Copper-67 Radioimmunotherapy and Growth Inhibition by Anti-L1-Cell Adhesion Molecule Monoclonal Antibodies in a Therapy Model of Ovarian Cancer Metastasis

Karin Knogler,1 Jürgen Grünberg,1 Kurt Zimmermann,1 Susan Cohrs,1 Michael Honer,2 Simon Ametamey,2 Peter Altevogt,3 Mina Fogel,4 P. August Schubiger,1,2 and Ilse Novak-Hofer1

Abstract

Purpose: We examined the tumor-targeting and therapeutic effects of 67Cu-labeled single amino acid mutant forms of anti-L1 monoclonal antibody chCE7 in nude mice with orthotopically implanted SKOV3ip human ovarian carcinoma cells.

Experimental Design: For radioimmunotherapy, chCE7 antibodies with a mutation of histidine 310 to alanine (chCE7H310A) and a mutation of asparagine 297 to glutamine (chCE7agl) were generated to achieve more rapid blood clearance. Biodistributions of 67Cu-4-(1,4,8,11-tetraazacyclotetradec-1-yl)-methyl benzoic acid tetrachloride (CPTA)–labeled mutant antibodies were measured in nude mice bearing SKOV3ip human ovarian cancer metastases. The effects of single i.v. injections of 67Cu-chCE7agl alone on tumor reduction and survival were investigated. In addition, a combination of low-dose 67Cu-radioimmunotherapy with unlabeled anti-L1 antibody L1-11A on survival was investigated.

Results: 67Cu-CPTA-chCE7agl showed high (up to 49% ID/g) and persistent (up to 168 h) uptake in SKOV3ip metastases, with low levels in normal tissues. 67Cu-CPTA-chCE7H310A revealed a shorter half-life in the blood and a lower tumor uptake and retention. A single low dose of 4 MBq of 67Cu-chCE7agl reduced tumor growth but did not prolong survival significantly, whereas a single 10.5 MBq dose of 67Cu-chCE7agl reduced tumor growth and prolonged survival significantly. The combination of unlabeled monoclonal antibody L1-11A with a subtherapeutic dose of 67Cu-radioimmunotherapy also prolonged survival significantly.

Conclusion: The results show improved pharmacokinetics and biodistributions as well as the therapeutic effect of the 67Cu-labeled single amino acid mutant chCE7agl. Therapeutic data indicate, for the first time, the feasibility of combining anti-L1-directed growth inhibition and 67Cu-radioimmunotherapy, thereby increasing the efficiency of antibody treatment of metastatic ovarian carcinoma.

Ovarian cancer is often detected late and late-stage disease is characterized by widespread peritoneal dissemination and ascites. Although therapies have been further developed in the past decade, all established therapies reveal a poor effectiveness in the late stage of the disease. Despite improved 5-year survival rates, recurrences are frequent, overall mortality remains unchanged, and new therapeutic strategies are urgently needed. Monoclonal antibodies (mAb) acting by stimulating the immune response such as anti–CA-125 oregovomab (1) and anti–vascular endothelial growth factor antibody bevacizumab, which inhibits angiogenesis, are in phase III trials as adjuvant therapies (1–3). At present, the use of radioimmunotherapy in the treatment of advanced ovarian cancer following cytoreductive surgery and chemotherapy is limited. In a large phase III study, i.p. administration of 90Y-labeled anti-MUC1 antibody, HMFG1, was not found to improve long-term survival (4). Also, radioimmunotherapy using 131I-labeled mAb, OC-125, showed little therapeutic benefit in patients with ovarian cancer (5). Antibodies used in radioactive form for the imaging and radioimmunotherapy of ovarian cancer were directed against MUC1 and CA-125 proteins. Both proteins have large, multivalent, and highly glycosylated mucin-type exodomains. They are shed in high amounts from tumor cells and therefore antibody targeting may be impaired. In addition, the anti-MUC1 and anti–CA-125 antibodies that were used are not actively internalized into target tumor cells, which may also limit their efficacy as targeting vehicles.

Authors’ Affiliations: 1Center for Radiopharmaceutical Science, ETH-PSI-USZ, Paul Scherrer Institute, Villigen, Switzerland; 2Federal Institute of Technology, Zürich, Switzerland; 3German Cancer Research Center, Heidelberg, Germany; and 4Department of Pathology, Kaplan Medical Center, Rehovot, Israel

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Note: K. Knogler and J. Grünberg contributed equally to this study.

Requests for reprints: Ilse Novak-Hofer, Center for Radiopharmaceutical Science, ETH-PSI-USZ, Paul Scherrer Institute, CH-5232 Villigen, Switzerland. Phone: 41-56-310-4067; Fax: 41-56-310-2849; E-mail: ilse.novak@psi.ch.

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Recently, antibody therapy directed against the L1-cell adhesion molecule protein has emerged as a new option for targeting ovarian cancer metastases (6). L1 is a cell surface protein, originally studied intensively in the nervous system (7–9), which is overexpressed in a number of different tumors, such as neuroblastomas (10), renal carcinomas (10, 11), ovarian, endometrial carcinomas, and melanomas (12). A soluble form of L1 was found in the serum of patients with ovarian cancer and may turn out to be a marker for progression of this disease (13). L1 is increasingly recognized as a signaling molecule which directly and indirectly transmits signals regulating proliferation, migration, and invasion of tumor cells (14–16). Anti-L1 antibodies were found to inhibit tumor cell proliferation in vitro (6, 17), and recently, it was shown that the anti-L1 antibody L1-11A inhibits the growth of SKOV3ip human ovarian cancer metastases in vivo in nude mice (6). Based on these results, we investigated in this study if the efficacy of antibody treatment was increased by combining the growth inhibition activity of anti-L1 antibodies with radioimmunotherapy using radiometal-labeled anti-L1 antibodies directed against another epitope.

For radioimmunotherapy, the metallic radionuclide $^{67}$Cu was selected. Its appropriate half-life of 2.6 days and high abundance of medium energy $\beta$-emission of 141 keV make it a suitable option to the high-energy $\beta$-emitter $^{90}$Y for the treatment of small tumor masses. The lower percentage of therapeutically useless gamma-emission (16% of 93 keV and 48% of 185 keV) compares favorably with the commonly used $^{131}$I (82% of 364 keV). Anti-L1 mAb chCE7 binds to target cells with picomolar affinity ($K_d \approx 10^{10}$ mol/L) and inhibits cell proliferation (6, 18). ChCE7 is internalized into target cells, shows high uptake in tumor xenografts in nude mice when used in radiiodinated or in radiocopper-labeled form (19, 20), and performs well in imaging of metastases in patients with neuroblastoma (21). For radioimmunotherapeutic applications, it was of interest to develop variant antibodies with more rapid clearance from the blood because dose-limiting toxicity is usually directed against the blood cells and the bone marrow. To circumvent the low tumor uptake and accumulation of radioactivity in the kidneys observed with rapidly clearing radiometal-labeled antibody fragments, chCE7 antibodies with single amino acid mutations were constructed with no overall change in molecular weight (150 kDa). An aglycosylated form of mAb chCE7 (chCE7N297Q), as well as a single amino acid mutant which had been shown to decrease the half-life of IgG1 by interfering with FcRn receptor binding (chCE7H310A), were produced (22, 23). Both single amino acid mutants cleared more rapidly from the blood than the parent mAb chCE7. Based on clearance properties and biodistribution data, we selected $^{67}$Cu-chCE7agl as radioimmunoconjugates for the therapy of nude mice with SKOV3ip human ovarian cancer metastases. We found that radioimmunotherapy using a single 10.5 MBq dose of the $^{67}$Cu-immunoconjugate prolonged survival for a significantly longer time than biweekly treatment with unlabeled mAb L1-11A alone. A single low 5 MBq dose of the $^{67}$Cu-immunoconjugate led to significantly increased survival when combined with biweekly treatment with unlabeled mAb L1-11A. Our study shows proof of principle of combining radioactive and inactive anti-L1-cell adhesion molecule mAbs for more efficient therapy of ovarian cancer.

Materials and Methods

Materials. Chemicals and solvents used were from Fluka (Buchs, SG, Switzerland) unless stated otherwise.

Cells and antibodies. HEK-293 cells were from the German Collection of Microorganisms (Braunschweig, Germany), SKOV3ip human ovarian carcinoma cells were previously described (6). HEK-293 and SKOV3ip cells were maintained in DMEM (4.5 g/L glucose). All media were supplemented with 10% FCS, 2 mmol/L of glutamine, 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of fungizone ("complete medium"). All media and additives were obtained from BioConcept (Allschwil, Switzerland). MAb L1-11A (subclone of UJ127.11) was purified by Invivo Biotech services GmbH (Henningsdorf, Germany).

Antibody design, plasminoids, and gene assembly. MAb chCE7 is composed of murine VI and murine VH fused to the Fc part of human IgG1. The CDNs for chCE7 light and heavy chain (a gift from J. Leonard, ETHZ, Switzerland) were cloned into the HindIII BamHI site of murine VL and murine VH fused to the Fc part of human IgG1. The CDNs for chCE7 light and heavy chain (a gift from J. Leonard, ETHZ, Switzerland) were cloned into the HindIII BamHI site of murine VL and murine VH fused to the Fc part of human IgG1.

Expression, selection, and purification of single amino acid mutants of mAb chCE7. HEK-293 human embryonic kidney cells (1 x 10⁷) were cotransfected with pcDNA3-chCE7L (light chain mutant) and pcDNA3chCE7H (heavy chain mutant), and clones were selected with G418 (25). Antibody production was verified with a Slot-Blot method (25). Mutant mAbs were purified from tissue culture supernatants either with protein G-Sepharose (chCE7E and chCE7agl), Amersham Biosciences, Dübenord, Switzerland) or with Ni-NTA-Sepharose [chCE7H310A, chCE7ΔCH2, and chCE7F(ab’’)]₂ followed by size exclusion chromatography using a Superdex 200 HR 10/30 column (Amersham Biosciences).

Characterization of purified chCE7 single amino acid mutants. Purified proteins were analyzed by SDS-PAGE under reducing and nonreducing conditions and by size exclusion chromatography on a Superdex 200 column at 0.5 mL/min flow rate using PBS buffer [0.1 mol/L NaCl and 0.05 mol/L sodium phosphate buffer (pH 7.4)].

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on a shaking platform for 2 h at 37°C. Nonspecific binding was determined by parallel incubations in the presence of 10 μg of unlabeled competitor. After incubation, cells were washed twice with PBS containing 0.5% bovine serum albumin to remove unbound immunon conjugates and cell-bound radioactivity was measured with a gamma counter (CobraII Packard, Canberra Packard GmbH, Frankfurt, Germany). Binding of 67Cu-labeled antibodies to SKOV3ip cells was measured by incubating cells with radioiodine-labeled traceres in a concentration range between 800 and 25 ng. Nonspecific binding in the presence of 10 μg of unlabeled mAb chCE7 was subtracted and data was analyzed with the GraphPad prism program using the Scatchard method.

Internalization of radiocopper-labeled immunon conjugates into SKOV3ip cells was measured as described earlier (19). Briefly, triplicate samples of adherent 2 x 10⁶ SKOV3ip cells were incubated with 3 kBq (150 ng) of 67Cu-labeled antibodies in complete DMEM medium for 4 h on ice. Unbound antibodies were washed off with PBS, fresh medium was added, and cells were incubated at 37°C for 2, 16, and 24 h. One set of cells (time 0) was processed directly by measuring acid- or released radioactivity using 0.1 mol/L of NaCl and 0.05 mol/L of glycine (pH 2.8). Cells were dissolved in 1 mol/L of NaOH and acid stable, cell-bound radioactivity was measured. Internalized radioactivity was defined as the percentage of acid stable activity of total cell-bound activity.

Ligand substitution of chCE7 antibodies. For labeling with 67Cu, the proteins were substituted with 4-(1,4,8,11-tetrazacyclododec-1-yl)-methyl benzoic acid tetrachloride (CPITA), which was synthesized and attached as a succinimide ester to chCE7 antibodies as previously described (26). Using an isotope dilution assay, it was estimated that there were four CPTA ligands for each mAb (26).

Radiolabeling. Radiodination of chCE7 antibodies (100 μg) in PBS was done with the iodogen method (Pierce, Perbio Science, Lausanne, Switzerland) as described previously (18).

67Cu was produced in-house by irradiating natZn with protons at the 72 MeV accelerator of the Paul Scherrer Institute (Villigen, Switzerland) as described before (27), and used for radiolabeling 1 day after production. For the positron emission tomography (PET) imaging experiments, 66Cu was produced at the Paul Scherrer Institute as described before (26).

Two hundred to 400 μg (250 μL) of the immunon conjugates in a total volume of 500 μL of 0.1 mol/L sodium acetate buffer (pH 5.5) 18.5 to 30 MBq (500-800 μCi) of neutralized 67Cu solution. For producing large amounts of radiolabeled conjugates for therapeutic experiments, up to 3 mg of ligand-substituted conjugates for therapeutic experiments, up to 3 mg of ligand-substituted conjugates were labeled with up to 300 MBq of 67Cu for 30 min at 22°C. After incubations, EDTA was added to a final concentration of 5 mmol/L for 5 min to complex unchelated copper. Purification of the labeled antibodies was achieved by fast protein liquid chromatography size exclusion chromatography on a Superdex 200 column (Amersham Biosciences) in PBS at a flow rate of 1.0 mL/min.

Quality control of radiolabeled preparations. Radiolabeled immunon conjugates were analyzed by size exclusion chromatography on a Superose 12 column (Amersham Biosciences) at 0.5 mL/min flow rate using PBS buffer. The major peaks of mAb eluted with a retention time of 22.50 min for 67Cu-chCE7, 22.50 min for 67Cu-chCE7-agl, and 23.60 min for 67Cu-chCE7H310A. The EDTA-metal complex appeared with a retention time of 34 to 35 min. The immunoreactivity of the 67Cu-chCE7 preparations were assayed with a cell-binding test using increasing numbers of SKOV3ip cells as described before (28). The stability of the labeled antibodies (6 μg, 160 MBq) was analyzed after incubation in human plasma of immunon conjugates in 2.0 mL of human plasma at 37°C. After the addition of 5 mmol/L of EDTA, samples (0.5 mL) were separated by fast protein liquid chromatography on a Superdex 200 HR 10/30 column (Amersham Biosciences) in PBS as described (29). Fractions (0.5 mL) were collected and radioactivity was measured in a gamma counter.

Pharmacokinetic and biodistribution studies. All animal studies were conducted in compliance with Swiss laws on animal protection. Housing and animal husbandry was done according to local laws on animal protection. For measuring the clearance of antibodies from the blood, groups of five female 6-week-old BALB/c mice were injected with ~370 kBq (10 μCi) of radiolabeled conjugates into the tail vein. Protein content was 7 μg in the case of 65Cu-chCE7, 30 μg in the case of 67Cu-chCE7-agl, and 80 μg in the case of 67Cu-chCE7H310A. At timed intervals after injection, blood samples (2 μL) were collected from the tail vein and measured together with an aliquot of the injected solution in a gamma counter. 151I emission was read at a window of 15 to 70 keV, whereas for 67Cu, an energy window between 160 and 210 keV was used. Results are expressed as the percentage of injected dose per total amount of blood (% ID/2 mL). Pharmacokinetic variables were determined using a two-compartment model and the equation (T) = A x e^-βt + A x e^-αt, where the first part of the equation corresponds to the distribution phase and the second part to the terminal elimination phase. The areas under the curve were calculated using the GraphPad Prism program.

For biodistribution studies, female nude mice, 5 weeks old (CD1-foxn1nu; Charles River, Inc., Sulzfeld, Germany) were injected i.p. with 5 x 10⁶ SKOV3ip human ovarian carcinoma cells. Two weeks later, 67Cu-labeled chCE7 antibody conjugates were injected into the tail vein at the concentrations indicated. At 16, 24, 48, 96, 120, and 168 h postinjection, groups of four animals were sacrificed and dissected. Tumors and major organs were weighed and measured together with an aliquot of the injected solution in a gamma counter. Results are expressed as the percentage of injected dose per gram (% ID/g). Statistical analysis of data was done with Student’s t test (two-tailed, unequal variance).

Therapy studies. Female CD1-foxn1nu mice, 5 weeks old, were injected i.p. with 7 × 10⁶ SKOV3ip cells. Two days postinoculation, 4 MBq (200 μg) or 10.5 MBq (330 μCi) of 67Cu-labeled chCE7 agl was injected in groups of eight to nine mice. Control animals (11) were left untreated. After 21 days, the animals were euthanized and dissected, tumor weight and ascites weight was measured. In the survival experiments, female CD1-foxn1nu mice, 5 weeks old, were injected i.p. with 7 × 10⁶ SKOV3ip cells or 14 × 10⁶ SKOV3ip cells. After 2 days, random groups of eight or nine animals were formed and 67Cu-labeled antibody conjugates were injected i.v. at the doses indicated. MAb L1-11A (in 300 μL of sterile PBS at a dose of 10 μg/kg) was injected i.p. biweekly. Animals were checked twice a week for weight, visible ascites, and behavioral signs of distress. In three “survival” experiments with 67Cu-labeled chCE7agl alone and in combination with mAb L1-11A, animals were kept until ascites was visible or a weight loss of >15% combined with behavioral signs of distress were observed. At this end point, animals were euthanized.

Statistical analysis. Statistical analysis of data was done using Student’s t test (two-tailed, unpaired) and by log-rank test in the case of the survival experiment. In the survival experiment, one animal in the control group and one animal in the 67Cu-chCE7agl–treated group were censored (unexplained death).

PET imaging. For the PET experiments, a tumor-free nude mouse was imaged after i.v. injection of 21 MBq (120 μCi) of 65Cu-CPITA-chCE7, prepared as described earlier (26). One mouse was imaged after the injection of 21.5 MBq (150 μCi) of 65Cu-CPITA-chCE7 22 days postinoculation of SKOV3ip cells, whereas one mouse was injected with 23 MBq (120 μCi) of 65Cu-CPITA mAb L1-11A also 22 days postinoculation of SKOV3ip cells. The animals were anesthetized 21 to 23 h after injection and scanned with a quad-HIDAC PET tomograph (Oxford Positron Systems, Oxford, United Kingdom). PET data were acquired in list mode for 60 to 90 min and reconstructed in a single time frame using the OPL-EM algorithm (0.5 mm bin size, 200-240 240 matrix size). Image files were analyzed using the dedicated software PMOD (30), and depicted as maximal intensity projections.

Immunohistochemistry. Immunostaining of paraffin sections from SKOV3ip tumors was done with anti-L1 antibody (L1-14.10) as described before (31).
Results

Pharmacokinetics of mAb chCE7 variants with single amino acid mutations. In order to select a mAb chCE7 antibody with more rapid clearance from the blood than the parental mAb, a number of chCE7 variant forms were constructed. Medium size divalent F(\(ab\))\(_2\) fragments (100 kDa) and antibodies deleted in the CH2 domain (110 kDa) were produced as described before (25). In addition, chCE7 forms with single amino acid mutations were constructed with no overall change in molecular weight (150 kDa). A mutant mAb with Asn\(^{310}\) replaced by Gln and thereby deleted in N-linked glycosylation (chCE7agl) as well as a mutant with His\(^{310}\) changed to Ala with decreased affinity for the FcRn receptor (chCE7H310A) was produced. The expression of recombinant proteins included treatment of transfected HEK-293 cells with sodium butyrate and yielded high amounts of antibodies (30 mg/L of purified protein). In a set of competition binding assays, the binding of \(^{125}\text{I}-\text{chCE7}\) to SKOV3ip cells in the presence of increasing concentrations of different antibodies was measured. \(\text{IC}_{50}\) values were 82 ng for chCE7agl, 33 ng for chCE7H310A, and in the same series, the \(\text{IC}_{50}\) of chCE7 was 75 ng (data not shown). These results indicated that binding affinities of the mutant antibodies were comparable to the wild-type chCE7 antibody.

We next tested the pharmacokinetics of mutant radioiodinated chCE7 antibodies. Figure 1A shows that the clearance from the blood of medium-sized mAb fragments chCE7\(\Delta\)CH2 and chCE7\((\text{ab})_2\) showed similar rapid clearance as expected, followed by chCE7H310A and chCE7agl. The single amino acid mutants were cleared with biphasic kinetics and the differences in \(t_{1/2}\) for the mutant proteins were not statistically different. The order of terminal half-lives \((t_{1/2,b})\) of blood clearance from the slowest to the fastest was as follows: intact chCE7 > chCE7agl > chCE7H310A. The spectrum of \(t_{1/2,b}\) of radioiodinated antibodies ranged from \(~6\) days \((^{125}\text{I}-\text{chCE7})\) to \(10\) h \((^{129}\text{I}-\text{chCE7H310A})\). The difference between radiiodinated chCE7 and the chCE7agl antibody was statistically significant at all time points \((P < 0.01)\). The blood clearance and biodistributions of the radiocopper-labeled chCE7agl and chCE7H310A conjugates were compared with mAb chCE7 (Fig. 1B). The \(^{67}\text{Cu}\)-labeled F(\(ab\))\(_2\) fragments were excluded because of the known accumulation of radiometal-labeled medium-sized F(\(ab\))\(_2\) fragments in the kidneys (27). The chCE7\(\Delta\)CH2 fragment was not chosen because of its tendency to aggregate and its inconvenient handling during ligand substitution and labeling. In contrast, the single amino acid mutant forms of chCE7 had little tendency to aggregate. In a parallel study on stability in human plasma, the \(^{67}\text{Cu}\)-CPTA-chCE7agl as well as the \(^{67}\text{Cu}\)-CPTA-chCE7 conjugates were stable at 37°C for at least 48 h with no evidence of fragmentation or binding to serum proteins (data not shown). Figure 1B shows clearance from the blood of the \(^{67}\text{Cu}\)-labeled immunonjugates. Statistical analysis of the blood clearance curves showed a significant difference \((P < 0.01)\) between radiocopper-labeled chCE7 and the chCE7H310A mutant at all time points, the difference between chCE7 and chCE7agl was significant \((P < 0.05)\) at the longest time points (72 and 96 h).

Table 1 summarizes the pharmacokinetic variables of different antibody formats in radioiodinated and radiocopper-labeled form. Table 1 shows, in addition to the \(\alpha\)-phase (distribution) and \(\beta\)-phase (terminal elimination) half-lives, the calculated area under the curve for each construct (% ID/2 mL \(\times h\)). The mean residence time was calculated as a single variable for blood clearance. Mean residence time as a single variable for blood clearance considers all pharmacokinetic processes which a mAb is exposed to in the body and was found to be 4- to 5-fold lower for the chCE7H310A mutant antibody than for the other antibodies. The difference between the mean residence time of chCE7 and chCE7agl was less pronounced than the difference in terminal half-life \(t_{1/2,b}\).

In vitro and in vivo evaluation of \(^{67}\text{Cu}\)-labeled single amino acid mutant of mAb chCE7. Both \(^{67}\text{Cu}\)-labeled chCE7agl and chCE7H310A showed more rapid pharmacokinetics than the parental mAb chCE7 and were potentially suitable for therapy. Tumor uptake and normal tissue distribution in nude mice with SKOV3ip metastases was investigated in order to select the radioimmunconjuate exhibiting the highest tumor accumulation and the lowest levels in liver and blood for therapy experiments. The nuclide we chose for radioimmunotherapy is the medium-range \(\beta\)-particle emitter, \(^{67}\text{Cu}\) (\(t_{1/2,\beta} = 2.6\) days). We had previously selected the CPTA chelate as the most stable ligand for copper labeling of intact antibodies (28). Catabolism...
of the internalizing chCE7 antibody exhibited high retention of the Cu-CPTA complex in target tumor cells (19). Approximately four CPTA ligands were substituted per antibody molecule. Immunoreactivity as determined by binding to SKOV3ip cells in vitro was 80% to 100% for 67Cu-labeled antibodies. We previously showed that high binding affinity and internalization of chCE7 antibody contribute to the excellent uptake of 67Cu-CPTA-chCE7 in tumor xenografts (18). We therefore investigated binding affinity and internalization of the radiocopper-labeled chCE7agl mutant antibody in SKOV3ip cells.

Figure 2 shows a direct comparison of the two radiocopper immunoconjugates. Binding affinities to SKOV3ip cells were found to be similar (Ka 6.7 × 10-10 mol/L for chCE7wt and 6.9 × 10-10 mol/L for chCE7agl; Fig. 2A). Internalization of the two conjugates was also similar, after 24 h, 80% of the cell-bound radioactivity was being recovered in the acid stable pool (Fig. 2B). Tumor and tissue uptake of 67Cu-CPTA-labeled mAb chCE7agl was measured in nude mice with SKOV3ip human ovarian cancer metastases.

Table 2 shows biodistributions measured starting 1 day post-i.v. injection up to 5 days for chCE7agl. The immunoconjugate reached a maximal tumor accumulation at 48 h with 49.2 ± 3.95% ID/g. Five days postinjection, the accumulation of radioactivity in normal tissues was low, similar to 67Cu-labeled chCE7 antibody. In the next step, 67Cu-chCE7H310A was compared with 67Cu-chCE7agl. Because of the more rapid blood clearance of chCE7H310A, 16 h was chosen as the starting point for measurements. 67Cu-labeled chCE7H310A reached maximal tumor accumulation at 16 h postinjection with 13.1 ± 2.39% ID/g. Liver uptake for this conjugate was high with 26.1 ± 3.68% ID/g and accumulation in the spleen was 9.2 ± 1.73% ID/g. Radioactivity cleared from the tumor rapidly and reached 2.17 ± 0.65% ID/g 120 h postinjection. When 67Cu-chCE7H310A was compared with 67Cu-chCE7agl at the 24-h time point, the latter showed higher and more persistent tumor uptake (39.7 ± 6.2 versus 9.67 ± 10.7; P = 0.035) and lower radioactivity levels in the liver (4.71 ± 0.74 versus 14.70 ± 3.3, P = 0.010) than the chCE7H310A mutant with impaired FcRn receptor binding. Because of the more favorable biodistributions 67Cu-CPTA-chCE7agl was selected for further therapy experiments. The chCE7agl antibody was found to inhibit growth of SKOV3ip cells in vivo to a similar extent as mAb chCE7 and mAb L1-11A (data not shown).

**Antitumor effects of 67Cu-labeled chCE7agl antibody.** The SKOV3ip metastasis model was used to assess antitumor effects. Radiocopper-labeled antibodies were injected i.v. 2 days post-i.p. inoculation of 7 × 106 SKOV3ip cells in groups of seven to nine nude mice. In the first experiment, the effect of radioimmunotherapy with two different doses of 67Cu-chCE7agl on progressive growth of SKOV3ip cells was investigated.

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**Table 1.** Pharmacokinetic variables of 125I- and 67Cu-labeled chCE7 single amino acid mutants and wild-type antibody

<table>
<thead>
<tr>
<th>Antibody formats</th>
<th>t1/2a (h)</th>
<th>Aa (%) ID/2 mL</th>
<th>t1/2b (h)</th>
<th>Ab (%) ID/2 mL</th>
<th>AUC</th>
<th>MRT (h)</th>
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<tbody>
<tr>
<td>125I-chCE7</td>
<td>0.70</td>
<td>55.4</td>
<td>138</td>
<td>45.4</td>
<td>4,015</td>
<td>39.1</td>
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<tr>
<td>125I-chCE7agl</td>
<td>0.59</td>
<td>68.5</td>
<td>96.3</td>
<td>27.8</td>
<td>2,437</td>
<td>35.6</td>
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<tr>
<td>125I-chCE7H310A</td>
<td>2.05</td>
<td>47.4</td>
<td>10.1</td>
<td>46.5</td>
<td>834</td>
<td>8.67</td>
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<tr>
<td>67Cu-chCE7</td>
<td>1.57</td>
<td>64.9</td>
<td>78.9</td>
<td>47.1</td>
<td>3,932</td>
<td>33.1</td>
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<tr>
<td>67Cu-chCE7agl</td>
<td>1.15</td>
<td>52.1</td>
<td>39.3</td>
<td>57.2</td>
<td>3,045</td>
<td>29.5</td>
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<tr>
<td>67Cu-chCE7H310A</td>
<td>0.81</td>
<td>77.6</td>
<td>11.2</td>
<td>31.5</td>
<td>712</td>
<td>9.41</td>
</tr>
</tbody>
</table>

*Y-axis intercept of the two components are given by Aa, and Ab, where the sum of Aa and Ab is the total % ID/2 mL.

1. The area under the curve (AUC) is a time integral of the blood concentration (% ID/2 mL × h).

2. The mean residence time (MRT) used to give a single variable for blood clearance.

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Fig. 2. Binding affinities and internalization of 67Cu-chCE7 and its aglycosylated variant into SKOV3ip cells. A, Scatchard plots of saturation binding to SKOV3ip cells, extrapolating to a Ka of 6.7 × 10-10 mol/L for chCE7wt and to 6.9 × 10-10 mol/L for chCE7agl. B, Intracellular radioactivity (percent of total cell-bound activity) after internalization of 67Cu-chCE7 and its aglycosylated variant into SKOV3ip cells. Cells were loaded with radiocopper-labeled antibodies at 4°C for 4 h, unbound antibodies were washed off and cells were incubated in complete medium for up to 24 h. Radioactivity in the acid-stable and acid-releasable fraction was measured at the indicated time points. Columns, percentage of acid-stable radioactivity of total cell-bound activity.
After 21 days, tumor masses and ascites were removed quantitatively.

Table 3 shows that in the control group, 7 of 11 animals developed ascites, whereas in the low-dose radioimmunotherapy group, no ascites had appeared, and in the high-dose radioimmunotherapy, 2 of 9 animals presented with ascites. Both doses led to a significant 70% decrease in overall tumor burden, the difference between the two dosages was not significant. In the next step, experiments were designed to investigate survival in this late-stage ovarian cancer model by using 67Cu-labeled chCE7agl alone and in combination with unlabeled anti-L1 mAb L1-11A. In a pilot experiment, mice with SKOV3ip metastases were imaged 21 to 23 h postinjection of 64Cu-labeled anti-L1 antibodies.

Table 2. Biodistributions of 67Cu-labeled chCE7 single amino acid mutants in nude mice with human ovarian cancer (SKOV3ip) metastases

<table>
<thead>
<tr>
<th>Organ (% ID/g)</th>
<th>24 h</th>
<th>48 h</th>
<th>96 h</th>
<th>168 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>67Cu-chCE7agl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>39.7 (6.23)</td>
<td>49.2 (3.95)</td>
<td>40.2 (4.75)</td>
<td>48.5 (13.4)</td>
<td>34.4 (0.80)</td>
</tr>
<tr>
<td>Blood</td>
<td>10.9 (1.70)</td>
<td>8.33 (3.28)</td>
<td>3.27 (2.15)</td>
<td>2.22 (1.17)</td>
<td>1.95 (1.15)</td>
</tr>
<tr>
<td>Liver</td>
<td>4.71 (0.74)</td>
<td>4.44 (1.07)</td>
<td>2.80 (0.57)</td>
<td>3.12 (0.87)</td>
<td>5.72 (1.39)</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.76 (1.53)</td>
<td>5.37 (1.07)</td>
<td>4.81 (1.18)</td>
<td>4.42 (3.38)</td>
<td>4.74 (2.18)</td>
</tr>
<tr>
<td>Kidney</td>
<td>6.02 (0.53)</td>
<td>5.94 (1.38)</td>
<td>4.09 (1.35)</td>
<td>4.36 (0.97)</td>
<td>2.22 (0.63)</td>
</tr>
<tr>
<td>Heart</td>
<td>5.53 (1.52)</td>
<td>3.42 (1.09)</td>
<td>1.46 (0.75)</td>
<td>1.72 (0.98)</td>
<td>1.07 (0.32)</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.06 (0.31)</td>
<td>1.18 (0.53)</td>
<td>0.89 (0.37)</td>
<td>0.92 (0.38)</td>
<td>0.79 (0.09)</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.69 (0.32)</td>
<td>1.42 (0.29)</td>
<td>0.92 (0.30)</td>
<td>0.67 (0.19)</td>
<td>0.91 (0.11)</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.34 (0.32)</td>
<td>1.20 (0.61)</td>
<td>0.84 (0.46)</td>
<td>0.41 (0.27)</td>
<td>0.25 (0.20)</td>
</tr>
</tbody>
</table>

Table 3. L1-directed therapy of orthotopic SKOV3ip tumors in nude mice treated with 67Cu-chCE7agl

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Number of mice injected</th>
<th>Mean tumor weight ± SD (g)</th>
<th>P*</th>
<th>Mean ascites (mL)</th>
<th>Mean body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>11</td>
<td>1.30 ± 0.69</td>
<td>0.003</td>
<td>1.61 (7/11)</td>
<td>23.1 ± 1.8</td>
</tr>
<tr>
<td>67Cu-chCE7agl4 MBq</td>
<td>8</td>
<td>0.51 ± 0.23</td>
<td>0.006</td>
<td>0 (0/8)</td>
<td>24.4 ± 1.9</td>
</tr>
<tr>
<td>67Cu-chCE7agl10.5 MBq</td>
<td>9</td>
<td>0.38 ± 0.20</td>
<td>0.006</td>
<td>0.30 (2/9)</td>
<td>24.2 ± 1.6</td>
</tr>
</tbody>
</table>

NOTE: Groups of four animals were injected i.v. with 225 kBq (10 μg) of 67Cu-chCE7agl, 141 kBq (25 μg) of 67Cu-chCE7 and 95 kBq (26 μg) of 67Cu-chCE7 H310A. Data are presented as % ID/g ± SD.

Table 2. Biodistributions of 67Cu-labeled chCE7 single amino acid mutants in nude mice with human ovarian cancer (SKOV3ip) metastases

<table>
<thead>
<tr>
<th>Organ (% ID/g)</th>
<th>16 h</th>
<th>24 h</th>
<th>48 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>67Cu-chCE7H310A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>13.1 (2.39)</td>
<td>9.67 (10.7)</td>
<td>6.81 (0.77)</td>
<td>2.17 (0.65)</td>
</tr>
<tr>
<td>Blood</td>
<td>1.10 (0.10)</td>
<td>0.34 (0.18)</td>
<td>0.26 (0.08)</td>
<td>0.19 (0.07)</td>
</tr>
<tr>
<td>Liver</td>
<td>26.1 (3.68)</td>
<td>14.7 (3.33)</td>
<td>10.7 (5.06)</td>
<td>8.58 (2.47)</td>
</tr>
<tr>
<td>Spleen</td>
<td>9.20 (1.73)</td>
<td>4.44 (3.81)</td>
<td>3.39 (1.48)</td>
<td>3.59 (0.25)</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.91 (0.49)</td>
<td>2.09 (0.37)</td>
<td>2.16 (0.46)</td>
<td>1.39 (0.22)</td>
</tr>
<tr>
<td>Heart</td>
<td>2.88 (0.74)</td>
<td>1.55 (0.34)</td>
<td>1.11 (0.53)</td>
<td>1.83 (0.33)</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.92 (0.38)</td>
<td>0.51 (0.24)</td>
<td>0.32 (0.07)</td>
<td>0.53 (0.08)</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.78 (0.41)</td>
<td>1.18 (0.54)</td>
<td>0.70 (0.40)</td>
<td>0.59 (0.14)</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.07 (0.33)</td>
<td>0.04 (0.19)</td>
<td>0.21 (0.14)</td>
<td>0.12 (0.07)</td>
</tr>
</tbody>
</table>

NOTE: Groups of four animals were injected i.v. with 225 kBq (10 μg) of 67Cu-chCE7agl, 141 kBq (25 μg) of 67Cu-chCE7 and 95 kBq (26 μg) of 67Cu-chCE7 H310A. Data are presented as % ID/g ± SD.

After 21 days, tumor masses and ascites were removed quantitatively.

Table 3 shows that in the control group, 7 of 11 animals developed ascites, whereas in the low-dose radioimmunotherapy group, no ascites had appeared, and in the high-dose radioimmunotherapy, 2 of 9 animals presented with ascites. Both doses led to a significant ~70% decrease in overall tumor burden, the difference between the two dosages was not significant. In the next step, experiments were designed to investigate survival in this late-stage ovarian cancer model by using 67Cu-labeled chCE7agl alone and in combination with unlabeled anti-L1 mAb L1-11A. In a pilot experiment, mice with SKOV3ip metastases were imaged 21 to 23 h postinjection of 64Cu-labeled anti-L1 antibodies.

Figure 3 shows 64Cu-PET imaging of a tumor-free control mouse with mAb chCE7 (A), a mouse with SKOV3ip metastases imaged with anti-L1 mAb chCE7 (B), and a mouse with SKOV3ip metastases imaged with anti-L1 mAb L1-11A (C). The abdominal tumor nodules were visualized well, demonstrating good tumor targeting ability of the two mAbs. Binding experiments to SKOV3ip cells in vitro showed that binding of mAb chCE7 was not inhibited by mAb L1-11A and vice versa, indicating that the mAbs bind to different epitopes on L1-cell adhesion molecules (data not shown), and the combination of the two mAbs did not impair their respective tumor-binding abilities. When paraffin sections of SKOV3ip tumors were stained with anti-L1 antibody, L1-14.10, the staining was found to be heterogeneous, with L1-positive tumor cells in the leading edge of the tumor and tumor cells invading and L1-negative cells in the tumor mass (Fig. 3D).

The decrease in tumor mass observed by 67Cu-radioimmunotherapy (Table 3) or by repeated administration of unlabeled mAb L1-11A (6) may not necessarily lead to increased survival. Therefore, survival experiments were done subsequently with groups of seven to nine mice. We defined the end points as the appearance of ascites, which is clearly visible in nude mice, or >15% weight loss, combined with behavioral signs of distress. At this end point, mice were dissected and the tumor mass was determined.

Good tumor targeting ability of the two mAbs. Binding experiments to SKOV3ip cells in vitro showed that binding of mAb chCE7 was not inhibited by mAb L1-11A and vice versa, indicating that the mAbs bind to different epitopes on L1-cell adhesion molecules (data not shown), and the combination of the two mAbs did not impair their respective tumor-binding abilities. When paraffin sections of SKOV3ip tumors were stained with anti-L1 antibody, L1-14.10, the staining was found to be heterogeneous, with L1-positive tumor cells in the leading edge of the tumor and tumor cells invading and L1-negative cells in the tumor mass (Fig. 3D).

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quantitatively removed and measured. Tumor masses of such moribund animals ranged between 0.9 and 2.2 g.

Figure 4 shows Kaplan-Meier survival curves. In the first experiment (A), treatment of mice with biweekly doses of unlabeled mAb L1-11A was assessed and was found to prolong survival from 42 days (untreated controls) to 65 days, the difference not being statistically different ($P = 0.1767$). In a second experiment (B) a single dose of 10.5 MBq $^{67}$Cu-CPTA–labeled chCE7agl prolonged the median survival from 31 to 51 days and showed significantly longer survival than controls ($P = 0.0335$). In contrast, the $^{67}$Cu-CPTA-labeled control antibody (Lym1) showed a median survival of 34 days compared with controls (31 days) and the difference in survival was not statistically significant ($P = 0.6572$). In the third experiment (C), $14 \times 10^6$ SKOV3ip cells were inoculated in contrast to the other experiments in which $7 \times 10^6$ cells were inoculated. In this experiment, untreated animals with a low, suboptimal (5 MBq) dose of $^{67}$Cu-chCE7agl and the combination of $^{67}$Cu-chCE7agl with biweekly treatment with mAb L1-11A were evaluated. When data was analyzed by Kaplan-Meier plots, controls showed a median survival of 29 days. Animals given a single 5 MBq dose of control mAb $^{67}$Cu-Lym-1 showed a median survival of 33 days, the same as animals treated with a single 5 MBq dose of $^{67}$Cu-chCE7agl. The combination of $^{67}$Cu-chCE7agl with biweekly doses of 10 mg/kg of mAb L1-11A extended median survival to 61 days. Mice treated with a single dose of 5 MBq $^{67}$Cu-labeled irrelevant mAb Lym1 showed no significant difference compared with mice with no treatment at all ($P = 0.0744$). Mice that received $^{67}$Cu-labeled chCE7agl in combination with unlabeled mAb L1-11A showed a statistically significant longer survival than the mice that received no treatment at all ($P = 0.0063$), and the difference between the combination therapy and therapy with a single low dose of $^{67}$Cu-chCE7agl was also significant ($P = 0.046$).

Taken together, the results show that high-dose radioimmunotherapy is more effective than single low-dose radioimmunotherapy and multiple dosing with unlabeled mAb L1-11A. A combination of low-dose radioimmunotherapy with unlabeled mAb L1-11A prolonged survival significantly compared with the single treatments.

**Discussion**

MAbs can exert antitumor activity by stimulating the cellular immune response and recruiting T cells to the tumor site or by directly inhibiting the growth of tumor cells via induction of apoptosis and/or inhibition of growth signaling. In addition, mAbs mediate target-specific cell killing when they are coupled with cytotoxic drugs or radionuclides. When used for therapeutic strategies, all of these antibody-induced antitumor effects have been observed, but none have been proved entirely satisfactory in eradicating tumors. Loss of the target antigen by mutation and outgrowth of antigen-negative tumor cells is not the only reason for suboptimal efficacy. Mutations in the target protein and in different signaling pathways also contribute to the reduced efficacy of antibody-mediated growth inhibition (32, 33). For this reason, antibodies loaded with cytotoxic radionuclides (radioimmunotherapy) could increase the efficacy of antibody therapies. For instance, in patients with non-Hodgkin’s lymphoma, $^{90}$Y-ibritumomab (Zevalin) produced significantly higher overall and complete response rates than did the corresponding unlabeled growth-inhibitory rituximab (Rituxan; refs. 34, 35). In contrast, the failure of i.p. therapy of
epithelial ovarian cancer with ^{90}Y-muHMFG1 antibody, which was reported recently in a large phase III study (4), indicates the importance of improved tumor targeting for this disease. Although the bulk of ovarian cancer resides in the i.p. space, sometimes, distant metastases can be detected in lymph nodes and bone marrow and i.v. application may be an advantage. For i.v. application, pharmacokinetics of the antibodies should exhibit a half-life in the blood sufficiently long to achieve high accumulation of radioactivity at the tumor site. At the same time, the levels in normal tissues such as kidney and liver should be minimized. The radiocopper-labeled chCE7agl construct we selected for therapy experiments combines these properties and shows consequently excellent tumor targeting in the SKOV3ip metastases model. Efficient internalization of the radiocopper-labeled chCE7agl mutant in target tumor cells contributes to the prolonged tumor residence.

We also evaluated mutations of residues in the Fc region which regulate binding to the FcRn receptor and had been found to reduce the half-life of the radiiodinated immunoglobulins in the blood (22, 36). A series of antibody fragments with β half-lives in the blood ranging from 83 to 8 h had been generated by this approach (22). Following this concept, we found that a H310A mutation reduced the β half-life in the blood of radioiodinated chCE7 (150 kDa) >10-fold, and for the ^{67}Cu-labeled immunonjugate, an ~7-fold reduced half-life was observed. An aglycosylated chCE7 mutant N297Q (chCE7agl), showed a modest ~50% reduction in half-life. The biodistributions of the two mutant mAbs were compared in ^{67}Cu-labeled form and the H310A mutant showed about four times lower tumor uptake and about two times higher levels of radioactivity in the liver. Consequently, the N297Q (agl) mutant was chosen for therapy experiments.

To test therapeutic efficacy, the SKOV3ip orthotopic metastases model was chosen. The fact that therapy had already started 2 days postinoculation of cells indicates that it is not a model for treating widespread ovarian cancer, but rather, that it may represent the situation of micrometastatic disease. In mouse models of i.p. metastases, therapeutic effects in terms of prolonged survival were observed with radioimmunotherapy using radiometal-labeled antibodies, including a domain-deleted mAb (37–39). No complete cures were achieved in these models of aggressive metastatic disease.

We tested single i.v. doses of radiocopper-labeled anti-L1 mAb chCE7agl. The i.v. route for injection of the radiocopper conjugate as opposed to the frequently propagated i.p. administration was chosen because it may be relevant for both imaging and treatment of distant metastases. PET imaging with ^{64}Cu-labeled antibodies would be very useful to select patients and to provide dosimetry and may be of value in following the course of the disease and efficacy of treatments. The shortened half-life in the blood of the ^{67}Cu-chCE7agl compared with the parent mAb is an improvement for both imaging as well as for therapy. We found that a low 4 MBq dose of ^{67}Cu-chCE7agl led to an already significant reduction of tumor burden similar to the effect achieved with a 10.5 MBq dose (Table 3). However, when survival was analyzed, this low dose did not prolong survival significantly, in contrast to the 10 MBq dose which led to significantly increased survival (Fig. 4). In addition, in these survival experiments, we asked how the efficiency of radioimmunotherapy with ^{67}Cu-labeled anti-L1 mAb chCE7agl compares with growth inhibition using anti-L1 mAb L1-11A. Many possible combinations could be envisaged. For a first evaluation of the feasibility of this approach, we chose a survival experiment combining biweekly treatment with unlabeled mAb L1-11A and low-dose radioimmunotherapy with 5 MBq of ^{67}Cu-chCE7agl. We found that the single treatments did not significantly prolong the survival of control animals. The heterogeneous expression of L1 observed in the SKOV3ip tumor masses (Fig. 3D) could explain why therapy with unlabeled mAb L-11A showed no significant increase in survival, whereas high-dose radioimmunotherapy with 10.5 MBq of ^{67}Cu-chCE7agl led to a significantly longer survival, the
effect of radiation being effective over several cell diameters. The specificity of radioimmunotherapy is shown in the experiment with the $^{67}$Cu-labeled Lym-1 antibody used as a nonbinding IgG1 control antibody which showed no significant effects on survival. To date, only one study on the efficacy of $^{67}$Cu-radioimmunotherapy in nude mice with s.c. xenografts of human Burkitt lymphoma has been done (40), and the maximal tolerated dose was ~20 MBq of $^{67}$Cu-labeled mAb Lym-1 dosing, starting at 12 MBq. Increasing doses to 18.5 MBq showed no dose-dependent effect in this experiment of this similar tumor model, and the authors discuss the potential adding synergistic agents. Our results in the SKOV3ip metastases model show significant tumor regression and increased survival with a single 10.5 MBq dose of $^{67}$Cu-chCE7Ag. A combination of unlabeled anti-L1 mAb L1-11A with a single low-dose $^{67}$Cu-radioimmunotherapy prolonged survival significantly, the data indicating the advantages of combining anti-L1-directed growth inhibition with radioimmunotherapy for the therapy of ovarian cancer metastases.

Acknowledgments

We thank Prof. B. Seifert (Department of Biostatistics, University of Zurich) for helping with the statistical analysis of the therapy studies. Support from Drs. Achim Kruger and Matthias Att (Technical University of Munich) with the SKOV3ip model in the initial phase of the study is highly appreciated.

References

Copper-67 Radioimmunotherapy and Growth Inhibition by Anti–L1-Cell Adhesion Molecule Monoclonal Antibodies in a Therapy Model of Ovarian Cancer Metastasis

Karin Knogler, Jürgen Grünberg, Kurt Zimmermann, et al.


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