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Human monoclonal antibodies to domain C of tenascin-C selectively target solid tumors *in vivo*

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We had previously reported that splice isoforms of tenascin-C containing the extra-domain C are virtually absent in normal adult tissues but are highly abundant in high-grade astrocytomas, with a prominent peri-vascular pattern of expression. We now report that the extra-domain C of tenascin-C is strongly expressed in the majority of lung cancers, with a vascular and stromal pattern of expression. Using antibody phage technology, we have generated a human monoclonal antibody (G11), with a dissociation constant K_D = 4.2 nM for the human domain C. The G11 antibody, expressed in scFv and in mini-antibody (SIP) format, as well as a scFv-interleukin-2 fusion protein, was then characterized in quantitative biodistribution studies using mice grafted subcutaneously with U87 gliomas, revealing a selective tumor uptake, with tumor/blood ratios up to 11.8:1 at 24 h. A radioiodinated preparation of SIP(G11) was also investigated in a double tracer study using an orthotopic rat glioma model, confirming the antibody's ability to preferentially localize at the tumor site, with tumor/brain ratios superior to the ones observed with ^{18}F-fluorodeoxyglucose. These tumor-targeting properties, together with the strong immunohistochemical staining of human tumor sections, indicate that the G11 antibody may be used as a portable targeting moiety for the selective delivery of imaging and therapeutic agents to gliomas and lung tumors.

Keywords: angiogenesis/antibody phage display/human monoclonal antibodies/tenascin-c/tumor targeting

Introduction

The targeted delivery of bioactive molecules (e.g. antibody constant regions, cytokines, drugs, radionuclides, photosensitizers, pro-coagulant factors, etc.) to the tumor environment by means of ligands specific to good-quality tumor-associated antigens and endowed with suitable pharmacokinetic properties is a promising avenue for the therapy of disseminated cancers (Carter, 2001; Neri and Bicknell, 2005).

Antigens, which are preferentially expressed in the modified tumor extracellular matrix (ECM), are, in many respects, ideal targets for tumor targeting applications (Brack *et al*., 2004; Neri and Bicknell, 2005). ECM components are often more abundant and more stable than antigens located on the surface of tumor cells. Furthermore, they typically exhibit a low shedding profile and are well accessible to agents (such as antibody derivatives) coming from the bloodstream. Our group has extensively demonstrated the tumor-targeting potential of antibodies directed against components of the tumor ECM, using as an example the L19 human monoclonal antibody (Pini *et al*., 1998), specific to the EDB domain of fibronectin, a marker of angiogenesis (Zardi *et al*., 1987; Kaczmarek *et al*., 1994; Castellani *et al*., 1994, 2002). The L19 antibody was shown to efficiently target tumor neo-vasculature and stromal structures in animal models of cancer (Tarli *et al*., 1999; Viti *et al*., 1999) and in patients with aggressive solid tumors (Santimaria *et al*., 2003), and is now in clinical development for cancer treatment as a radioiodinated antibody (Berndorff *et al*., 2005), as a fusion protein with human interleukin-2 (Menrad and Menssen, 2005) and as a fusion protein with human TNF (Borsi *et al*., 2003; Balza *et al*., 2006).

We have previously reported the over-expression of a splice variant of another ECM component (tenascin-C containing the extra-domain C) in high-grade astrocytomas, with a prominent pattern of staining around tumor neo-vasculature. In contrast, domain C was undetectable in normal human tissues by immunohistochemistry and even at the level of Northern blot analysis (Carnemolla *et al*., 1999). For practical applications, tumor-associated antigens and the corresponding antibodies are particularly useful if they can be used to target at least certain tumor classes with high incidence.

We report here that the extra-domain C is strongly expressed in the majority of lung cancers, with a vascular and stromal pattern of expression. Using state-of-the-art antibody phage technology, we have generated a high-affinity human antibody ('G11'), which was shown to selectively target solid tumors in rodents both as a recombinant antibody fragment and as fusion protein with interleukin-2. These results pave the way for the clinical development of the G11 antibody for the imaging and therapy of gliomas and lung tumors.

Results

Isolation of the G11 antibody

We have recently described the construction of a large synthetic human antibody library (termed 'ETH2-GOLD') and the isolation of scFv(A12), a human antibody fragment specific to domain C of tenascin-C, with a dissociation constant, K_D, of ~670 nM for the antigen (Silacci *et al*., 2005). In order to improve the binding affinity of scFv(A12), an *in vitro* affinity maturation procedure was performed by randomizing residues in the CDR1 and CDR2 of the antibody heavy and light chains according to a procedure previously described by our group (Pini *et al*., 1998). In a first step a new antibody repertoire

Table I. Sequences of the scFv fragments specific to the extra-domain C of tenascin-C

scFv	VH chain			VL chain		
	31–33[a]	52–56[a]	95–100[a]	31–32[a]	50–53[a]	91–96[a]
A12	SYA	SGSGGS	HPPHRP	SYY	GKNN	HGPRRP
E10	**GSR**	**NEE**GG**Q**	HPPHRP	SYY	GKNN	HGPRRP
G11	**GSR**	**NEE**GG**Q**	HPPHRP	**LYY**	**GKNN**	HGPRRP

Relevant amino acid positions of antibody clones isolated from the designed synthetic libraries. Positions that are mutated in the primary antibody library are underlined. Residues in E10 and G11, mutated during the affinity maturation procedure, are in boldface. Single amino acid codes are used according to standard IUPAC nomenclature.
[a]Numbering according to Tomlinson *et al*. (1992) and Williams *et al*. (1996).

based on the sequence of the scFv(A12) was constructed by combinatorially mutating positions 31, 32 and 33 of the VH CDR1 and positions 52, 52a, 53 and 56 of the VH CDR2 (Table I). The residues chosen for randomization are found to frequently contact the antigen in the known three-dimensional structures of antibody–antigen complexes. Moreover, in natural antibodies they are frequently found to be diverse and to be subjected to somatic hypermutation (Tomlinson *et al*., 1992). This repertoire containing 2×10^6 individual antibody clones was used for selections. Two rounds of panning under stringent conditions were performed and yielded several different scFv fragments specific to the C domain of tenascin-C, whose monomeric binding affinity to the antigen was characterized using BIAcore technology. The antibody fragment scFv(E10) (Table I), which displayed the lowest kinetic dissociation constant, was further characterized by SDS–PAGE analysis, size exclusion chromatography and by BIAcore analysis (Figure 1), which revealed a $K_D = 93$ nM for the human antigen (Table II). This corresponds to a factor 7 improvement, compared with the affinity of the parental antibody fragment scFv(A12).

ScFv(E10) was further combinatorially mutated at positions 31, 31b and 32 in the VL CDR1 and at positions 50, 52 and 53 in the VL CDR2 (Table I). These residues were chosen on the basis of the high sequence variability observed in natural antibodies (Ignatovich *et al*., 1997). An antibody repertoire containing 2.2×10^8 individual clones was obtained and used for two rounds of panning under stringent conditions. The clone scFv(G11) isolated from this library (Table I), which showed the slowest kinetic dissociation from the antigen, was purified and further characterized by SDS–PAGE analysis, by size-exclusion chromatography and by BIAcore (Figure 1). The monomeric dissociation constant K_D of scFv(G11) is equal to 4.2 nM for the human antigen (Table II), representing an affinity improvement by a factor 22, compared with scFv(E10), and a factor 160, compared with the parental antibody scFv(A12).

Immunohistochemistry with scFV(G11) on tumor sections

ScFv(G11) was extensively characterized by immunohistochemistry on cryosections of human lung tumors and of normal tissues. Figure 2 shows representative sections from different types of lung cancer including: squamous cell carcinoma, bronchial alveolar carcinoma, small cell carcinoma, large cell carcinoma, pulmonary metastasis of renal cell carcinoma, mesothelioma and lung adenocarcinoma. In all the lung tumor sections studied, a moderate to very strong staining was observed, with staining patterns ranging between a vascular-type in certain tumors (e.g. squamous cell carcinoma) and a predominantly stromal in others (e.g. lung adenocarcinoma). The G11 antibody did not exhibit any detectable staining in normal tissues (see representative examples in Figure 2), confirming the very restricted expression pattern of tenascin-C isoforms containing the extra-domain C.

Cloning and expression of the G11 antibody in different formats

We have previously reported that certain antibody formats (e.g. mini-antibodies, cytokine fusions) may display certain advantages for tumor-targeting applications *in vivo* (Borsi *et al*., 2002; Carnemolla *et al*., 2002). For this reason, we cloned scFv(G11) in small immunoprotein (SIP) format by genetically fusing the scFv at the N-terminus of a human εCH4 domain of the secretory isoform S2 of human IgE (Figure 1; Borsi *et al*., 2002). This domain promotes the formation of homodimers that are further stabilized by disulfide bonds between the C-terminal cysteine residues, resulting in a 75 kDa bivalent miniantibody. This format has been shown to be superior to IgG and to scFv for radioimmunotherapeutic applications (Borsi *et al*., 2002; Berndorff *et al*., 2005). Furthermore, we fused scFv(G11) with human interleukin-2 and produced the resulting fusion protein in mammalian cells (Figure 1). In contrast to scFv(L19)-IL2, which can be produced as a non-covalent homodimer (Carnemolla *et al*., 2002; Menrad and Menssen, 2005), scFv(G11)-IL2 proved to be exclusively monomeric in physiological conditions (data not shown).

Quantitative biodistribution studies in tumor-bearing rodents

The *in vivo* tumor targeting properties of the different anti-domain C of tenascin-C antibodies and antibody formats were evaluated using radioiodinated protein preparations in both mouse and rat tumor models. Biodistribution experiments were performed in nude mice bearing U87 human glioblastoma xenografts. This tumor model was shown to be strongly positive for the domain C-containing TN-C isoform in immunohistochemical analysis (Figure 2), with the G11 antibody strongly staining the tumor vascular structures. ^{125}I-labeled antibodies [scFv(E10), scFv(G11) and scFv(G11)-IL2] were injected intravenously; 24 h later, the animals were sacrificed, the organs excised, weighted and radioactivity was counted.

While the antibody fragment scFv(E10), obtained prior to the last affinity maturation step (Table I and Figure 1), failed to preferentially localize to the tumor, all antibody formats of G11 showed a selective accumulation at the tumor site (Table III).

The tumor/blood ratios at 24 h for scFv(G11), SIP(G11) and scFv(G11)-IL2 were 4.8, 5.6 and 11.8, respectively. Tumor/organ ratios at 24 h ranged between 5 and 30 for scFv(G11)-IL2, while being slightly lower for the other two formats.

In order to confirm the ability of the G11 antibody to target tumors also in the orthotopic setting, we injected rats carrying orthotopic C6 gliomas with a radioiodinated preparation of SIP(G11) and ^{18}F-fluorodeoxygluclose (FDG). Figure 3 shows a side-by-side comparison of brain autoradiograms obtained with the two tracers at 24 h and 15 min following injection, respectively. The shorter imaging time used for FDG was

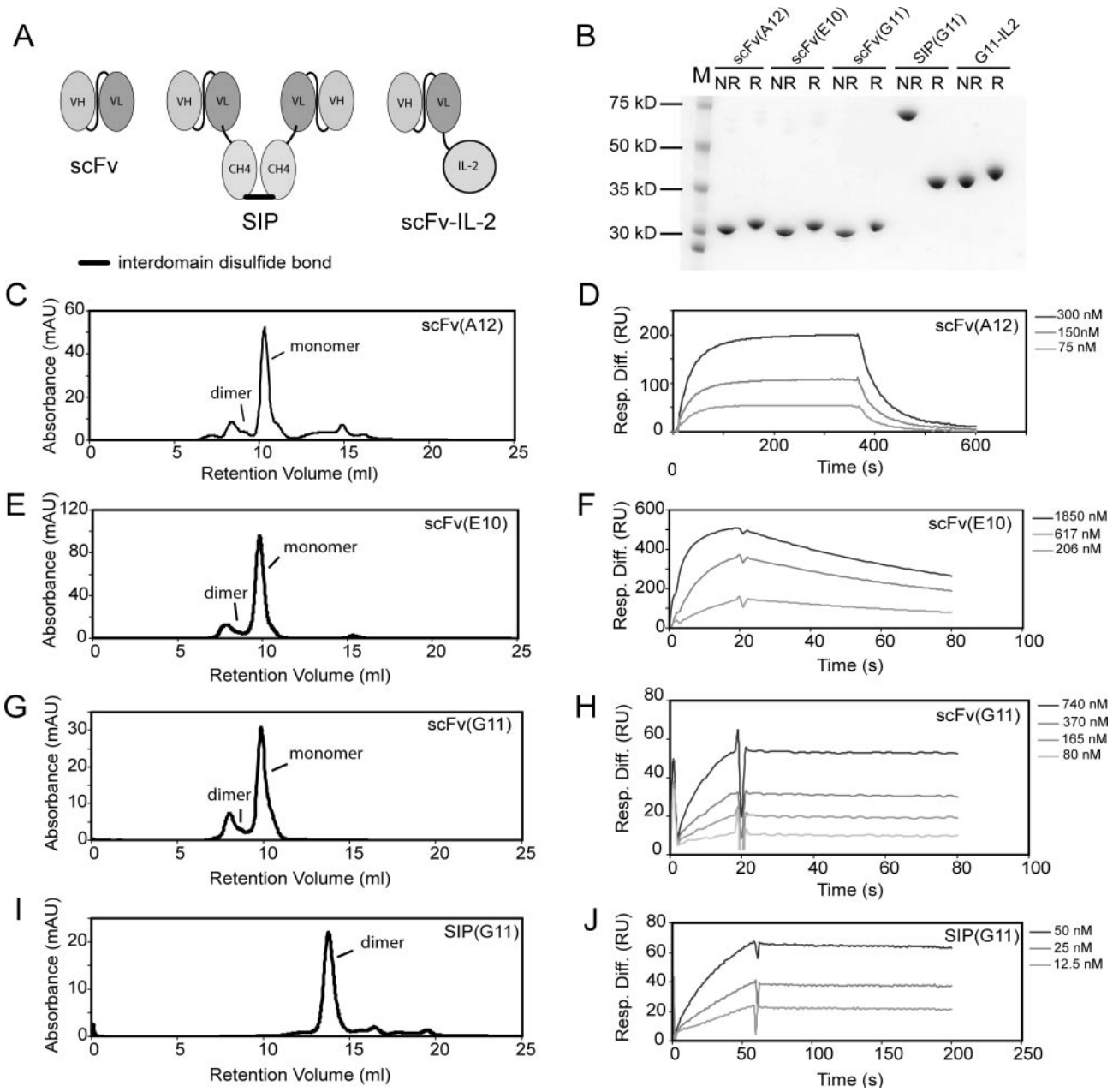

Fig. 1. Anti-domain C of tenascin-C antibodies. (**A**) Schematic representation of the different antibody formats and fusion protein used in this study. ScFv fragment consisting of a variable heavy and a variable light chain connected by a peptide linker, the small immunoprotein (SIP) format, a disulfide-linked homodimer and a scFv-IL2 fusion protein. (**B**) SDS–PAGE analysis of purified scFv(A12), scFv(E10), scFv(G11), SIP(G11) and scFv(G11)-IL2 under non-reducing (NR) and reducing (R) conditions. (**C, E, G**) Size-exclusion chromatography profiles of the purified scFv(A12), scFv(E10) and scFv(G11), respectively. The retention volume (ml) of the major peak corresponds to the monomeric form of the scFv fragments. (**I**) Size-exclusion chromatography profile of the purified SIP(G11). The retention volume (ml) of the major peak corresponds to the disulfide-linked homodimer. (**D, F, H, J**) BIAcore analysis of the binding of scFv(A12), scFv(E10), scFv(G11) and SIP(G11) to the extra-domain C of tenascin-C. Purified monomeric preparations of the scFv fragments and SIP(G11) were injected at different concentrations and the kinetic constants were calculated with the BIAevaluation 3.1 software.

necessary in view of the faster clearance and shorter half-life of this radiopharmaceutical. The autoradiographic analysis clearly confirms the ability of SIP(G11) to localize to the brain tumor, with a heterogenous accumulation of radioactivity within the tumor mass, and its superior tumor to normal brain ratio compared with FDG.

Discussion

We have generated a high-affinity antibody (G11) specific to the extra-domain C of tenascin-C. We have demonstrated the ability of different formats of this antibody (scFv fragment, SIP and scFv-interleukin-2 fusion) to target tumors *in vivo* in two different rodent models of cancer, with tumor to blood ratios at 24 h up to 11.8:1 and tumor to organ ratios up to 32:1. Moreover, the G11 antibody has allowed us to discover that the extra-domain C of tenascin-C is strongly expressed in virtually all types of lung cancer and mesothelioma, thus, opening targeted therapeutic strategies for this class of neoplasias. Lung cancer is the leading cause of cancer death in the world. With the existing therapeutic efforts, patients with lung cancer still have a poor prognosis and <15% live 5 years. This dismal

Table II. Affinities of the antibody fragments specific to the extra-domain C of tenascin-C

Antibody	k_{on} ($s^{-1}M^{-1}$)	k_{off} (s^{-1})	K_D (M)
scFv(A12)	5.4×10^4	6.0×10^{-2}	6.7×10^{-7}
scFv(E10)	1.0×10^5	9.3×10^{-3}	9.3×10^{-8}
scFv(G11)	6.7×10^4	2.9×10^{-4}	4.2×10^{-9}

The affinity measurements were obtained using a BIAcore 3000 instrument (according to Neri *et al.*, 1997). k_{off}, kinetic dissociation constant; k_{on}, kinetic association constant; K_d, dissociation constant. The affinity of scFv(G11) to the murine isoform of the extra-domain C of tenascin-C was also measured, $K_d = 54$ nM. Values are accurate to ±50%, on the basis of the precision of concentration determinations.

statistic has changed minimally in the last 20 years, and, therefore, new therapeutic strategies are clearly needed.

In the recent past, other types of cancer have benefited from the availability of monoclonal antibodies specific to tumor-associated antigens, even when they would cover only a small portion of the patients population [e.g. Herceptin (Mokbel and Hassanally, 2001)]. Interestingly, G11 appears to cover a much broader population and may, therefore, be used for the construction of antibody-based pharmaceuticals, with a substantial therapeutic potential in oncology.

A number of laboratories have compared the tumor-targeting properties of affinity variants of the same monoclonal antibody. For instance, the groups Marks/Adams/Weiner have

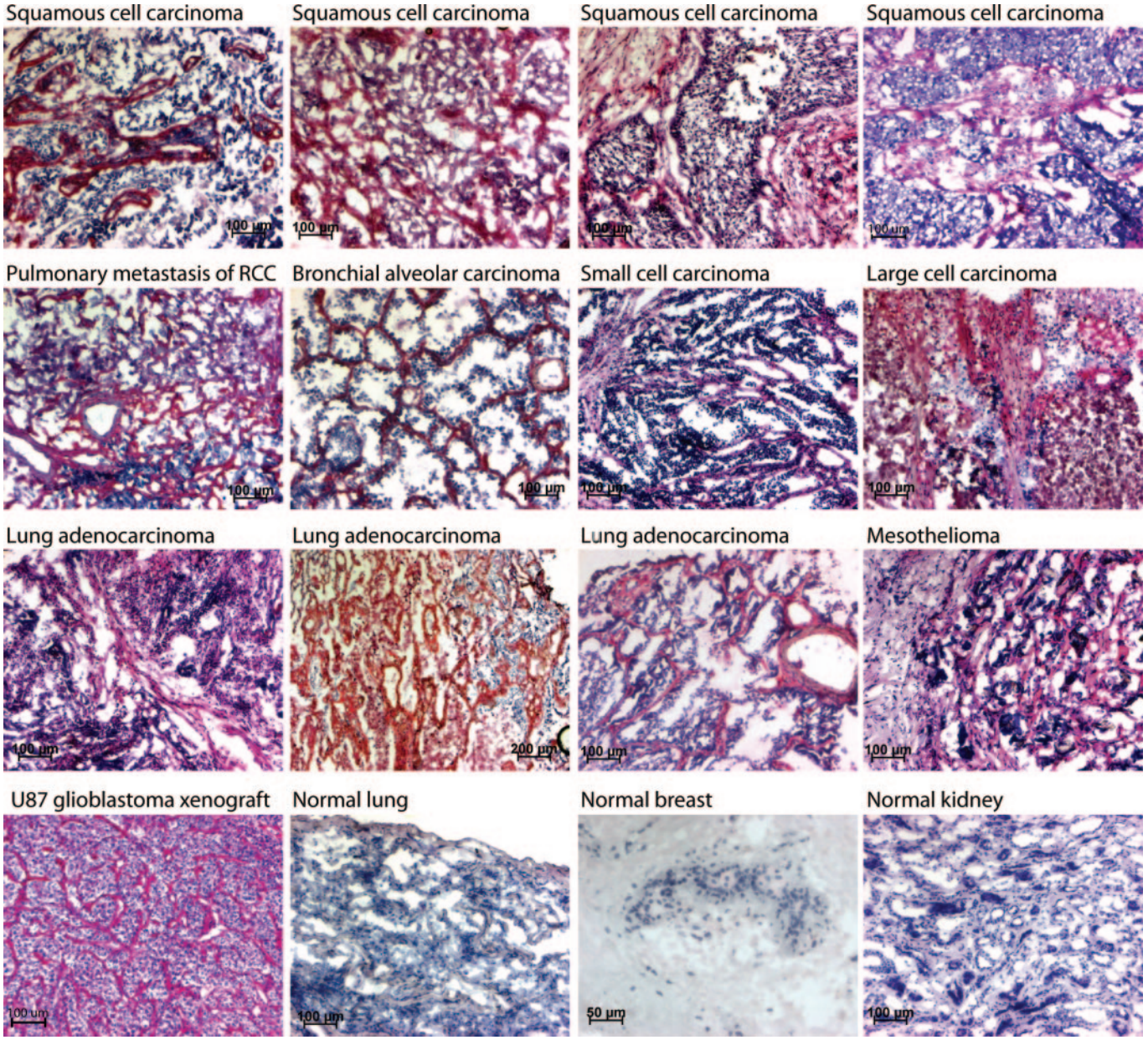

Fig. 2. Representative findings of an immunohistochemical analysis performed on different types of human lung tumor sections obtained from surgically resected tumors from patients, using the scFv(G11) as primary antibody (red staining, over the blue hematoxylin staining of cell nuclei). An intense vascular and/or stromal pattern of staining was observed in virtually all surgical specimens analyzed in this study. In addition, the figure presents the strong vascular staining of scFv(G11) of U87 human glioblastoma xenografts grown in nude mice, the model that was used for biodistribution analysis. In contrast, G11 staining was undetectable in normal lung, normal breast and normal kidney sections. RCC = renal cell carcinoma.

Table III. Biodistribution experiments of radiolabeled anti-domain C of tenascin-C antibodies in nude mice bearing U87 human glioblastoma xenografts

	scFv(E10)	scFv(G11)	SIP(G11)	scFv(G11)-IL2
Organs				
Tumor	**1** (0.07 ± 0.02)	**1** (0.71 ± 0.03)	**1** (0.85 ± 0.07)	**1** (0.48 ± 0.03)
Blood	**0.7** (0.11 ± 0.01)	**4.8** (0.15 ± 0.02)	**5.6** (0.15 ± 0.02)	**11.8** (0.04 ± 0.01)
Liver	**0.5** (0.15 ± 0.02)	**6.6** (0.11 ± 0.01)	**5.3** (0.17 ± 0.02)	**5.3** (0.09 ± 0.01)
Kidney	**0.1** (0.77 ± 0.18)	**1.4** (0.51 ± 0.03)	**3.7** (0.23 ± 0.01)	**4.8** (0.10 ± 0.01)
Intestine	**2.3** (0.03 ± 0.01)	**20** (0.04 ± 0.01)	**10.2** (0.08 ± 0.01)	**32.1** (0.02 ± 0.01)
Spleen	**1.8** (0.04 ± 0.02)	**21.8** (0.03 ± 0.02)	**6.5** (0.13 ± 0.01)	**8.3** (0.06 ± 0.01)
Heart	**1.5** (0.05 ± 0.01)	**12.6** (0.06 ± 0.01)	**14.9** (0.06 ± 0.01)	**29** (0.02 ± 0.01)

Tumor/organ ratios, at 24 h after intravenous injection, are indicated in boldface. The numbers in brackets correspond to the percent antibody injected dose per gram of tissue (%ID/g) ± standard deviation.

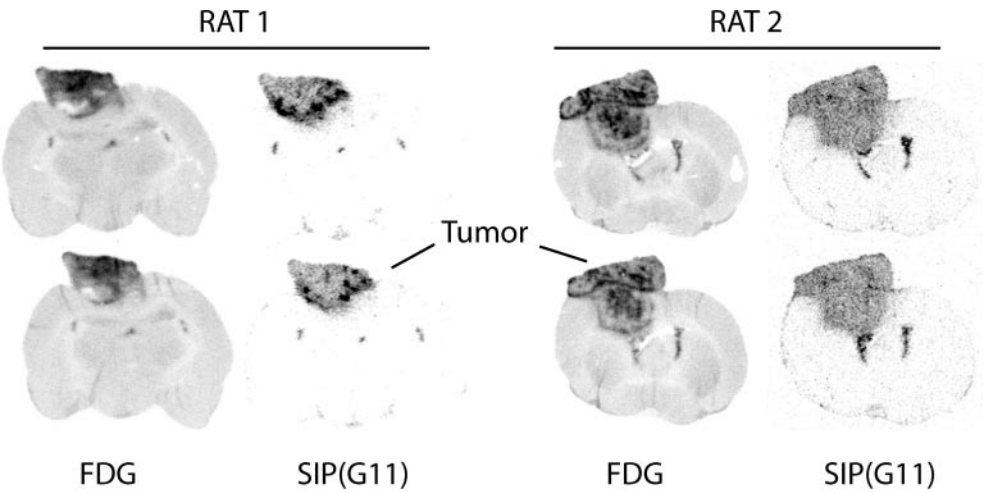

Fig. 3. Double tracer study with ^{18}F-Fluorodeoxyglucose (FDG) and ^{131}I-SIP(G11) in C6 glioma bearing rats. Autoradiography of rat brain sections. SIP(G11) was conjugated to ^{131}I and injected (500–600 μCi) i.v. in the animals. After 24 h the animals were sacrificed. Because of the short half-life of ^{18}F-Fluorodeoxyglucose (110 min), 1.2–1.7 mCi were injected just 15 min before sacrificing the animals. To quantitate the radioactive signal of the two tracers two successive autoradiography were performed. First, brain sections were incubated 4 h to detect the ^{18}F-Fluorodeoxyglucose signal. Then, the phosphoimager screen was erased and the same sections incubated again 4 days, to reveal the ^{131}I-SIP(G11) signal. The analyses of two different rats are compared here.

nicely shown that affinity maturation of a monomeric scFv fragment specific to HER2-neu would correlate with improved targeting until a threshold value (~1 nM), after which targeting performance did not appear to further increase (Adams *et al*., 1998). Similarly, our group has previously demonstrated that the high-affinity human antibody L19, specific to the EDB domain of fibronectin, was capable of improved tumor targeting, compared with the parental antibody E1 (Viti *et al*., 1999). In the present study, biodistribution data clearly show that the high-affinity antibody fragment scFv(G11) targets tumors more efficiently than the parental antibody scFv(E10), thus, strengthening the concept that nanomolar binding antibodies may be needed for efficient and selective tumor uptake. However, the use of bivalent antibody formats, with their enhanced functional affinity, may improve the tumor-targeting performance even of antibodies with moderate affinity for the antigen (Adams *et al*., 1998).

In our biodistribution analysis, scFv(G11)-IL2 exhibited the best tumor-targeting performance. This may be due to the vasoactive properties of the pro-inflammatory cytokine IL2 (Epstein *et al*., 1995; Carnemolla *et al*., 2002). Our group has recently developed scFv(L19)-IL2, a tumor-targeting immunocytokine, which is currently in clinical trials. The newly described scFv(G11)-IL2, which binds with high affinity both to the human and to the murine domain C of tenascin-C, appears to have a therapeutic potential for the treatment of patients with lung cancer. It remains to be seen whether a non-covalent homodimeric preparation of scFv(G11)-IL2, obtained by shortening the polypeptide linker between VH and VL (Holliger *et al*., 1993), may display superior tumor-targeting properties compared with the monomeric format described in this article.

The use of interleukins in cancer therapy has been hindered, until now, by unacceptable toxicities already at relatively low doses, thus, limiting dose escalation and the therapeutic benefit for the patients. The use of human monoclonal antibody fragments appears to be a logical avenue for the targeted delivery of anti-cancer cytokines to neoplastic sites, with an impressive therapeutic potential in animal models of cancer (Becker *et al*., 1996; Penichet *et al*., 1997; Carnemolla *et al*., 2002; Helguera *et al*., 2002; Menrad and Menssen, 2005). The development of scFv(G11) and the discovery that this human antibody strongly stains lung tumors paves the way for the clinical development of novel immunocytokines to be used either alone or in combination with chemotherapy (Ebbinghaus *et al*., 2005) for the therapy of lung cancer. In this respect, detailed pharmacological studies will be required in order to learn about chemotherapeutic regimens (doses, schedules), which are synergistic with scFv(G11)-IL2 and other immunocytokines.

Materials and methods

Selections of antibodies from the ETH-2 gold library

The domain C of tenascin-C was biotinylated with EZ-Link™ Sulfo-NHS-SS-Biotin (Pierce) according to manufacturer's instructions. The biotinylated antigen (final concentration 10^{-7} M) was incubated with 10^{12} phage particles in 1 ml 1% BSA for 40 min on a shaker at room temperature. The phage–antigen complex was captured in previously avidin-coated (first and third round) or streptavidin-coated (second round) Maxisorp wells (Nunc, Germany). The phage–antigen mix was distributed in eight wells and incubated for 20 min on a shaker at RT. The wells were rinsed 10 times with phosphate-buffered saline (PBS) 0.1% Tween-20 and 10 times with PBS. Bound phage was eluted by incubation with 1 ml 50 mM dithiotreitol for 5 min. The eluted phage was used to infect exponentially growing *Escherichia coli* TG-1.

Construction of the affinity maturation libraries

In the first step of affinity maturation the scFv(A12) clone was used as template for the construction of the library. Sequence variability in the VH CDR1 and CDR2 of the library was introduced by PCR using partially degenerate primers DP47CDR1 for (5′-CTGGAGCCTGGCGGACCCAGCTCAT-MNNMNNMNNGCTAAAGGTGAATCCAGA-3′) (all oligonucleotide used in this work were purchased from Operon Biotechnologies, Cologne, Germany) and DP47CDR2for (5′-GCCCTTCACGGAGTCTGCGTAGTATGTMNNACC-ACCMNNMNNMNNAATAGCTGAGACCCACTCC-3′), in a process that generates random mutations at positions 31–33 of the VH CDR1 and at positions 52, 52a, 53 and 56 of the VH CDR2. VHVL combinations were assembled in scFv format by PCR assembly using the primers LMB3long (5′-CAGGAAACAGCTATGACCATGATTAC-3′) and fdseqlong (5′-GACGTTAGTAAATGAATTTTCTGTATGA-GG -3′), using gel-purified VH and VL segments as templates. The assembled VH–VL fragments were doubly digested with *Nco*I/*Not*I and cloned into *Nco*I/*Not*I-digested pHEN1 phagemid vector (Hoogenboom *et al*., 1991). The resulting ligation product was electroporated into electrocompetent *E.coli* TG-1 cells according to (Viti *et al*., 2000), giving rise to a library containing 2×10^6 individual antibody clones.

In the construction of the second affinity maturation library scFv(E10) clone was used as template. Sequence variability in the VL CDR1 and CDR2 of the library was introduced by PCR using partially degenerate primers DPL16CDR1for (5′-TCCTGGCTTCTGCTGGTACCAGCTTGCMNNMNN-MNNTCTGAGGCTGTCTCCTTG -3′), in a process that generates random mutations at positions 31, 31b and 32 of the VL CDR1 and at positions 50, 52, and 53 of the VL CDR2. VH–VL combinations were assembled in scFv format by PCR assembly LMB3long (5′-CAGGAAACAGCTATGACCAT-GATTAC-3′) and fdseqlong (5′-GACGTTAGTAAATGAAT-TTTCTGTATGAGG-3′), using gel-purified VH and VL segments as templates. The assembled VH–VL fragments were doubly digested with *Nco*I/*Not*I and cloned into *Nco*I/*Not*I digested pHEN1 phagemid vector (Hoogenboom *et al*., 1991). The resulting ligation product was electroporated into electrocompetent *E.coli* TG-1 cells according to (Viti *et al*., 2000), giving rise to a library containing 2.2×10^8 individual antibody clones.

Selection of antibodies from the affinity maturation libraries

Biotinylated domain C of tenascin-C was used at a final concentration of 10^{-8} M with 10^{12} transforming units (t.u.) of phage antibodies in 1 ml 1% BSA for 40 min. The phage–antigen complex was then captured using 5.3×10^7 streptavidin-coated magnetic beads (Dynal). The beads were washed five times with 1 ml 0.1% Tween-20 in PBS (100 mM NaCl, 50 mM phosphate, pH 7.4) followed by five times washing with 1 ml PBS. Bound phage was eluted with 1 ml 50 mM dithiothreitol (Applichem, Germany) and amplified in *E.coli* TG-1, using VCS-M13 (Stratagene) as helper phage. Phage particles were purified from culture supernatant by PEG precipitation (Viti *et al*., 2000).

Screening of supernatants by ELISA and by biacore

Induced supernatant of individual clones were screened by ELISA as described by Viti *et al*. (2000) on high-bind StreptaWell plates (Roche) coated with biotinylated antigen. Bound antibody was detected by means of anti-peptide tag antibodies, anti-myc tag antibody 9E10 (Marks *et al*., 1991), followed by an anti-mouse immunoglobulin horse radish peroxidase (HRP) conjugate (SIGMA). The assay was developed by a colorimetric reaction using BM-Blue POD soluble substrate (Roche). Supernatants of ELISA positive clones were further screened by surface plasmon resonance (SPR) real-time interaction analysis on a high-density coated antigen chip, using a BIAcore3000 instrument (BIAcore AB, Sweden).

Sequencing of scFV antibody genes

Antibodies were sequenced using Big Dye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) on an ABI PRISM 3130 Genetic analyzer. Templates for termination reactions were either miniprep DNA or PCR products. Primers used for sequencing were LMB3 long (5′-CAGGAAACAGCTATGA-CCATGATTAC-3′; annealing upstream of scFv gene), fdseq long (5′-GACGTTAGTAAATGAATTTTCTGTATGAGG-3′, annealing downstream of scFv gene), and DP47CDR2ba (5′-ACATACTACGCAGACTCCGTGAAGGGC-3′, annealing 100 bp upstream of VH CDR3).

Characterization of scFV antibodies

ScFv antibody fragments were expressed in *E.coli* TG-1 and purified from culture supernatant and from periplasmic preparations by affinity chromatography, using either protein A sepharose (Amersham Biosciences) or antigen-coupled sepharose obtained by coupling recombinant antigen to CNBr-activated sepharose (Amersham Biosciences). Purified antibodies were analyzed by size-exclusion chromatography on superdex 75 HR10/30 columns (Amersham Biosciences), peaks representing monomeric fractions were collected and used for affinity measurements by BIAcore on a low-density coated antigen chip.

Immunohistochemistry on frozen tissue sections

Sections of 8–12 μm thickness were treated with ice-cold acetone, rehydrated in TBS (50 mM Tris, 100 mM NaCl, pH 7.4) and blocked with 20% fetal bovine serum (Invitrogen). Affinity-purified scFv fragments (final concentration 2–10 μg/ml) carrying an myc-tag were added onto the sections, together with secondary antibody was monoclonal anti-myc 9E10 antibody (5 μg/ml). Bound antibodies were detected with rabbit anti-mouse immunoglobulins antibody

(Dakocytomation, Denmark) followed by mouse monoclonal alkaline phosphatase–antialkaline phosphatase complex (APAAP; Dakocytomation, Denmark). Fast Red (Sigma) was used as phosphatase substrate, and sections were counterstained with Gill's hematoxilin No2 (Sigma).

Cloning, expression and purification of SIP(G11)

The SIP secretion sequence, which is required for secretion into the extracellular medium, was amplified from the construct SIP(L19)-pcDNA3.1 (Borsi *et al*., 2002) using primers 23paba (5′-CTGAAGCTTGTCGACCATGGGCTGGAGCC-3′) and 29pafo (5′-CAGCTGCACCT CCGAGTGCACACCTGT-GGAGAG-3′). The human εCH4 domain was amplified from the same construct using primers 34paba (5′-CTGACCGTCC-TAGGCTCCGGAGGCTCTGGGGCCCCGCGTGCT-3′) and 28pafo (5′-CTGGAATTCGAGCTCGGTACCTAGCAGCCA-CCC-3′). ScFv was amplified with 30paba (5′-GGTGTGCAC-TCGGAGGTGCAGCTGTTGGAGTCT-3′) and 33pafo (5′-CCCAGAGCCTCCGGAGCCTAGGACGGTCAGCTTG-GTCCCTCC-3′). The resulting PCR fragments were gel purified, assembled by PCR and cloned into vector pcDNA3.1 (Invitrogen) by means of *Hin*dIII and *Eco*RI digestion. The constructs were used to transfect HEK293 cells using FuGENE 6 transfection reagent (Roche). Transfected HEK293 cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and selected using 500 μg/ml of Geneticin (G418, Calbiochem). Monoclonal cultures were obtained by limited dilution. SIP(G11) was purified from supernatant by affinity chromatography using antigen-coated sepharose resin. The purified protein was analyzed by SDS–PAGE, size-exclusion chromatography using a superdex 200 HR 10/30 column and by BIAcore.

Cloning, expression and purification of scFV(G11)-il2

Cloning of the scFv(G11)–IL2 fusion protein was performed as previously described for the scFv(L19)–IL2 fusion protein (Carnemolla *et al*., 2002). IL2 was obtained by digestion of the scFv(L19)–IL2 containing pcDNA3.1 vector using the restriction enzymes *Eco*RI and *Bam*HI. The scFv(G11) was amplified by PCR using the primers PG39 (5′-AAACTTA-AGCTTGTCGACCATGGG-3′) and PG40 (5′-ATAGAATT-CGCCTAGGACGGTCAGCTTGG-3′). The amplified DNA was digested with *Hin*dIII and *Eco*RI. The gel-purified DNA fragments [scFv(G11) and IL2] were ligated into the *Hin*dIII/*Bam*HI double-digested pcDNA3.1 vector. The constructs were used to transfect CHO cells using FuGENE 6 transfection reagent (Roche). Transfected CHO cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% FBS (Invitrogen), 4 mM L-Glutamine (Invitrogen) and selected using 500 μg/ml of Geneticin (G418, Calbiochem). Monoclonal cultures were obtained by limited dilution. scFv(G11)–IL2 was purified from supernatant by affinity chromatography using antigen-coated sepharose resin. The purified protein was analyzed by SDS–PAGE and size-exclusion chromatography using a superdex 200 HR 10/30 column.

Biodistribution in U87 human glioblastoma model

U87 human glioblastoma cells (HTB-14, ATCC) were cultured in MEM (Minimal Eagles Medium, Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen) and antibiotics at 37°C in an atmosphere of 5% CO_2. 3×10^6 U87 human glioblastoma cells were injected subcutaneously into the right flank of 6–8-week-old female Balb-C nu/nu mice (Charles River Laboratories). The tumors were allowed to grow for 20–25 days. By then, tumors reached a size of typically 200 mg and were used for biodistribution studies.

The *in vivo* targeting performance of the different antibody formats was evaluated by biodistribution analysis as described by (Tarli *et al*., 1999). Briefly, the different purified antibody formats were radioactively labeled with ^{125}I using the Iodogen method (Fraker and Speck, 1978; Salacinski *et al*., 1981) and injected i.v. into U87 human glioblastoma xenograft bearing nude mice. About 4 μg of each protein was injected. Animals were sacrificed 24 h after injection. Organs were excised, weighed and the radioactivity was counted. Targeting results of representative organs are expressed as percentage of injected dose per gram of tissue (%ID/g).

Double tracer study in an orthotopic rat glioma model

0.5 mg of SIP(G11) was coupled to ^{131}I according to the chloramine-T method (Visser *et al*., 2001). The radiolabeled antibody was then purified from the free iodine by gel filtration on a PD-10 desalting column (Amersham Biosciences). 0.5–0.6 mCi of ^{131}I-SIP(G11) was injected i.v. in rats bearing an orthotopic brain tumor, C6 malignant glioma (Sherburn *et al*., 1999). To assess the targeting performance of the iodinated antibody G11 autoradiography analysis of brain sections was performed 24 h after injection. For comparison the animals were also injected with 1.2–1.7 mCi of ^{18}F-Fluorodeoxyglucose (FDG) 15 min before being sacrificed. To quantitate the radioactive signal of the two tracers two successive autoradiography were performed. First, brain sections were incubated 4 h to detect the ^{18}F-Fluorodeoxyglucose signal. Then, the phosphoimager screen was erased and the same sections incubated again 4 days, to reveal the ^{131}I-SIP(G11) signal.

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