td>2.93 ± 0.80</td>
<td>2.26 ± 0.53</td>
<td>1.95 ± 0.37</td>
<td>1.13 ± 0.08</td>
<td>1.47 ± 0.32</td>
</tr>
<tr>
<td>SW2 tumor/COLO320 tumor</td>
<td>1.28</td>
<td>1.48</td>
<td>1.94</td>
<td>2.76</td>
<td>1.90</td>
<td>2.95</td>
<td>2.05</td>
<td>ND</td>
</tr>
<tr>
<td>Ratios^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COLO320 tumor/blood</td>
<td>0.03</td>
<td>0.10</td>
<td>0.13</td>
<td>0.48</td>
<td>2.09</td>
<td>1.57</td>
<td>2.61</td>
<td>ND</td>
</tr>
<tr>
<td>SW2 tumor/blood</td>
<td>0.04</td>
<td>0.14</td>
<td>0.24</td>
<td>1.31</td>
<td>3.97</td>
<td>4.64</td>
<td>5.38</td>
<td>5.25</td>
</tr>
<tr>
<td>SW2 tumor/COLO320 tumor</td>
<td>1.28</td>
<td>1.48</td>
<td>1.94</td>
<td>2.76</td>
<td>1.90</td>
<td>2.95</td>
<td>2.05</td>
<td>ND</td>
</tr>
</tbody>
</table>

4D5MOCB-ETA immunotoxin was determined after i.v. injection in mice bearing SW2 and COLO320 tumor xenografts at contralateral sites. Data represent the percentages of injected dose (%ID) of tissue ND, not determined.

Ratios presented were calculated from averages of tumor/blood or tumor:tumor ratios of individual mice.

From Ref. 38 for comparison.

Antitumor Activity of 4D5MOCB-ETA. An essential requirement for the clinical use of antitumor agents is a significant antitumor activity demonstrated in animal models of human tumors treated with 4D5MOCB-ETA. The tumor burden was reduced, and tumor growth was markedly inhibited in these xenografts (11). At the doses used, 4D5MOCB-ETA did not cause significant toxicity to mice, and the animals showed no signs of toxicity or morbidity.

Transaminase activity in plasma [U/L]

![Graph showing changes in transaminase activity over time](image)
man tumor xenografts. To investigate whether the potent in vitro cytotoxicity and the favorable tumor localization properties of 4D5MOCB-ETA translate into antitumor activity, mice bearing established SW2, HT29, or CAL27 tumor xenografts were treated by i.v. injection of the immunotoxin with two different dose schedules: (a) 45-μg total, with 5 μg given every second day for 3 weeks; and (b) 30-μg total, with 10 μg given every second day for 1 week. Mice bearing Ep-CAM-negative COLO320 tumor xenografts were used as controls. All treatment doses were well tolerated, and mice did not show any signs of toxicity such as weight loss or impaired liver function (Fig. 4).

As depicted in Fig. 5, a significant inhibition of the growth of all Ep-CAM-positive tumors was achieved by treating mice with either the 5- or the 10-μg dose schedule. Treatment of mice bearing SW2 xenografts resulted in a shrinkage of the tumor volume to maximal 20% of the initial size and a slight resumption of growth to a final 2.6-fold size increase at the end of the monitored period. A similar effect was achieved upon treatment of CAL27 tumors, which were reduced to maximal 60% of the initial volume. Fifty days after start of treatment, the median tumor volume did not exceed 1.4-fold the initial size. Two of 7 mice treated with the 5-μg dose showed complete tumor regression and remained tumor free. Neither CAL27 nor SW2 tumors showed a significant difference in their tumor response to the two treatment schedules.

For HT29 tumors, strong growth inhibition was achieved with the 5-μg dose given for 3 weeks when sizes decreased to 0.7-fold of the initial volume. As already observed for CAL27 tumors, 3 of 7 mice showed complete regression of their HT29 tumors. Unexpectedly, the efficacy of the 10-μg schedule was comparatively lower, indicating that for these tumors, a long-term treatment is more effective. No antitumor effect of 4D5MOCB-ETA was seen in mice bearing Ep-CAM-negative COLO320 control tumors (Fig. 5).

**DISCUSSION**

The lack of significant advances in the treatment of metastatic or refractory cancers has stimulated the design of novel approaches to targeted cancer therapy such as the use of antibody-based cancer therapeutics. Here, we summarize our results obtained with a humanized scFv-based immunotoxin specific for Ep-CAM. Because of their different mechanism of action, and especially because of their built-in targeting function that conventional anticancer agents do not have, immunotoxins may add new options for the treatment of malignancies resistant to conventional treatments (26, 28). ETA and its homologues irreversibly block protein synthesis in cells by ADP-ribosylating a posttranslationally modified histidine residue of elongation factor 2, called diphthamide, which ultimately triggers apoptosis (48). Although resistance of cells to ETA was described as a consequence of the mutation of the crucial histidine residue or loss of enzyme activity required for diphthamide synthesis (49,
A number of chemical and recombinant immunotoxins that use either plant or bacterial toxins as effector domains and that target distinct cell surface antigens associated with tumor cells have been shown to be potent and selective anticaner agents in preclinical studies (28). However, only few of them proved to be promising candidates for clinical use.

Major responses have been reported thus far only for leukemias. In Phase I studies using an ETA-based scFv immunotoxin that targets IL-2 receptor α chain termed LMB-2, responses have been observed treating hematological malignancies (51, 52). Moreover, two-thirds of the patients with refractory hairy-cell leukemia involved in a Phase I study showed complete remission after treatment with BL22, a recombinant ETA-based dsFv immunotoxin specific for the CD22 surface antigen (53). Both immunotoxins showed minor or reversible toxic side effects and thus merited to be involved in Phase I studies for which a large number of patients have been evaluated.

Two recombinant ETA-based dsFv immunotoxins are also currently being evaluated for the treatment of advanced solid tumors. The immunotoxin SS1(dsFv)-PE38 is directed against cells expressing mesothelin, a normal protein normally produced by mesothelial cells and expressed also on malignant mesotheliomas and ovarian carcinomas (54). The immunotoxin LMB-9 has been derived from the monoclonal antibody B3 that targets the LewisY antigen (55), which is also widely expressed in epithelial tissues, a fact that contributes to safety concerns. LMB-9 and SS1(dsFv)-PE38 are currently being tested in Phase I clinical trials in patients with advanced solid tumors, and first results are eagerly awaited. In a previous study with 38 patients suffering from advanced carcinomas, the chemical conjugate of monoclonal antibody B3 and ETA induced one complete and one partial response (56). In these studies, vascular leakage because of capillary damage was found to be dose limiting, and subsequent preclinical investigations revealed significant binding of the B3 antibody to LewisY expressed on endothelial cells (57).

In addition to HER-2/neu (25), Ep-CAM represents another more promising target for antibody-based therapy of solid tumors because of its abundant expression in many carcinomas and its limited distribution in normal epithelial tissues (5). Although, Ep-CAM expression is not exclusively restricted to tumor cells, Riethmüller et al. (7) found that application of the anti-Ep-CAM monoclonal antibody 17-1A in patients with rectal colorectal carcinoma or minimal residual disease reduced the overall mortality by 32%, decreased the recurrence rate by 23%, and reduced the number of distant metastases (8).

In previous studies, two Ep-CAM-specific chemically conjugated immunotoxins have been described that both exploit the favorable tumor targeting properties of monoclonal antibody MOC31. One of these first generation conjugates is based on a full-length ETA and was used as a powerful agent to purge bone marrow from metastatic breast carcinoma cells (58). The second one was developed by our group and consists of MOC31 chemically linked to a truncated form of ETA lacking the cell-binding domain I (18). Despite its potent cytotoxicity in vitro, the MOC31-ETA conjugate could only significantly affect the growth of relatively small tumor xenografts (<100 mm³) in mice. The immunotoxin 4DSMOCB-ETA was developed by fusing the highly stable humanized scFv 4DSMOCB (38) to a truncated form of ETA comprising amino acids 252–608 and the COOH-terminal eukaryotic ER retention sequence KDEL. The 4DSMOCB-ETA is extremely potent in the femtomolar range and potently inhibits the growth of carcinoma cells of diverse histological origins in a highly antigen-specific manner as demonstrated by an increase in the cytotoxic potency by more than four orders of magnitude, compared with antigen-negative cells. Moreover, the antigen-specific action of 4DSMOCB-ETA was additionally corroborated in competition assays using an excess of scFv 4DSMOCB. Similar to the findings reported from immunotoxins targeting other tumor-associated antigens, including HER-2/neu and EGF receptor (25, 59), the cytotoxic activity of 4DSMOCB-ETA did not correlate with the amount of target antigen expressed on the tumor cell surface. Thus, it is likely that other cell type-specific parameters such as rate of internalization, intracellular trafficking, and fate of the enzyme domain are additional determinants of immunotoxin efficacy. In terms of its in vitro cytotoxicity, 4DSMOCB-ETA is the most potent Ep-CAM-specific immunotoxin that has been reported in the literature and was 1000-fold more potent than the chemical immunotoxin conjugate MOC31-ETA (18).

A prerequisite for the optimal binding of antibody-based therapeutics to target antigens expressed on the surface of tumor cells and for efficient tumor localization is protein stability under physiological conditions. The extremely potent cytotoxicity shown by 4DSMOCB-ETA may at least partly be attributable to the stability of the targeting scFv (38). The immunotoxin itself was obtained to >90% from the soluble fraction after bacterial lysis, was monomeric, and could be expressed and purified with a yield of ~0.5 mg/liter bacterial culture from simple shake flasks. These are excellent prospects for scale-up by high cell density fermentation (60). The high stability of the immunotoxin was confirmed by the large proportion of molecules that eluted in monomeric form after 20 h of incubation at 37°C in PBS, a result that was also obtained by incubating the radioactive-labeled immunotoxin in serum. Addition of protease inhibitors prolonged the stability for >48 h, indicating that protein degradation was not a consequence of intrinsic molecule instability but rather was a side effect occurring during purification. The addition of ETA and a second hexahistidine tag at the NH₂-terminal end of the scFv did not interfere with the binding properties of this ligand.

The antitumor activity of 4DSMOCB-ETA was demonstrated by the significant growth inhibition upon systemic administration to mice bearing established tumor xenografts (160 mm³) from colorectal, small cell lung, or squamous cell carcinoma of the head and neck. Both dose schedules were well tolerated and proved to be very effective in inhibiting tumor growth. The 3-week treatments with a total dose of 45 μg eradicated a significant fraction of the tumors, and some mice remained tumor-free during the whole study. In contrast, after completion of the shorter 1 week treatment with a total dose of 30 μg of 4DSMOCB-ETA, HT29 tumors rapidly resumed their growth.
The systemically administered 4DSMOCB-ETA was cleared from the blood with slightly slower kinetics when compared with the scFv, probably as a consequence of its increased molecular size. The blood clearance rate inversely correlated with the amount of radioactivity in the kidney which was lower for the immunotoxin than for the scFv (ID/g tissue 22.79 versus 300%), while in the liver the values were higher for the immunotoxin.

Although all doses of immunotoxin were well tolerated and mice did not show any signs of illness such as weight loss and the accumulation of 4DSMOCB-ETA in liver, spleen, and bone raised the issue of potential toxicity to these tissues. The inhibitory effect of ETA on protein synthesis is known to induce severe hepatotoxicity by sensitizing hepatocytes to the action of TNF, which is released by Kupffer cells upon ETA-mediated T-cell stimulation and induces liver cell necrosis (46, 47). To assess the degree of liver damage upon 4DSMOCB-ETA treatment, immunoconompetent mice received repeated doses of 5 μg (250 μg/kg⁻¹) or 10 μg (500 μg/kg⁻¹) of immunotoxin every other day for three cycles. In both cases, the level of ALT/AST activity in the plasma of treated mice did not change significantly compared to untreated controls. First signs of impaired liver function only appeared after treatment with a 20-μg (1 mg/kg⁻¹) dose given twice every other day (7.5-fold increase over control). In line with these findings, histological analysis of liver specimens did not reveal any signs of ETA-induced pathological changes, except for the two 20-μg treatments, which induced moderate hepatocyte necrosis. A recent study has shown that ETA-induced hepatotoxicity and vascular leak syndrome can be circumvented and enlarged the therapeutic window by pretreatment with anti-inflammatory agents such as indomethacin or soluble TNF receptor and nonsteroidal drugs, respectively (27, 29). The lack of correlation between organ-specific accumulation and toxicity of the immunotoxin strongly suggests that the radioactivity in these highly perfused organs simply reflects the presence of noncell bound and noninternalized immunotoxin in the large blood pool and capillary network. On the other hand, it is unclear whether in specialized phagocytes such as Kupffer cells of the liver, internalized proteins can escape into the cytoplasm, e.g., to interact with the elongation factor-2, or are rapidly directed to lysosomal degradation (61). In addition, it remains to be determined whether hexahistidine tags can affect the biodistribution behavior of recombinant proteins in vivo. In the kidney, the level of radioactivity is always higher with metal-labeled than with iodinated proteins that are prone to dehalogenation (62). Therefore, metal-labeled proteins probably more accurately reflect the real picture of in vivo biodistribution (63).

In this study, we describe the development and preclinical testing of a humanized recombinantly expressed Ep-CAM-specific immunotoxin. Potent and selective cytotoxicity in vitro, favorable tumor localization properties, as well as the powerful antitumor effect of 4DSMOCB-ETA in vivo suggest its clinical use in the treatment of Ep-CAM-positive carcinomas. A Phase I study with 4DSMOCB-ETA in patients with refractory squamous cell carcinoma of the head and neck is currently being initiated to investigate safety, toxicity, and therapeutic potential of our Ep-CAM-specific immunotoxin.

ACKNOWLEDGMENTS

We thank Dr. Mathias Schmidt for helpful discussions and Anne-marie Honegger for Fig. 18. We also thank Brigitte Sigrist and Christine de Pasquale for their excellent technical assistance. We thank Hans Lutz (Veterinärmedizinisches Labor, Tierspital, Zürich, Switzerland) and Dr. Pete Ossett (Institute of Veterinary Pathology, University of Zürich, Zürich, Switzerland) for their support with the toxicology experiments.

REFERENCES


Correction

In the article by C. Di Paolo et al., which appeared in the July 2003 issue of Clinical Cancer Research (pp. 2837–2848), figure 1 appeared with an error in coloring. The correct figure appears below.

Fig. 1 The recombinant 4DSMOCB-ETA immunotoxin. A, schematic representation of the scFv-toxin fusion protein, which includes the ompA signal sequence for periplasmic expression. The scFv antibody fragment 4DSMOCB is fused to the Pseudomonas exotoxin A (ETA252–608) by the linker shown. The protein is flanked by two hexahistidine tags, the C-terminal of which precedes the ER retention signal KDEL. B, three-dimensional model of the mature 4DSMOCB-ETA. The structure of the scFv (\( V_L \) in pink, \( V_H \) in cyan), of ETA252–608 (domain II in orange, domain Ib in magenta and domain III in red) and of the linking peptides (green) are shown. Both hexahistidine tags are indicated in yellow. C, total extract of SB536 bacterial culture samples before (−) and after (+) IPTG induction and 10 μg of 4DSMOCB-ETA immunotoxin purified by Ni\(^{2+}\)-IDA and anion exchange affinity chromatography columns coupled in series (P), were analyzed on 10% SDS-PAGE under reducing conditions. D, the immunotoxin proteins present in the same samples were visualized on a Western blot using a HRP-conjugated anti-tetrahistidine antibody. Markers are shown in lane M: myosin (\( M_r 208,000 \)), β-galactosidase (\( M_r 119,000 \)), bovine serum albumin (\( M_r 94,000 \)), ovalbumin (\( M_r 51,100 \)), carbonic anhydrase (\( M_r 35,400 \)) and soyabean trypsin inhibitor (\( M_r 28,800 \)).
A Recombinant Immunotoxin Derived from a Humanized Epithelial Cell Adhesion Molecule-specific Single-Chain Antibody Fragment Has Potent and Selective Antitumor Activity

Claudio Di Paolo, Jörg Willuda, Susanne Kubetzko, et al.


Updated version  Access the most recent version of this article at: 
http://clincancerres.aacrjournals.org/content/9/7/2837

Cited articles  This article cites 60 articles, 29 of which you can access for free at: 
http://clincancerres.aacrjournals.org/content/9/7/2837.full.html#ref-list-1

Citing articles  This article has been cited by 13 HighWire-hosted articles. Access the articles at: 
/content/9/7/2837.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.