

# A New Anticancer Glycolipid Monoclonal Antibody, SC104, which Directly Induces Tumor Cell Apoptosis

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## Abstract

**A novel monoclonal antibody was raised by immunization of mice with colorectal tumor cell lines. The fusion was screened by immunohistochemistry for binding to primary colorectal tumors. Subsequent analysis on primary disaggregated colorectal tumors show that the antibody recognizes a cell surface antigen expressed by the majority of colorectal tumors. Antigen characterization has shown that the antibody recognizes a sialyltetraosylceramide but does not bind to GMI, GD1a, GT1b, or sialyl Lewis<sup>x</sup> antigens. Binding to a frozen panel of tumor and normal tissue sections revealed that the antigen was also strongly expressed on esophageal, gastric, and endometrial tumors. Its normal tissue distribution was largely restricted to moderate staining of large intestine. Surprisingly, SC104 antibody directly induces tumor cell death without the need for immune effector cells or complement. This may be related in part to its homophilic binding properties that allow cross-linking of antibody and receptors on the cell surface. Caspase activation can be detected following SC104 treatment of colorectal cells, and cotreatment with caspase inhibitors has been shown to inhibit cell death. This suggests that SC104 induces death by a classic apoptotic pathway. Furthermore, SC104 antibody shows additive killing with complement and 5-fluorouracil/leucovorin *in vivo*, suggesting a new therapeutic approach for this class of antibodies.** (Cancer Res 2006; 66(11): 5901-9)

## Introduction

Monoclonal antibodies (mAb) have recently proved their clinical use with Rituximab, being approved for treatment of non-Hodgkin's lymphoma (NHL; ref. 1), and Herceptin, being approved for advanced chemotherapy-refractory breast cancer (2). These antibodies act alone by directly inducing apoptosis, blocking growth factors, and/or stimulating antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). More recently, radiolabeled antibodies, Bexxar and Zevalin (3), have also been approved for treatment of NHL. Alternatively, Mylotarg, a mAb linked to the toxin calicheamicin, has also been approved for treatment of advanced acute myeloid lymphoma (4). One of the problems with mAbs is, if they do not directly or indirectly (via radiolabeled or toxin) induce cell death, they rely on immune effector mechanisms for efficacy, and most tumors have developed a variety of mechanisms to avoid this means of attack (5).

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This was elegantly illustrated by the mouse mAb 17-1A (Panorex), which recognizes EpCam that is overexpressed by the majority of colorectal tumors. This antibody does not induce direct killing but can mediate ADCC. Initial clinical trials showed a reduction in recurrence and enhanced survival for patients treated with 17-1A and lead to its approval in Germany. However, a large randomized clinical trial of Panorex, Panorex with 5-fluorouracil (5-FU)/leucovorin, and 5-FU/leucovorin alone failed to show a significant benefit (6). This may be related to the observation that 80% of colorectal tumors overexpress the complement regulatory protein CD55, restricting C3b deposition and complement-mediated lysis. Anaphylatoxins and C3b are also important cofactors for ADCC, synergizing to give enhanced killing (7). In contrast, Erbitux, a chimeric mAb that binds to the epidermal growth factor receptor (EGFR), showed a significant survival benefit in Irinotecan failed colorectal cancer patients and has recently received approval for this indication. Erbitux does not just rely on ADCC and CDC but directly inhibits cell proliferation, angiogenesis, and survival by blocking EGF binding (8). In this study, we describe a novel antibody that targets a gastrointestinal glycolipid, which directly induces tumor cell apoptosis.

## Materials and Methods

### Cell Lines and Antibodies

C170 and C146 are colorectal cell lines derived from primary tumors (9). Colo205, JW, HT29, LoVo, and P3NSO are colorectal cell lines, and a myeloma cell line was obtained from the American Type Culture Collection (Rockville, MD).

### Extranuclear Membranes

Fresh surgically resected colorectal adenocarcinoma or normal colonic mucosa were homogenized in a buffer containing 2 mmol/L NaHCO<sub>3</sub>, 2 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub>, and phenylmethylsulfonyl fluoride (pH 7.6; 4 volumes of buffer per g of tissue) to prepare a crude membrane preparation as described previously (10).

### Production of SC104 mAb

Four colorectal tumor cell lines (C170, C146, Colo205, and JW) were used to immunize BALB/c 5 days after the final immunisation; the spleen cells were harvested and fused with P3NSO cells. Hybridoma supernatant was screened by fixed-cell ELISA against C170 cells, and any positive wells were then screened for binding to frozen colorectal tumors but not adjacent normal mucosa.

### Immunohistochemistry Staining of Fixed Normal and Tumor Tissues

Human tissues were obtained from an approved supplier. Each tissue used was snap frozen in liquid nitrogen and stored at approximately  $-70 \pm 10^\circ\text{C}$ . All tissue samples were treated with an antigen marker appropriate for each tissue to confirm the preservation of antigens in that tissue. The markers used were keratin for epithelial bearing tissues, CD45

for all lymphoid tissues, and desmin for all cardiac and skeletal muscle. Each normal tissue was examined from three unrelated donors.

The method of staining employed was an indirect, two-stage method using secondary antibodies together with an avidin-biotin-peroxidase complex. Endogenous peroxidase activity was blocked using hydrogen peroxide. Endogenous biotin was blocked by treating all tissue sections with a sequence of avidin-biotin. Validation of the immunohistochemical staining method was determined by staining on positive control tissues, absence on negative controls, and effect of fixation on staining of control tissues. SC104 was tested against six donors of colon tumor and one of heart at concentrations of 0 or 50 µg/mL. Tissues were fixed in neutral buffered formalin. Two samples of colon tumor stained well enough for use as a positive control, and there was no staining in heart. The lowest concentration of SC104 giving the maximum staining intensity was 1 µg/mL, and this concentration was used for subsequent work.

Sections were incubated with normal swine serum (NSS) for 10 minutes to block nonspecific antibody-binding sites. After that, primary antibody was incubated on the slides for 1 hour, with the optimal dilution found to be 1:200. Primary antibody was omitted from the negative control, which was left incubating in NSS. The sections were then incubated in biotinylated goat anti-mouse/rabbit IgG (DakoCytomation Ltd., Cambridgeshire, United Kingdom) for 30 minutes followed by streptavidin-biotinylated horseradish peroxidase complex (SA-HRP; DakoCytomation) for 1 hour at room temperature with the addition of 3,3'-diaminobenzidine with 0.03% hydrogen peroxidase (DakoCytomation) to achieve visualization of the antigen. The sections were lightly counterstained with haematoxylin (DakoCytomation), dehydrated in alcohol, cleared in xylene (Genta Medical, York, United Kingdom), and mounted with DPX (Distyrene, Plasticiser, and Xylene; BDH, Poole, England).

**Indirect immunofluorescence.** C170, Colo205, LoVo, and HT29 cells ( $10^5$ ) were resuspended in 50 µL of SC104 (0-20 µg/mL) and incubated on ice for 20 minutes. After washing the samples thrice in RPMI/10% FCS, cells were incubated with FITC labeled rabbit anti-mouse antibody (1:50; DakoCytomation) and incubated on ice for a further 30 minutes before analysis on a FACScan (Becton Dickinson, Sunnyvale, CA). Results are expressed as mean linear fluorescence.

### Binding to Primary Tumor Cells

Tumor specimens were obtained at the time of colorectal cancer resection. Specimens were finely minced and disaggregated with 0.05% collagenase (type IV; Boehringer Mannheim, Lewes, United Kingdom) for 20 minutes at 37°C and stained by indirect immunofluorescence with SC104 as previously described (10).

### Lipolipid Identification

**Lipid extraction from C170 tumor cells.** A pellet of C170 cells (2 mL packed cell volume) was extracted with chloroform/methanol (3:1, v/v, 19 mL). The resultant emulsion was centrifuged at 8,000 rpm in a 50-mL solvent-resistant (Tefzel) centrifuge tube for 15 minutes at 4°C. The supernatant was dried down using a rotary evaporator at 30°C and resuspended in a small volume of chloroform (~1 mL). A 10-mL bed volume silica column (150 mm inner diameter) was prepared in 100% chloroform. The sample was added to the column, which was subsequently washed under gravity in the following solvents: 2 column volumes chloroform (elutes simple lipids), 5 column volumes acetone (elutes neutral glycolipids), 3 column volumes chloroform/methanol/acetone/acetic acid/water (52:8:8:18:4) followed by 10 column volumes chloroform/methanol (4:1; elutes phospholipids), 3 column volumes chloroform/methanol (2:3; elutes monosialylated glycolipids), 3 column volumes chloroform/methanol/water (65:25:4; elutes disialylated glycolipids), and finally 3 column volumes chloroform/methanol/water (60:35:8; elutes polysialylated glycolipids). Washes were collected separately, dried down by rotary evaporator, resuspended in a small volume of chloroform, and stored at 4°C before analysis.

Chemical desialylation was achieved by heating the antigen solution in 0.05 mol/L sulfuric acid at 80°C for 2 hours. The sample was then allowed to cool and stored at 4°C before analysis.

**High-performance TLC analysis of lipid extracts.** The sample was multiply spotted onto a Merck high-performance TLC (HPTLC) plate and

developed in chloroform/methanol/0.5% CaCl<sub>2</sub>(aq) (50:40:10) as standard. The plates were dried and then placed in an iodine vapour tank to stain for the presence of lipids. Bands were marked in pencil, and the iodine was allowed to sublimate off the plate overnight. For SC104 immunostaining, the plates were immersed in polyisobutyl methylmethacrylate (0.1% w/v solution) hexane/chloroform (9:1) then allowed to air-dry. The plates were then blocked in 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature followed by incubation in either test antibody solution (10 µg/mL) or BSA solution for 1 hour at room temperature. The plates were then washed thrice in PBS/Tween 20 (0.1%), before being incubated in rabbit anti-mouse HRP conjugate (DakoCytomation; 1:250 in PBS) for 1 hour at room temperature. The plates were subsequently washed thrice in PBS/Tween 20 (0.1%) and once in 10 mmol/L Tris, 100 mmol/L NaCl (pH 7), Tween 20 (0.01%) and developed in Sigma-FAST 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium reagent.

Orcinol staining was employed to detect the presence of carbohydrate moieties. Developed HPTLC plates were allowed to dry before spraying with orcinol reagent (Sigma, Poole, Dorset, United Kingdom) until fully coated. The plates were dried in a stream of hot air and incubated at 100°C for 15 minutes. Ninhydrin staining was used to detect molecules containing free amino groups. Developed HPTLC plates were first allowed to dry and then dipped in ninhydrin solution (Sigma; 0.25 % ninhydrin w/v in acetone) until fully wetted. The plate was then allowed to develop at room temperature for several hours.

**Comparison of antigen to commercially available lipid standards.** The SC104 antigen has been compared with a number of commercially available lipid standards. Examples of each standard were spotted onto HPTLC plates and developed in 50:40:10 chloroform/methanol/CaCl<sub>2</sub> (0.5% w/v). Plates were also spotted with partially purified SC104 antigen. Migration of each standard was detected by iodine vapour staining, and the location of each was marked on the plate. Plates were then probed with SC104.

### Cell Death Assays

**Annexin V/propidium iodide.** Tumor cells ( $10^5$ ) in suspension were incubated with various dilutions of SC104 antibody or appropriate controls for 1 hour at room temperature. The cells were then washed in ice cold PBS, stained for 20 minutes at room temperature in the dark with 5 µL FITC labeled Annexin V and 10 µL propidium iodide (BD Biosciences, Cowley, Oxford, United Kingdom), and analyzed by dual colour flow cytometry.

### Caspase Activation

**Detection of pan caspase activation.** C170 cells ( $2 \times 10^5$ ) were exposed to 30 µg/mL SC104 for up to 6 hours. Pan-caspase FITC-FMK-vad inhibitor (Promega, Southampton, United Kingdom) was added at a final concentration of 10 µmol/L, and the cells were incubated in the dark for a further 20 minutes. Cells were then harvested and washed in PBS. Activated caspases irreversibly bind the fluorescent labeled inhibitor, and this was detected by flow cytometry. Controls included a negative murine control antibody and a Fas antibody at 100 ng/mL.

**Inhibition of SC104 cell death using z-FMK-vad caspase inhibitor.** C170 cells ( $2 \times 10^5$ ) were exposed to 3 µg/mL SC104 overnight in the presence or absence of 3 µmol/L z-FMK-vad caspase inhibitor. Cell viability was then determined by FITC-labeled Annexin V and propidium iodide staining as described above and analyzed by dual-color flow cytometry.

**Inhibition of cell growth.** Colorectal cell lines C170, LoVo, Colo205, and HT29 were aliquoted ( $10^3$ ) into individual wells of a flat-bottomed 96-well plate and left to adhere overnight at 37°C. The following day, the cells were treated with 100, 30, 10, 3, and 1 µg/mL of SC104 or murine IgG (negative control; Sigma) in the presence of an additional 1% or 10% heat inactivated FCS (negative control), mouse serum (Cambridge Biosciences, Cambridge, United Kingdom) or human serum. Cells were left for 5 days at 37°C before the addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent to each well, and absorbance was determined at 490 nm.

### Homophilic Binding

**Indirect immunofluorescence staining of fresh and fixed cells.** C170 cells ( $10^5$ ) either fresh or fixed with 0.5% glutaraldehyde or 10% cell fix for 1

hour were resuspended in 100  $\mu$ L of SC104 antibody (0-100  $\mu$ g/mL) and incubated on ice for 1 hour. After washing the samples thrice in RPMI/10% FCS, cells were incubated with FITC-labeled rabbit anti-mouse antibody and incubated on ice for 30 minutes before analyzing by fluorescence-activated cell sorting as described above.

**ELISA.** ELISA plates were coated with 3  $\mu$ g/mL (100  $\mu$ L/well) of rabbit anti-mouse or C14 antigen in PBS and dried down overnight before blocking with PBS/1% BSA. SC104 antibody (0-50  $\mu$ g/mL) was added on ice for 1 hour. Plates were washed, and biotinylated SC104 (1  $\mu$ g) was added and incubated on ice for 30 minutes. Following a further three washes in PBS/Tween 20 (0.05%), anti-mouse SA-HRP (1:1,000) was added and left on ice for 30 minutes. Plates were washed five times in PBS/Tween 20 (0.05%) before adding 100  $\mu$ L/well of TMB substrate and reading at either 570 or 650 nm or ABTS substrate and reading at 405 nm.

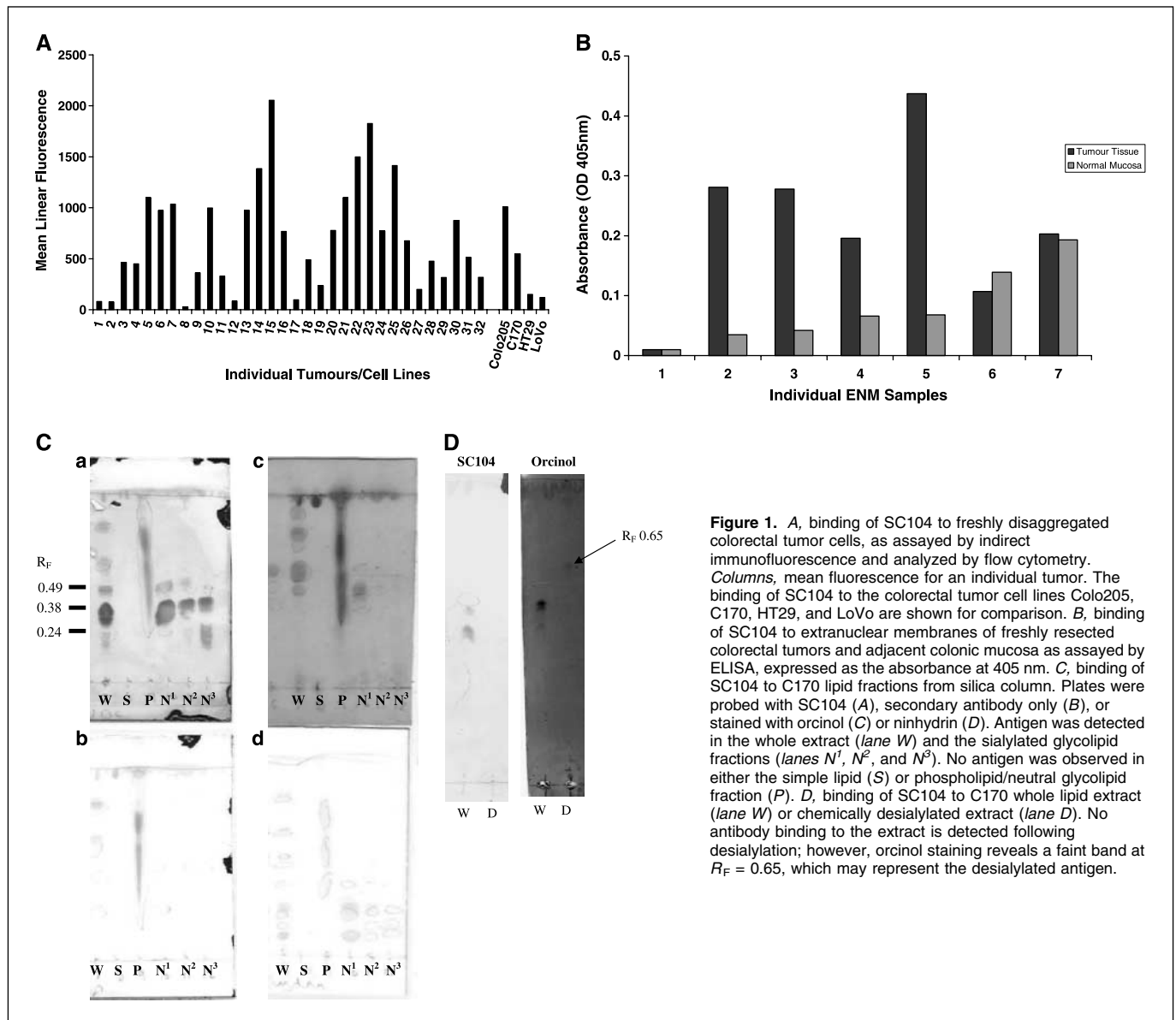
**In vivo Studies**

The colorectal tumor cell line C170 was maintained in serial passage in nude mice. For therapy, the mice were sacrificed, and the tumors were excised. The tumor was finely minced, and 3-mm<sup>2</sup> pieces were implanted, under anesthetic, s.c., into 40 male mice, which had been randomly

allocated to four experimental groups. Mice were either treated immediately (prevention) or 1 week later (therapeutic). Groups of mice were treated with 5-FU/leucovorin (12.5 mg/kg) by i.v. infusion on days 1, 3, 5, and 7. Three times weekly, mice were also injected i.p. with 0.2 mg of SC104 mAb. Control mice received either SC104 alone or control mouse IgG antibody with 5-FU/leucovorin or control mouse IgG antibody. Tumor size was measured by calipers, and tumor cross-sectional area was calculated on days 7, 9, 12, 14, and 16. Animals were weighed to assess the toxicity of treatment. Tumor growth was analyzed for statistical significance by ANOVA, and survival was analyzed by log-rank test.

**Results**

SC104 is a IgG1 mAb that was raised by immunization of mice with four colorectal cancer cell lines. SC104 antibody bound strongly to the cell surface of C170 and Colo205 cells and with lower intensity to HT29 and Lovo (Fig. 1A). It also bound to R1D9, CaCo2, and MKN45 cells of gastrointestinal origin but failed to bind to any breast, ovarian, or bladder cell lines. More importantly, it



**Figure 1.** A, binding of SC104 to freshly disaggregated colorectal tumor cells, as assayed by indirect immunofluorescence and analyzed by flow cytometry. Columns, mean fluorescence for an individual tumor. The binding of SC104 to the colorectal tumor cell lines Colo205, C170, HT29, and LoVo are shown for comparison. B, binding of SC104 to extranuclear membranes of freshly resected colorectal tumors and adjacent colonic mucosa as assayed by ELISA, expressed as the absorbance at 405 nm. C, binding of SC104 to extranuclear membranes of freshly resected colorectal tumors and adjacent colonic mucosa as assayed by TLC. Plates were probed with SC104 (A), secondary antibody only (B), or stained with orcinol (C) or ninhydrin (D). Antigen was detected in the whole extract (lane W) and the sialylated glycolipid fractions (lanes N<sup>1</sup>, N<sup>2</sup>, and N<sup>3</sup>). No antigen was observed in either the simple lipid (S) or phospholipid/neutral glycolipid fraction (P). D, binding of SC104 to C170 whole lipid extract (lane W) or chemically desialylated extract (lane D). No antibody binding to the extract is detected following desialylation; however, orcinol staining reveals a faint band at R<sub>F</sub> = 0.65, which may represent the desialylated antigen.

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was shown to bind strongly to >80% of freshly disaggregated colorectal tumor cells (Fig. 1A).

To verify if this antigen was truly gastrointestinal specific, SC104 was screened for binding to a range of frozen tumor and normal tissue sections (Table 1). An anti-EGFR antibody was also included for comparative purposes. SC104 mAb showed positive staining of the neoplastic epithelium of the colon, endometrial, esophageal, parotid salivary gland, and stomach and less intense staining of small numbers of epithelial cells in three of the six breast tumors. Positive staining was recorded in the epithelium of normal large intestine, parotid salivary gland, tonsil, and uterine cervix. Less intense staining was recorded in small numbers of transitional epithelial cells in the urinary bladder, scattered thymic lymphocytes of a single donor, glandular epithelial cells of the skin, epithelial cells of the prostate, breast and fallopian tube, ovarian follicular cells, and alveolar lining cells of the lung and small numbers of glial cells from the brain of one donor. Mucus stained positively in the stomach and small intestine. No specific staining was recorded in normal human heart, kidney, placenta, or spleen. In comparison, an anti-EGFR antibody stained breast, large intestine, placenta, prostate, parotid salivary gland, skin bladder, and cervix. These results suggest that the SC104 antigen was

mainly restricted to the normal colon; however, it was expressed more strongly on colorectal tumors than adjacent normal colon. To further quantify this differential staining, extranuclear membrane preparations of primary colorectal tumors and normal colon from the resection margin were produced from tumors with similar levels of expression to HT29 (weak) or C170 cells (moderate). ELISA staining of these membranes with SC104 revealed weak staining of the normal colon at lower or similar levels to the weakly expressing tumors. In contrast, there was stronger staining of the moderately expressing tumors with the mean T/N ratio being 7:1 (Fig. 1B).

To try and identify the nature of the antigen recognized by SC104 antibody, it was used to stain two different antigen preparations (data not shown). The first was a Lewis<sup>y/b</sup> expressing glycoprotein extracted from saliva. This antigen is a 90-kDa glycoprotein that expresses a wide range of carbohydrate residues. Both the anti-Lewis<sup>y/b</sup> antibody and SC104 antibody bound to this glycoprotein, whereas the anti-carcinoembryonic antigen antibody failed to bind. A similar result was obtained when a methanol/chloroform tumor glycolipid extract was assayed, suggesting that SC104 was recognizing a carbohydrate residue expressed on both glycoproteins and glycolipids, and that it may be recognizing Lewis<sup>y/b</sup>.

**Table 1.** Immunoreactivity of SC104 antibody with frozen tumor and normal tissue sections

Tissue	Anti-EGF mAb	SC104 binding	Comments on staining
<b>Tumors</b>			
Lung	6/6	0	
Esophageal	4/4	4/4	
Breast	0	3/6	
Renal	ND	0	
Colon	3/8	8/8	
Ovary	0	0	
Parotid	0	2/2	
Prostate	0	0	
Stomach	0	4/4	
Testes	ND	0/1	
Endometrial	1/2	2/2	
<b>Normal tissues</b>			
Brain	0	1/2	Small number of scattered glial cells from the brain of one donor
Breast	3/3	2/3	Epithelial cells, scattered moderate
Fallopian tube	0	2/3	Epithelial cells, scattered moderate
Heart	0	0	
Kidney	0	0	
Large intestine	3/3	3/3	Epithelial cells, mucous, diffuse mild to moderate
Lung	0	2/3	Alveolar lining cells, one donor minimal and one donor mild
Ovary	0	3/3	Follicular cells, scattered and mild
Placenta	3/3	0	
Prostate	3/3	3/3	Epithelial cells, scattered and moderate
Parotid salivary gland	3/3	3/3	Moderate
Skin	3/3	2/3	Diffuse, minimal staining
Small intestine	0	3/3	Mucus, superficial, diffuse minimal
Spleen	0	0	
Stomach	0	2/3	Mucus, superficial, diffuse mild
Thymus	0	3/3	Epithelial cells, Hassall's corpuscle, membrane scattered mild
Tonsil	0	3/3	Keratinized epithelium, membrane, scattered mild to moderate.
Urinary bladder	2/3	2/2	Epithelial cells, transitional epithelium, membrane scattered mild
Uterine cervix	3/3	3/3	Epithelial cells, membrane, diffuse mild to moderate

Abbreviation: ND, not done.

**Table 2.** Immunostaining of SC104 antigen and comparison with commercially available lipid standards

Standard	Structure	$R_F$	SC104 $R_F$
Lactosyl ceramide	Gal $\beta$ 1,4-Glc $\beta$ 1,1-Cer	0.79	
Gb <sub>3</sub>	Gal $\alpha$ 1,4-Gal $\beta$ 1,4-Glc $\beta$ 1,1-Cer	0.71	
Globoside	GalNAc $\beta$ 1,3-Gal $\alpha$ 1,4-Gal $\beta$ 1,4-Glc $\beta$ 1,1-Cer	0.62	
Forrsmann	GalNAc $\alpha$ 1,3-GalNAc $\beta$ 1,3-Gal $\alpha$ 1,4-Gal $\beta$ 1,4-Glc $\beta$ 1,1-Cer	0.56	
GM <sub>3</sub>	NeuAc-Gal $\beta$ 1,4-Glc $\beta$ 1,1-Cer	0.53	0.53
			0.49
Sialyl Le <sup>a</sup>	NeuAc-Gal $\beta$ 1,3-[Fuc $\alpha$ 1,4]GlcNAc $\beta$ 1,3-Gal $\beta$ 1,4-Glc $\beta$ 1,1-Cer	0.46-0.51	
AGM <sub>1</sub>	Gal $\beta$ 1,3-GalNAc $\beta$ 1,4-Gal $\beta$ 1,4-Glc- $\beta$ 1,1-Cer	0.48	
GM <sub>1</sub>	Gal $\beta$ 1,3-GalNAc $\beta$ 1,4-(NeuAc)Gal $\beta$ 1,4-Glc $\beta$ 1,1-Cer	0.39	0.39
			0.36
GD <sub>3</sub>	NeuAc-NeuAc-Gal $\beta$ 1,4-Glc $\beta$ 1,1-Cer	0.33	
			<0.24
GD <sub>1A</sub>	NeuAc-Gal $\beta$ 1,3-GalNAc $\beta$ 1,4-(NeuAc)Gal $\beta$ 1,4-Glc $\beta$ 1,1-Cer	0.22	
GD <sub>1B</sub>	Gal $\beta$ 1,3-GalNAc $\beta$ 1,4-(NeuAc-NeuAc)Gal $\beta$ 1,4-Glc $\beta$ 1,1-Cer	0.16	
GT <sub>1B</sub>	NeuAc-Gal $\beta$ 1,3-GalNAc $\beta$ 1,4-(NeuAc-NeuAc)Gal $\beta$ 1,4-Glc- $\beta$ 1,1-Cer	0.08	

No reactivity was observed between SC104 and the blood group antigens Lewis<sup>y</sup> and H type I and H type II blood group haptens.

Silica column chromatography was then employed to fractionate a lipid extract from C170 tumor cells. The fractions were then analyzed by HPTLC followed by SC104 immunostaining and chemical staining (Fig. 1C). No antigen was detected in the simple lipid fraction (S) or the phospholipid/neutral glycolipid fraction (P), but SC104 positive staining was observed in the whole extract (W) and mono-sialylated (N<sub>1</sub>), di-sialylated (N<sub>2</sub>) and poly-sialylated (N<sub>3</sub>) fractions of the lipid extract. Orcinol or ninhydrin staining was used to detect the presence of carbohydrates or free amino groups, respectively. This staining showed that some phospholipids were present with the glycolipids in the sialylated glycolipid fractions. However, the antigen seemed to comigrate with the orcinol positive glycolipids only, indicating that the antigen is a sialylated glycolipid.

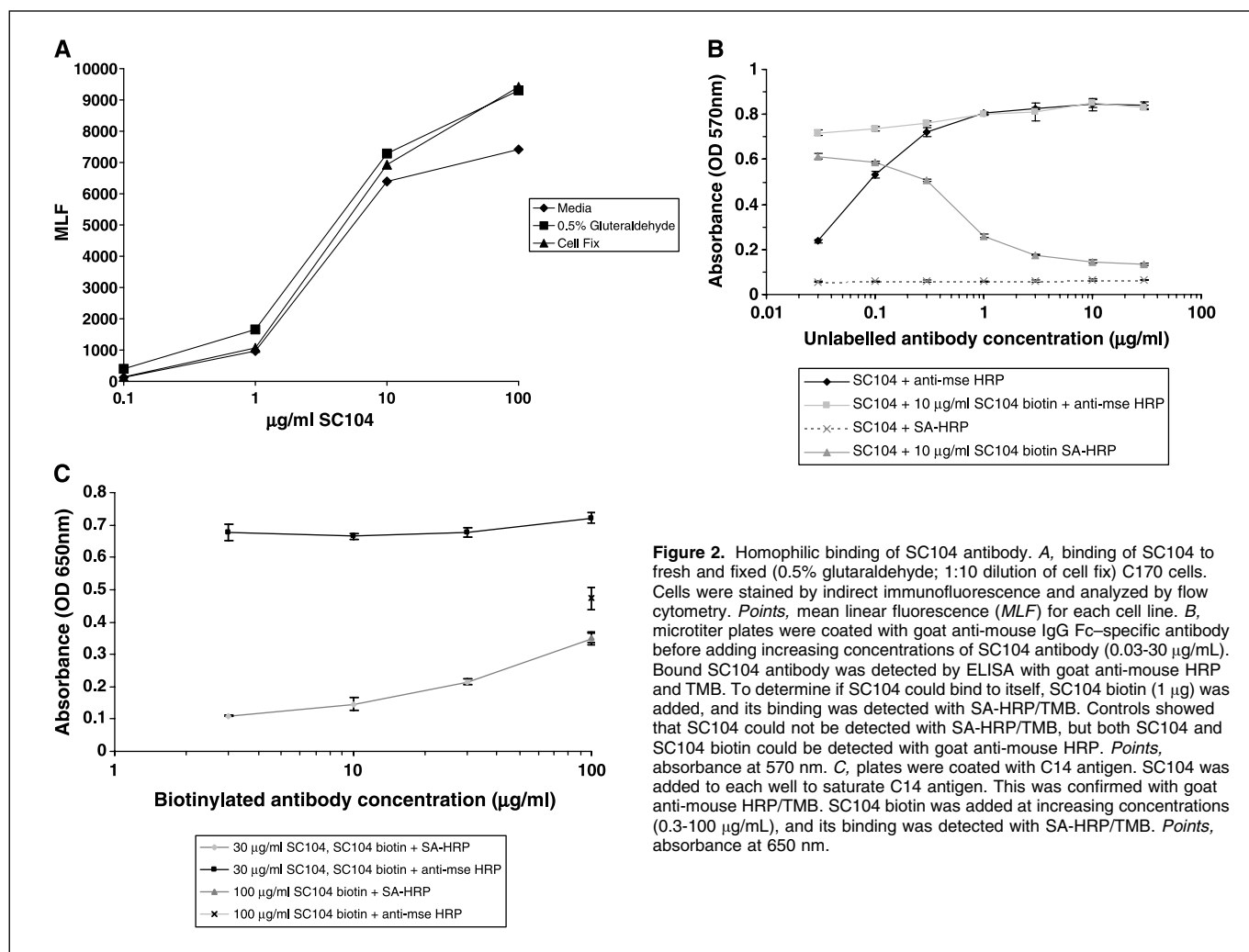
Acid hydrolysis was then used to chemically desialylate the antigen. HPTLC and SC104 immunostaining showed that chemical removal of sialic acids resulted in the complete loss of SC104 binding (Fig. 1D), providing further evidence that sialylation of the antigen is necessary for recognition. Comparison of the orcinol stained samples before and after acid hydrolysis revealed bands, which colocalized with the antigen before removal of sialic acids. After desialylation, these bands were replaced by a faint band (at  $R_F = 0.65$ ), which may represent the desialylated version of the antigen. The observed  $R_F$  value is very similar to that seen for the globoside glycolipid standard (Table 2), which has a structure comprised of four neutral oligosaccharides attached to ceramide. The data, therefore, indicate that the SC104 antigen must be monosialylated, although the presence of further sialic acids does not impinge on the binding of SC104.

The SC104 antigen has been compared with a number of commercially available lipid standards. The  $R_F$  values are shown in Table 2 and are also compared with the range of  $R_F$  values obtained for the variably sialylated SC104 antigen. In all cases, the standards are themselves not recognized by SC104. The comigration of SC104 with these standard lipids suggests that, along with the requirement for sialylation, the antigen has a short neutral oligosaccharide backbone that consists of three or four monosaccharides. It is most probable that this is actually a tetraosyl structure, with four

monosaccharides attached to ceramide, as this would more readily allow multiple sialylation. The least polar SC104 antigen that is detected is probably a partial antigen structure, consisting of a shorter oligosaccharide backbone.

The number of SC104 haptens at the surface of a C170 tumor cells is  $\sim 4 \times 10^5$  sites per cell. However, this number of antigens would easily be saturated by 1  $\mu$ g of SC104. It was, therefore, difficult to explain why a 10-fold excess of antibody was required for cell killing. It could be that upon antibody binding more sites were revealed. Antibody saturation curves were, therefore, generated on fresh and fixed cells. Figure 2A shows that antigen was not saturated even at a SC104 concentration of 100  $\mu$ g/mL, and curves were similar on fresh and fixed cells, making it unlikely that further antigen was being revealed upon antibody binding. These results are similar to previously reported data on R24, a mouse mAb recognizing GD3 ganglioside (11). This antibody shows nonsaturable antibody binding and in a series of elegant experiments was shown to be a homophilic binding antibody with the capacity to bind to both antigen and itself. SC104 was, therefore, screened for the ability to bind to itself by coating an ELISA plate with unlabeled SC104 and measuring the binding of SC104 biotin HRP-avidin (Fig. 2B). The plates were saturated with 1  $\mu$ g/mL of SC104, and addition of biotinylated SC104 did not show any further binding, showing that SC104 was not binding to itself. However, when purified glycoprotein expressing SC104 haptens was used to coat the plates, in the presence of saturating concentration of SC104, biotinylated SC104 continued to bind and failed to reach saturation even at 100  $\mu$ g/mL (Fig. 2C).

This study has shown strong staining of freshly disaggregated colorectal tumors by SC104. However, during these studies, it was observed that SC104 antibody binding seemed to accelerate tumor cell death. To determine if the antibody was inducing apoptosis or necrosis, cells exposed to SC104 antibody (30  $\mu$ g/mL) for 4 hours were counterstained with Annexin V and propidium iodide (Fig. 3A). Less than 1% of the cells exposed to control antibody showed staining with Annexin V alone. In contrast, cells exposed to SC104 showed 8% staining with Annexin V alone and 30% with both Annexin V and propidium iodide. Cells stained with Annexin V alone are described as being in early-stage apoptosis, whereas



**Figure 2.** Homophilic binding of SC104 antibody. **A**, binding of SC104 to fresh and fixed (0.5% glutaraldehyde; 1:10 dilution of cell fix) C170 cells. Cells were stained by indirect immunofluorescence and analyzed by flow cytometry. *Points*, mean linear fluorescence (*MLF*) for each cell line. **B**, microtiter plates were coated with goat anti-mouse IgG Fc-specific antibody before adding increasing concentrations of SC104 antibody (0.03-30  $\mu\text{g/ml}$ ). Bound SC104 antibody was detected by ELISA with goat anti-mouse HRP and TMB. To determine if SC104 could bind to itself, SC104 biotin (1  $\mu\text{g}$ ) was added, and its binding was detected with SA-HRP/TMB. Controls showed that SC104 could not be detected with SA-HRP/TMB, but both SC104 and SC104 biotin could be detected with goat anti-mouse HRP. *Points*, absorbance at 570 nm. **C**, plates were coated with C14 antigen. SC104 was added to each well to saturate C14 antigen. This was confirmed with goat anti-mouse HRP/TMB. SC104 biotin was added at increasing concentrations (0.3-100  $\mu\text{g/ml}$ ), and its binding was detected with SA-HRP/TMB. *Points*, absorbance at 650 nm.

cells stained with both Annexin V and propidium iodide are in late-stage apoptosis/necrosis.

Flow cytometry experiments were done, which aimed to further characterize the apoptotic mechanism of SC104. C170 colorectal cells were treated with SC104 for up to 6 hours before incubation with a FITC-labeled pan-caspase inhibitor FITC-FMK-vad. The fluorescent inhibitor binds irreversibly to any activated caspases within the cells and can then be detected by flow cytometry. After 5 hours exposure to 30  $\mu\text{g/ml}$  SC104, it can be seen that pan caspases have been activated (Fig. 3B).

The effects of SC104 mAb on cells when treated in the presence of a pan-caspase inhibitor z-FMK-vad were also examined. Cells were treated overnight with 3  $\mu\text{g/ml}$  SC104 in the absence or presence of the z-FMK-vad caspase inhibitor. Cell viability was then examined via Annexin V/FITC and propidium iodide staining. It was observed that the presence of the caspase inhibitor protected colorectal tumor cells from apoptosis (Fig. 3C).

The effect of SC104 on adherent cell proliferation was measured in an MTS assay. An  $\text{IC}_{50}$  of around 30  $\mu\text{g/ml}$  was obtained for C170 and Colo205 cells that strongly express SC104 antigen (Fig. 4A); similar results were obtained with other strongly expressing colorectal cells lines C146, C168, and CaCo2 (data not shown). In contrast, cell lines expressing low levels of SC104 antigen (HT29 and

LoVo) failed to show any significant reduction in cell proliferation, even when treated with 100  $\mu\text{g/ml}$  antibody for 5 days (Fig. 4A). The level of antigen expressed by HT29 and LoVo was similar to levels expressed by low-expressing tumors (20%) and normal colonic mucosa as shown in Fig. 1. As the large intestine showed the strongest binding of the normal tissues, these results suggest that there may be a therapeutic window, whereby SC104 mAb will kill tumor cells but avoid undue toxicity.

SC104 is a mouse IgG1 antibody and can mediate both CDC and ADCC (data not shown). To see if there was any additional killing in these assays with complement, the assays were repeated in the presence of human or mouse serum as a source of complement. Figure 4B shows that the presence of both mouse and human complement decreased the number of viable cells to a greater extent than SC104 alone. To determine if the effects of SC104 could be translated to inhibition of tumor growth *in vivo*, the antibody was given (200  $\mu\text{g/dose}$ ) to mice either transplanted with 3  $\text{mm}^2$  extracts of C170 tumors or to C170 tumors that had been allowed to grow for 1 week before administration of the antibody. Animals were treated either with SC104 alone, 5-FU/leucovorin at the maximum tolerated dose, or a combination of both. Figure 4C shows that from day 9, the treatment groups showed significant inhibition of growth when compared with controls. The group

receiving SC104 and 5-FU/leucovorin showed significant tumor inhibition (76% inhibition,  $P \leq 0.001$ ). Similarly, treatment with 5-FU/leucovorin (62% inhibition,  $P \leq 0.001$ ) or SC104 (62% inhibition,  $P \leq 0.001$ ) alone also inhibited tumor growth. SC104 was well tolerated with all mice showing no loss of weight or any other gross pathology. Finally, when SC104 was given therapeutically to mice expressing C170 tumors in combination with 5-FU/leucovorin, it both significantly inhibited tumor growth and enhanced survival ( $P = 0.0433$ , log-rank; Fig. 4D).

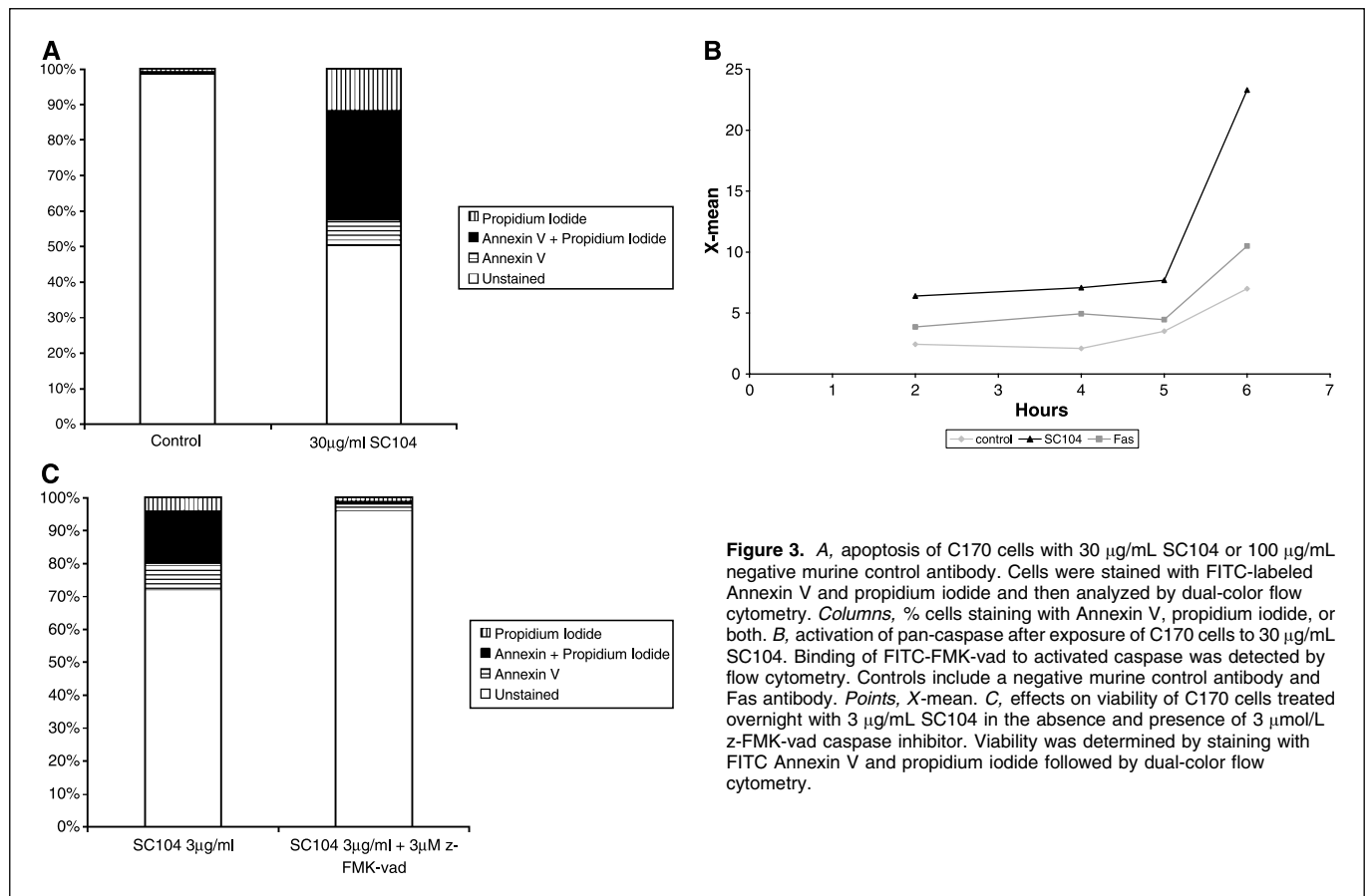
## Discussion

mAbs have recently proved their clinical use. These antibodies act alone by directly inducing apoptosis and/or stimulating ADCC or CDC. In this study, we describe a novel antibody that targets a gastrointestinal related glycolipid and which directly induces apoptosis but also synergizes with chemotherapy *in vivo*.

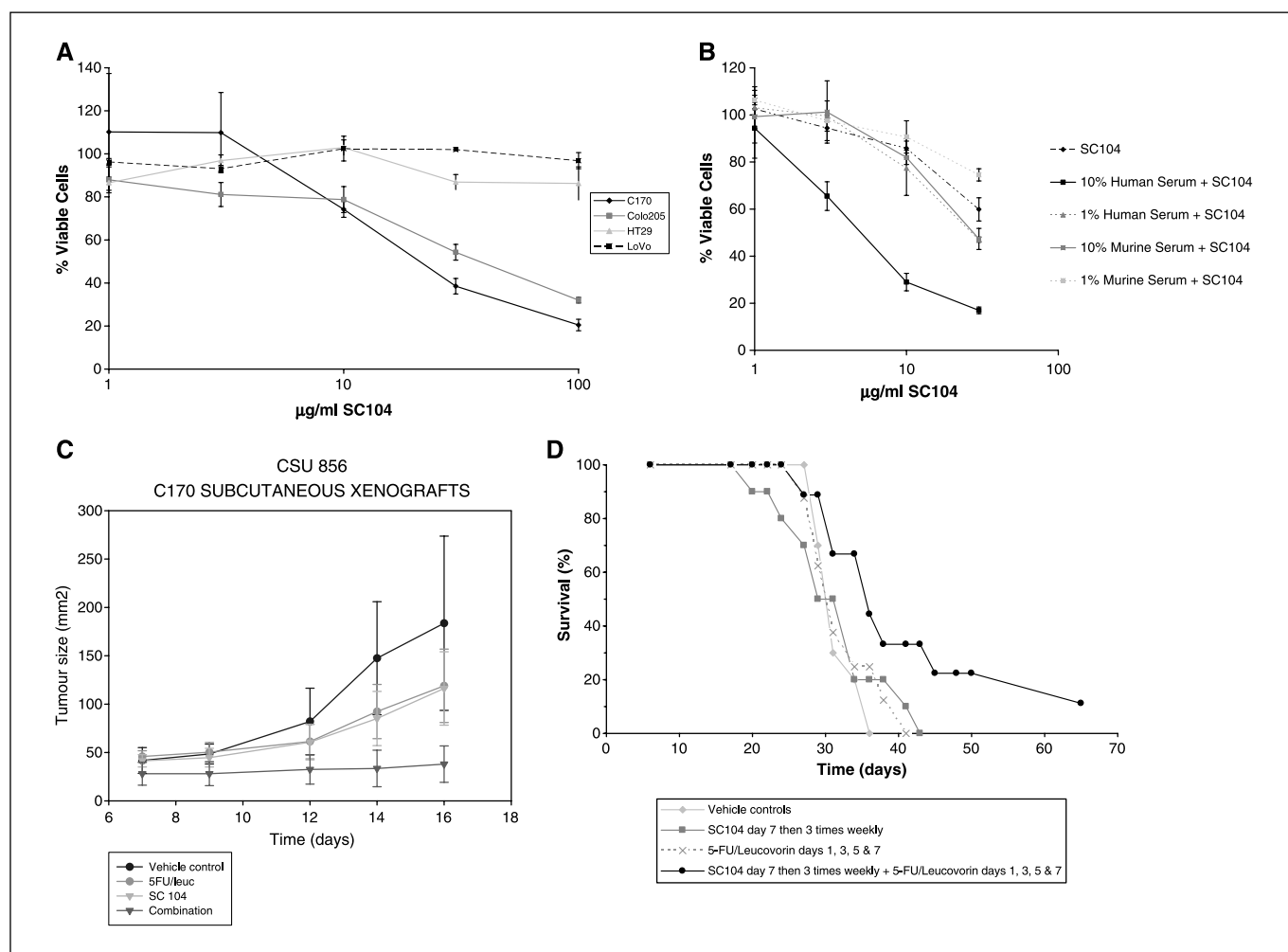
SC104 was raised against a panel of colorectal cancer cell lines with the intention of producing a pan-reactive, anti-colorectal tumor antibody. This was confirmed by staining of primary, disaggregated colorectal tumors, where 80% of the tumors showed strong cell surface expression. This was largely confirmed by immunohistochemistry on a panel of tumor and normal tissues with the staining being predominantly gastrointestinal related but with some weaker staining of other tissues. SC104 did not bind to Lewis<sup>y</sup> or H blood group antigen, which are all very immunogenic antigens expressed by the majority of colorectal cancers. Furthermore, antigen characterization revealed that the mAb recognized

an epitope on both a glycolipid extract and a glycoprotein antigen, suggesting that it recognized a carbohydrate determinant. Extensive glycolipid analysis has revealed that a good candidate structure for the antigen is a sialyltetraosylceramide. The antibody could bind in such a way that additional and even multiple internal sialic acids would not prevent antibody recognition of the antigen. However, no binding of SC104 to the gangliosides GM1, GD1a, GT1b, or sialyl Lewis<sup>x</sup> was observed. Overexpression of GD3 and GD2 in human melanoma, GM3 in mouse melanoma, GD2 in neuroblastoma, Gg3 in mouse lymphoma, human Hodgkin's lymphoma and Burkitt's lymphoma, fucosyl-GM1 in small cell lung cancer, and Xbo-H in breast and ovarian carcinoma are examples of high accumulation of specific glycosphingolipids in specific types of cancer (12). R24 mouse mAb recognizing GD3 has been used successfully in treatment of melanoma; however, the human anti-mouse antibody response limited repeated use (13, 14). A humanized diabody has recently been produced and will shortly enter clinical trials. Mitumomab is an anti-idiotypic antibody that mimics GD3 a ganglioside overexpressed on melanomas and small cell lung cancers (SCLC; ref. 15). Initial studies showed that the vaccine improved the overall survival of patients suffering from small cell lung cancer (16, 17). Unfortunately, a phase III study involving 800 patients with SCLC failed to show a significant survival advantage.

During staining of the primary colorectal tumors, it was observed that SC104 seemed to accelerate tumor cell death. These studies suggest that it may be inducing apoptosis as measured by Annexin V assays. Pan-caspase activation and more specifically a



**Figure 3.** A, apoptosis of C170 cells with 30 µg/mL SC104 or 100 µg/mL negative murine control antibody. Cells were stained with FITC-labeled Annexin V and propidium iodide and then analyzed by dual-color flow cytometry. Columns, % cells staining with Annexin V, propidium iodide, or both. B, activation of pan-caspase after exposure of C170 cells to 30 µg/mL SC104. Binding of FITC-FMK-vad to activated caspase was detected by flow cytometry. Controls include a negative murine control antibody and Fas antibody. Points, X-mean. C, effects on viability of C170 cells treated overnight with 3 µg/mL SC104 in the absence and presence of 3 µmol/L z-FMK-vad caspase inhibitor. Viability was determined by staining with FITC Annexin V and propidium iodide followed by dual-color flow cytometry.



**Figure 4.** The antiproliferative effect of SC104, both *in vitro* (A and B) and *in vivo* (C and D). A, % viability (cells in treated wells/cells in control  $\times$  100) C170, Colo205, HT29, and LoVo cells. B, % increase in cell mortality in response to the addition of either human or murine serum indicative of CDC-assisted cell death. The number of viable cells was determined by MTS and absorbance reading at 490 nm. C, the effect of SC104, 5-FU/leucovorin and a combination of SC104 and 5-FU/leucovorin on the growth of C170 xenografts growing in nude mice. Growth of C170 xenografts was measured at days 7, 9, 12, 14, and 16 by measurement of cross-sectional area (mm<sup>2</sup>) when animals were treated with either SC104 i.p. (0.2 mg), 5-FU/leucovorin (12.5 mg/kg, i.v.), and control antibody or SC104 i.p. (0.2 mg) and 5-FU/leucovorin (12.5 mg/kg, i.v.) or control antibody. D, survival data of animals with C170 xenografts treated with SC104 i.p. (0.2 mg), 5-FU/leucovorin (12.5 mg/kg, i.v.), or SC104 (0.2 mg) and 5-FU/leucovorin (12.5 mg/kg, i.v.) in combination or vehicle control. SC104 was given on day 7 then thrice weekly. 5-FU/leucovorin was administered on days 1, 3, 5, and 7.

pan-caspase inhibitor could prevent the SC104-induced apoptosis. This provides a strong indication that SC104 mAb kills colorectal tumor cells via a "classic" apoptotic pathway. Although the cell death initiated by SC104 is direct apoptosis, it has been shown *in vitro* that CDC may also have a role to play in further reducing the limited number of viable cells remaining after mAb treatment.

The cytotoxicity of the SC104 antibody seems to be related to its homophilic binding as the antibodies only induce cell death on cells that overexpress the antigen. This is very desirable as it should avoid toxicity to normal tissues that express similar levels of antigen to LoVo and HT29 cells that were not killed by the antibody. At low antigen density, low-affinity monomeric binding may result in poor antigen recognition, or it may be sterically impossible to achieve cross-linking between antigen and SC104 molecules if the antigen is sparsely spread over the surface of a cell. However, at high antigen density cross-linking of the SC104 glycolipids may accelerate internalisation and ceramide accumulation. Homophilic antibodies have been described, recognizing a

range of carbohydrate antigens (18–21). It has been suggested that they are induced to bacterial carbohydrates that do not provide T-cell help and cannot therefore induce affinity maturation. The relative low affinity of these antibodies is, therefore, compensated by a high functional avidity at high antigen density (11, 22). Antibodies that induce cell death without immune effector mechanisms may be very important in the treatment of solid tumors that have evolved complex mechanisms to protect themselves from CDC and ADCC (5).

The *in vivo* studies show that SC104 alone could inhibit tumor growth; however, at the maximum tolerated dose of 5-FU/leucovorin, the addition of SC104 almost completely prevented tumor growth with no associated toxicity. Similar doses of Herceptin, an antibody that inhibits cell proliferation and mediates ADCC, have been shown to be effective in both mice and man (2). Although the combination of SC104 and 5-FU/leucovorin was also effective against established tumors resulting in improved survival, the inhibition was more dramatic in the tumor prevention model. If



phase I clinical trials show that SC104 antibodies are safe, subsequent trials of the combination of 5-FU/leucovorin and SC104 compared with drug alone in colorectal cancer patients following surgery would be indicated. More support for this approach has been provided by the dramatic reduction in recurrence in the recent adjuvant studies with Herceptin and chemotherapy given directly after resection of primary breast tumors.

In conclusion, SC104 is a novel antibody recognizing sialyltetraosylceramide but does not bind to GM1, GD1a, GT1b, and sialyl

Lewis<sup>x</sup>. It binds to this glycolipid and directly induces tumor cell death. It shows additive killing with 5-FU/leucovorin and may be used in combination with these drugs to increase efficacy of treatment of colorectal cancer.

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