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Inhibition of xenograft tumor growth and downregulation of ErbB receptors by an antibody directed against Lewis-Y antigen

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Running title: Inhibition by Lewis-Y antibodies of tumor growth

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Abbreviations:

ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; EGFR, epidermal growth factor receptor (ErbB1) ; LeY, Lewis-Y antigen ; MAPK, mitogen-activated protein kinase

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ABSTRACT

The blood group-related Lewis Y antigen is expressed on the majority of human cancers of epithelial origin with only limited expression on normal tissue. Therefore, the Lewis-Y antigen represents an interesting candidate for antibody-based treatment strategies. Previous experiments showed that the humanized Lewis-Y-specific monoclonal antibody IGN311 reduced ErbB-receptor mediated stimulation of MAP-kinase by altering receptor recycling. Here, we tested whether binding of IGN311 to growth factor receptors is relevant also to inhibition of tumor growth *in vivo*. Prolonged incubation with IGN311 of human tumor cell lines which express high levels of ErbB1 (A431) or ErbB2 (SK-BR-3) resulted in down-regulation of the receptors and inhibition of cell proliferation. IGN311 inhibited the growth of tumors derived from A431 cells xenografted in nude mice. Treatment with IGN311 was associated with a downregulation of ErbB1 in the excised tumor tissue. Importantly, these effects of IGN311 were also mimicked by the Fab-fragment of IGN311. These data indicate that tumor cell growth inhibition by IGN311 cannot solely be accounted for by invoking cellular and humoral immunological mechanisms. A direct effect on signalling via binding to Lewis Y glycosylated growth factor receptors on tumor cells is also likely to contribute to the therapeutic effect of IGN311 *in vivo*.

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INTRODUCTION

The Lewis Y (LeY) antigen is a blood group-related difucosylated oligosaccharide with the chemical structure Fucα1→2Galβ1→4[Fucα1→3]GlcNAcβ1→R. While LeY is expressed at high levels during embryogenesis, its expression on adult tissue is restricted to epithelial surfaces and activated granulocytes. However, the vast majority of human carcinomas show an overexpression of the LeY antigen (Hellstrom et al., 1990) either as a glycolipid in the cell membrane or linked to cell surface proteins (Basu et al., 1987).

Growth factor receptors have been selected as targets for anti-tumor therapy using monoclonal antibodies against their extracellular domains. The best studied example is the ErbB-receptor family. The aberrant expression of ErbB receptors tyrosine kinases has been implicated in tumor growth and progression (Yarden and Sliwkowski, 2001). This family includes receptors of the epidermal growth factor (EGFR, ErbB1), ErbB2, and the heregulin-receptors ErbB3 and ErbB4. Upon ligand binding these receptors may homo- or heterodimerize, which leads to phosphorylation of intracellular tyrosine residues. These results in the recruitment of SH2 domain containing adaptor proteins that transduce the signal to mitogen activated protein kinases (MAPK) and phosphoinositide 3-kinase (Ullrich and Schlessinger, 1990). The use of trastuzumab, a humanized monoclonal antibody directed against ErbB2, represented a major breakthrough in the therapy of breast cancer. Nevertheless, targeting growth factor receptors can also lead to inhibition of physiological responses that these receptors elicit. In the case of trastuzumab, inhibition of cardiac ErbB2 may cause heart failure (Slamon et al., 2001).

The frequent overexpression of Lewis Y on cancer cells has stimulated the testing of monoclonal anti-LeY antibodies in animal cancer models (Wahl et al., 2001; Clarke et al., 2000; Scott et al., 2000). The observed growth inhibition has been attributed to recruitment of effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Co et al., 1996). Recently we have shown that the Lewis Y-specific humanized monoclonal antibody IGN311 inhibited EGF-mediated signaling in the

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epidermoid cancer cell line A431 as well as in the breast cancer cell line SK-BR-3 (Klinger et al., 2004). The effect of IGN311 (or its murine parent antibody ABL364) was not due to competition with binding of EGF to the receptor, but rather to altered kinetics of EGF-receptor recycling. In the present work, we extended these *in vitro* investigations and tested whether this mechanism may also contribute to inhibition of tumour growth *in vivo*. The observations show that, upon prolonged exposure to IGN311, tumor cells downregulate the expression of ErbB-receptors *in vitro* as well as *in vivo* in a xenograft tumor model. These data and the efficacy of Fab-fragments of IGN311 suggest that inhibition of tumor growth is, at least in part, due to an effect of IGN311 on signaling via binding to Lewis-Y glycosylated growth factor receptors present on cancer cells.

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METHODS

Materials. Foetal calf serum was purchased from PAA Laboratories (Linz, Austria), Dulbecco's modified Eagle medium (DMEM), RPMI-1640 medium, non-essential amino acids, β-mercaptoethanol were obtained from GIBCO-BRL (Grand Island, NY). A431 and SK-BR-3 cell lines were purchased from ATCC (Manassas, VA). SK-BR-5 cells were kindly provided by Novartis Institute for Biomedical Research, Vienna. The anti EGF-receptor antibody (Ab-2, clone 225) were from NeoMarkers (Fremont, CA). The antibodies recognizing ErbB1 and ErbB2 were from Cell Signaling Technologies Inc. (Beverly, MA, USA), the antibody against cytokeratin 8 from Abcam (Cambridge UK M20 ab 9023-1) and the antiserum recognizing the carboxyl terminus of erk1/erk2 from Santa Cruz (Santa Cruz, CA). The anti-Lewis murine monoclonal antibody ABL364 was provided by Novartis (Basel, Switzerland), and its humanized version IGN311 was produced under GMP conditions by BioInvent (Lund, Sweden). Trastuzumab (Herceptin) was a gift from Roche (Basel, Switzerland).

Cell culture. A431, SK-BR-3 and SK-BR-5 cells were propagated in Dulbecco´s modified Eagle medium (DMEM) at 5% CO_2 and 37°C supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 units/ml penicillin G and 100 µg/ml streptomycin. For cell proliferation assays, A431, SK-BR-5 or SK-BR-3 cells (10^5 cells/dish) were plated into 3 cm culture dishes in the absence or presence of indicated concentrations of antibody. Media were changed daily with addition of fresh antibodies. After 24, 48 and 72 hours, the cells were detached with trypsin and counted.

Determination of ErbB receptor levels in cell lysates and in tumor tissues samples. Cell culture conditions were as outlined above (10^5 cells/ 3 cm dish). Media were changed daily with fresh addition of antibodies. After washing with PBS, cells were lysed in lysis buffer (20

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mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 40 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1% Triton X-100, 250 units/ml aprotinin, and 40 µg/µl leupeptin; pH adjusted to 7.5 with HCl). The cellular debris was removed by centrifugation at 10,000 × *g* for 10 min, and the total protein content was measured photometrically using the bicinchoninic acid method (Micro-BCA kit, Pierce). Equal amounts of protein (20 µg) were resolved on SDS polyacrylamide gels. Subsequently, proteins were transferred to nitrocellulose membranes, which were probed with antibodies against ErbB1 and ErbB2; immunoreactive bands were visualized by chemoluminescence using a secondary antibody coupled to horseradish peroxidase. The loading control was done by staining for p42/p44 MAP-kinase (erk1/erk2).

ErbB1 levels in the excised xenograft tumors (see below) were determined as follows: the frozen tissue samples (see below) were pounded with a mortar; the resulting powder was resuspended in two volumes of buffer (20 mM Tris.HCl, pH 7.4, 1% SDS); insoluble material and debris were removed by centrifugation at 10,000 × *g* for 10 min. The amount of material applied onto SDS-polyacrylamide gels (~5% of original tissue) was normalized by determining the content of cytokeratin-8 in each sample. Immunoblotting for cytokeratin-8 and ErbB1 was done as outlined above. Immunoreactive bands were quantitated by densitometry using the Scion Image software (Scion Corporation, Frederick, MD).

Animal maintenance and xenograft inoculation. The experiments were approved by the Animal Welfare Committee of the Medical University of Vienna; animals were maintained according to the standards set forth by the *Good Scientific Practice*-regulations of the Medical University of Vienna. Female nude mice (Balb/c *nu/nu*) were purchased from the *Institut für Versuchstierzucht Himberg (Austria)*; they were typically 5 to 6 weeks old. The animals were allowed to adapt for 1 week under sterile conditions (Seal-Safe-IVC-Cages, Techniplast, Munich). On the 1st experimental day, A431 cells (1 to $3*10^6$ suspended in 0.2 mL phosphate

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buffered saline) were injected subcutaneously. Prior to injection, the presence of Lewis Y-antigen on the cell surface was verified by FACS. On the next day, the appropriate antibody or vehicle was injected intraperitoneally: The injection of antibody was repeated every 72 h. The tumor size was measured and the tumor volume computed from the formula for an ellipsoid body (volume = length * breadth * height * $\pi/6$), where – for small tumors – height (=thickness) was assumed to correspond to the smaller of the two diameters. At the end of the experiment mice were sacrificed and tumors were excised, shock frozen in liquid N_2 and stored at -80°C for further analysis.

Preparation of Fab

Antibody IGN311 (63 mg) was dialyzed against 20mM sodium phosphate buffer (pH=7.0) supplemented with 10mM EDTA. Aliquots of 0.5 ml (containing 5 mg IGN311) were added to 0.5 ml immobilized papain (Immuno Pure Fab-Kit ;Pierce) equilibrated against digestion buffer cystein-monohydrochloride / phosphate buffer pH=7.0 for 24 hours at 37 °C. Fab fragment was purified by negative Protein G affinity chromatography (HiTrap, Amersham Pharmacia) after separation of immobilized Papain. Flow-through was concentrated to 3.6 mg/ml by Macrosep OMEGA 30K (Pall) against PBS. Cleavage efficiency and fragment purity of >95% were confirmed by SDS-polyacrylamide gel electrophoresis and size exclusion chromatography.

Statistical analysis

Statistical comparisons were done by ANOVA followed by Tukey's multiple comparison test using the statistical package implemented in the Graph-Pad Prism® software.

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RESULTS

IGN311 inhibits cell growth of A431 and SK-BR-3 cells.

The anti-LewisY-antibodies IGN311 and ABL364 have been shown to block signaling via binding to Lewis-Y glycosylated ErbB-receptors and other cell surface receptors (Klinger et al., 2004). *In vivo*, tumor cells may be stimulated by a cocktail of growth factors rather than by a single paracrine factor. We mimicked this situation by allowing the LewisY-positive cancer cell lines A431 or SK-BR-3 to grow in the presence of serum and by testing the ability of IGN311 to block serum-driven growth. When added at 100 nM, IGN311 caused growth inhibition that was only observed after a delay (Fig. 1A). In subsequent experiments, the response to the antibodies was therefore assessed at ≥72 h. IGN311 and ABL364 exert their half-maximum inhibitory effect on EGF-dependent signalling at ~10 nM (Co et al., 1996), which is consistent with the affinity of these antibodies for their respective epitope Lewis Y (Klinger et al., 2004). Accordingly, there was no appreciable inhibition of cell proliferation at 1 nM IGN311 (full triangles in Fig. 1C). In contrast, trastuzumab (Herceptin®) caused about 50% of its antiproliferative effect at 1 nM (empty triangles in Fig. 1C), a concentration which is in the range of its affinity for ErbB2. When employed at the saturating concentration of 100 nM, IGN311 and trastuzumab inhibited growth to a comparable extent (Fig. 1C&D); there was not any additional inhibition, if trastuzumab and IGN311 were combined (full squares in Fig. 1C). The data in Fig. 1C were obtained with SK-BR-3 cells; in contrast trastuzumab did not inhibit the growth of A431 cells (Fig. 1D). A431 cells express large amounts of ErbB1 but only very small amounts of ErbB2; thus, in A431 cells, the efficacy of IGN311 was compared to that of the anti-erbB1-antibody 2C225 (the parent antibody of cetuximab). At 100 nM, IGN311 and 2C225 were equieffective.

IGN311 downregulates ErbB1 receptors in A431 cells.

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The mechanism of action that is generally considered relevant for clinical action of trastuzumab is downregulation of ErbB2 (Baselga et al., 2001). We recently showed that IGN311 alters the kinetics of ErbB receptor recycling. This change in the cycle of endocytosis and externalization is causally related to supression of EGF-dependent stimulation of mitogen-activated protein kinase (MAPK) (Klinger et al., 2004). However, this blockage of signal transduction occurs on a short time scale. It is, in particular, not clear, if these early inhibitory actions can be directly linked to the growth inhibitory effect of the anti-LeY antibody, which required several days to become manifest (see Fig. 1A). Thus, we asked whether prolonged treatment with IGN311 altered the levels of ErbB receptors. A431 or SK-BR-3 cells, respectively, were treated for 72 h with 100 nM IGN311 (or its murine counterpart ABL364) as well as control antibodies (trastuzumab and 2C225). Cellular lysates were prepared and ErbB receptors were detected by immunoblotting. Incubation in the presence of IGN311 and of ABL364 led to a substantial reduction of ErbB1 and ErbB2 protein levels in both, A431 (Fig. 2A, top) and SK-BR-3 cell lines (Fig. 2B, top), respectively. The anti-EGFR antibody (2C225) was more effective in reducing ErbB1 expression in A431 cells (Fig. 2A, top), but failed to alter the levels of of ErbB2 in SK-BR-3 cells (Fig. 2B, top). In contrast and as expected, trastuzumab caused down-regulation of ErbB2 levels in SK-BR-3 cells (Fig. 2B, top). Noteworthy, the downregulation of ErbB2 induced by IGN311 or by ABL364 was comparable in magnitude to that induced by trastuzumab (Fig. 2B). Finally, the changes observed cannot be attributed to variations in the amount of protein that were loaded in individual lanes, because we detected comparable levels of MAPK in all samples; this determination was done with the lower part of the same gel (Fig. 2A,B, bottom).

IGN311 inhibits growth of A431 xenografts in nude mice.

IGN311 inhibited serum-induced growth of cancer cells and caused downregulation of ErbB-receptors under cell culture conditions. We tested whether both phenomena can be reproduced

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in vivo, which would further indicate a causal relation. When injected subcutaneously into nude mice, SK-BR-3 cells failed to grow and to give rise to measurable tumors. Hence, the *in vivo* experiments were limited to A431 cells. In the first experiment, we injected a tumor cell load of $1*10^6$ A431 cells/nude mouse. The experiment was terminated when the tumor volume was estimated to be larger than 1 mL. In untreated controls, this threshold was reached 25 days after injection. When administered intraperitoneally at 10 mg/kg and every 3rd day, IGN311 (open circle in Fig. 3A) and ABL364 (closed triangle in Fig. 3A) inhibited tumor growth to a similar extent. We verified that the antibodies were also efficacious at a higher tumour cell load by injecting $3*10^6$ A431 cells (Fig. 3B , C). In this case, the latency was shortened and the tumor volume reached the threshold of 1 mL after 16 days in the control animals. Fig. 3B also shows that IGN311 was more effective at the highest dose of 30 mg/kg compared to the lower dose of 10 mg/kg (see also Fig. 3D). In contrast, while ABL364 suppressed growth of the xenograft when applied at 10 mg/kg (open circle in Fig. 3C), growth was actually enhanced at the higher dose of 30 mg/kg and, with some of the animals developing large tumors on day 12; this is not readily evident from Fig. 3C (closed triangle), because all animals were sacrificed on this day and thus not subjected to further evaluation. We observed previously that at high concentrations ABL364 was *per se* capable of inducing MAP kinase stimulation; this effect was thought to result from the fact that ABL364 is an IgG3 isotype; hence, it tends to aggregate and this may support crosslinking and thus activation of growth factor receptors (Klinger et al., 2004; Greenspan and Cooper, 1992). This mechanism may also be responsible for the stimulation of xenograft growth that we observed at 30 mg/kg ABL364.

Estimation of tumor growth by determination of tumor volume may not always be completely accurate, because it assumes that the shape of all tumors are approximated by an ellipsoid body; tumors also may differ with respect to the depth at which they infiltrate the subcutaneous tissue and the height of tumors is notoriously difficult to measure. We therefore

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also assessed the effect of IGN311 on tumor weight as a more objective parameter (Fig. 3D). The data were pooled from four independent experiments; in each of these, at least two different treatment groups were compared to a (vehicle-treated) control group. The number of injected tumor cells varied between $1*10^6$ and $3*10^6$ cells in these experiments. This resulted in variability of tumor weight. To account for this variability, the average control value was set 100% for each individual experiment and the weight of each tumor was related to this reference value. It is evident from Fig. 3D that IGN311 caused a dose-dependent reduction in tumor weight, *i.e.* IGN311 did not cause any appreciable effect at 3 mg/kg, but an intermediate effect and maximal efficacy were seen at 10 mg/kg and 30 mg/kg, respectively.

IGN311 exerts effects that are not dependent on its effector functions.

When tested with human peripheral mononuclear effector cells, IGN311 supports a higher antibody-dependent cellular cytotoxicity (ADCC) than the parental murine antibody ABL364 (Co et al., 1996). The efficacy of ADCC in a given model, however, eventually depend on the degree of fitting between the humanized or murine antibody Fc part with the respective Fcγ receptors on the effector cells of the host. There was no appreciable difference between the growth of the tumors in animals treated with 10 mg/kg ABL364 or with 10 mg/kg IGN311 (Fig. 3D). Furthermore, IGN311 inhibited proliferation of tumor cells under cell culture conditions in the absence of any Fcγ receptor–bearing effector cells or complement source (Fig. 1D) indicating that this effect cannot be explained by ADCC or CDC. If IGN311 exerted its effect, at least in part, in a manner independent on effector functions, i.e. ADCC or CDC, a Fab fragment ought to be also active in the tumor xenograft model. We verified that the Fab of IGN311 was also capable of reducing cell growth *in vitro*. Under conditions comparable to those outlined in Fig. 1, 300 nM were equieffective to 100 nM IGN311 (data not shown). Next we compared the efficacies of the Fab fragment of IGN311 and uncleaved IGN311 *in vivo* by injecting nude mice with $3*10^6$ A431 cells and treating them with either IGN311 (10

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mg/kg) and Fab (20 mg/kg). As shown in Fig. 3D, 20 mg/kg of its Fab exerted a growth inhibitory effect that was similar in magnitude to that elicited by 10 mg/kg IGN311. Similarly, when monitored by tumor volume based on the measured dimensions, the time course of tumor growth was comparable in mice treated with IGN311 or the Fab fragment thereof (data not shown). While this observation does not rule out effector functions as a mechanism of action, it clearly shows that there are effects other than antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity that contribute to the action of IGN311.

Prolonged treatment with trastuzumab predisposes patients to develop heart failure (Slamon et al., 2001); signaling via ErbB2 is also required to maintain normal cardiac performance, for tissue-specific deletion of ErbB2 in the adult mouse heart results in dilated cardiomyopathy (Ozcelik et al., 2002). We did not observe any macroscopic change in the heart and lung that was suggestive of dilated cardiomyopathy. Likewise, the ratio heart and lung weight to body weight was not affected by treatment with IGN311 (data not shown).

IGN311 downregulates ErbB1 receptor expression in vivo.

In vitro, prolonged incubation of A431 and SK-BR-3 cells with IGN311 down-regulated the levels of ErbB1 and ErbB2, respectively (Fig. 2). If the action of IGN311 *in vivo* was also related – in part - to binding of LeY-antigen carrying ErbB-receptors, IGN311 should also downregulate ErbB1 in A431 xenografts. This prediction was verified by comparing the levels of ErbB1 in xenograft tumors excised from control animals and from animals treated with IGN311 or its Fab fragment. Tumor lysates were prepared and receptor expression was determined by immunoblotting. ErbB1 protein levels were markedly reduced in tumors from mice that had been treated with 10 mg/kg and 30 mg/kg IGN311 or with 20 mg/kg of the Fab (Fig. 4, upper blots). The tumors excised from treated and control animals may differ in their cellular composition, in particular the relative proportion of stromal cells. Hence we used

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cytokeratin 8 as a loading control (Fig. 4, bottom blots). This protein is only expressed in cells of epithelial origin. Hence, in the subcutaneous tissue, the xenografted A431 cells can be considered the only candidate source of cytokeratin 8. The levels of cytokeratin 8 can therefore serve as a marker for the proportion of tumor cell proteins in the total lysate. As can be seen in Fig. 4, the level of cytokeratin 8 were reasonably comparable in all samples. Thus we rule out that the differences in ErbB1 levels are due to variation in the amount of tumor cell protein applied to individual lanes.

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DISCUSSION

While growth factor receptors have been proposed as targets for tumour therapy more than two decades ago (Masui et al., 1984), breakthroughs have been only achieved in recent years and the concept has been vindicated by the success of trastuzumab (Herceptin®) (Slamon et al., 2001). We have developed a Lewis-Y specific humanized monoclonal antibody IGN311 which recently has been tested in a Phase I dose escalation study in biopsy-proven Lewis-Y positive carcinoma patients (Oruzio et al., manuscript in preparation). There are several reasons for choosing the LeY-antigen as a target for therapeutic antibodies: Lewis Y has been found to be expressed on the majority of human cancers of epithelial origin (Blaszczyk-Thurin et al., 1987). Expression on normal tissue is limited to epithelial surfaces and activated granulocytes (Dettke et al., 2001). In contrast, LeY was found to be present on up to 70-90% of adenocarcinomas of the lung, breast, colorectal, gastric, pancreatic, prostate and ovarian cancers (Sakamoto et al., 1986; Steplewski et al., 1990; Inagaki et al., 1990; Murata et al., 1992). Furthermore, the levels of Lewis Y expression have been shown to correlate with survival in patients with lung carcinoma (Miyake et al., 1992).

There are several lines of experimental evidence for proof of principle for Lewis Y targeted antibody therapy: Anti-LeY antibodies have been used for the delivery of chemotherapeutic drugs (e.g. doxorubicin, (Wahl et al., 2001)), or used in radioactive form ([^{131}I]-labeled anti-LeY antibodies to enhance the effect of Taxol tumor chemotherapy; (Clarke et al., 2000). The humanized anti-LeY antibody 3S193 was also shown to exert growth inhibitory effects on MCF7 cells xenografted into nude mice (Scott et al., 2000). Antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, have been proposed as the mechanism of action (Co et al., 1996; Klinger et al., 2004). Activation of CDC and ADCC has been demonstrated ex vivo in patients' serum after intravenous infusion of IGN311, and was found to correlate with the kinetics of IGN311 levels in the serum (Oruzio et al., manuscript in preparation). Here we provide evidence for an additional mechanism of action, namely

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blockage of growth factor receptor-dependent signaling. This interpretation is based on the following observations: (i) growth factor receptors, including ErbB1 and ErbB2 are Lewis Y glycosylated, (ii) in vitro, IGN311 caused growth inhibition with a delayed onset; which is inconsistent with cell death due to any residual complement-dependent toxicity. Furthermore, Fab prepared from IGN311 also inhibited cell growth *in vitro* indicating that the Fc-portion was dispensable for growth inhibition under cell culture conditions. (iii) addition of IGN311 (and its murine counterpart) to tumor cells caused loss of ErbB-receptors. This reduction in ErbB1 was also seen in xenograft tumors of recipient mice that had been treated with IGN311 (or alternatively its Fab-fragment). Thus, long term exposure to IGN311 can cause downregulation of LewisY-antigen modified cell surface receptors. Loss of growth factor receptors is likely to contribute to an antiproliferative effect as is the previously documented blockage by IGN311 of growth factor-induced signaling (Co et al., 1996). (iv) Nude mice - while lacking T-cell mediated immunity - still have NK-cells, monocytes and complement. which are candidate effectors by which IGN311 may blunt xenograft growth in nude mice. However, the fact that the Fab fragment of IGN311 also inhibited xenograft growth is difficult to reconcile with ADCC. We therefore conclude that a direct action on growth factor receptors contributes to the antitumor effect of IGN311 *in vivo*.

The precise mechanism by which long term exposure to IGN311 causes down-regulation of ErbB-receptors is not known. However, our earlier work demonstrated that short term exposure of cells to IGN311 (and its murine counterpart ABL364) altered the kinetics of ErbB1-receptor recycling and affected the distribution of the internalized receptors (Klinger et al., 2004). It is likely that these short term-effects, in particular the altered intracellular compartmentalization of the receptors, are causally related also to long term down-regulation. Trastuzumab (Herceptin®) is also thought to elicit its therapeutic effect - at least in part - by downregulating ErbB2 (Baselga et al., 2001); the underlying mechanism still remains a matter of controversy (Austin et al., 2004).

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The concentration of IGN311 at its site of action is not known; it can, however, be roughly estimated based on the following considerations: 10 mg/kg correspond to 0.2 mg/20 g mouse or 1.3 nmol/mouse; the systemic bioavailalibilty of intraperitoneally administered antibody is about 0.3 (Flessner, 2001). The intravascular space in a 20 g mouse is ~1 mL. The half-life of a humanized IgG1 in mice is in the range of 3 to 7 d (Vieira and Rajewski, 1998; Bazin et al., 1994). If, for the sake of simplicity, the half-life of IGN311 is assumed to be equal to the dosing interval (that is 3 days), the steady state plasma concentration is about 0.3 µM. The permeability of the vasculature within the tumor is not known; if it corresponds to that of normal subcutaneous tissue (O'Connor and Bale, 1984), the concentration of IGN311 in the extracellular space is about 10% of that in the plasma, that is ~30 nM and ~100 nM upon repeated administration of 10 and 30 mg/kg IGN311. It is obvious that this is but a rough estimate because of several uncertainties (in particular, the permeability of the capillaries in the tumor). Nevertheless the concentration is within the range, in which the antibody was active in inhibiting cell growth *in vitro*. We note that the effect of 20 mg/kg IGN311 Fab fragments was comparable in magnitude to that of 10 mg/kg IGN311. In other words, on a molar basis the IGN311 was more potent than the Fab fragments thereof. This however does not necessarily argue against our interpretation for the following reasons. First, in vitro, the potency of IGN311 also exceeded that of the Fab fragment. More importantly, the pharmacokinetics of IgG1 and of Fab fragments prepared therefrom have been reported to differ substantially, with considerably longer half-life of IgG1 compared to its Fab fragments (Covell et al., 1986). However, Fab fragments have a larger volume of distribution, i.e. they permeate better into the interstitial fluid and do so more rapidly. Thus, it is not surprising that, on a molar basis, higher amounts of Fab fragments have to be administered to elicit the same effect as that of IGN311. Finally, the quantitative differences seen between whole IGN311 and its Fab when calculated at the molar basis may reflect the contribution of the effector functions, i.e. ADCC and CDC, in the overall antitumor efficacy of IGN311 in this model.

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In conclusion, the presented data indicate that interfering with cell signaling via binding to Lewis Y-glycosylated growth factor receptors present on cancer cells may be one of the mechanisms for the antitumor activity of the Lewis Y-specific humanized monoclonal antibody IGN311 *in vivo*.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. **Growth inhibition of cancer cells by anti-LeY antibodies.** *A & B,* A431 cells (10^5 cells/dish) were plated in 3 cm dishes in the absence (diamonds) and presence of 100 nM IGN311 (triangles in A) or 2C225 (circles in B). After 24, 48 and 72 hours cells were trypsinized and counted. *C,* SK-BR-3 cells (10^5 cells/dish) were seeded into 3 cm dishes in the presence of 1 or 100nM of either IGN311 or herceptin or the combination thereof. An irrelevant IgG was used as a negative control. Data are means from triplicate determinations in a single experiment, which was repeated twice with similar results. *D,* summary of the cell counts obtained after 72 hours of antibody treatment. Results are expressed as % of control in order to correct for interassay differences. Data are means ± S.D. from 3 independent experiments.

Fig. 2. **Downregulation of ErbB receptor expression in A431 and SK-BR-3 cells**. A431 (A) or SK-BR-3 cells (B) were plated into 3 cm culture dishes (10^5 cells/dish) in the absence (control) or presence of the 100 nM trastuzumab (Herceptin), ABL364, IGN311 or 2C225 as indicated. After 72 hours, cellular lysates were prepared as described in “Materials and Methods”. A431 and SK-BR-3 lysated were immunoblotted for ErbB1 (A) and ErbB2 (B) respectively. Equal loading was assessed by blotting for p42/p44 MAPK (bottom).

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Fig. 3**. Inhibition by IGN311 of tumor xenograft growth.** A431 cells (10^6 cells/0.2 mL (A); $3*10^6$ cells/0.2 mL (B, C)) were subcutaneously injected into nude mice. On the next day mice were intraperitoneally injected with 10 mg/kg or 30 mg/kg of IGN311 or of ABL364. Control mice were injected with 100 µl PBS. Injection was repeated every third day. Tumor volume was assessed as mentioned in "Materials and Methods". *D,* summary of tumor weights obtained at the end of the treatment regimens similar to those shown in Panels A-C. Mice were treated with 3, 10 and 30 mg/kg of IGN311, 10 mg/kg of ABL364 or 20 mg/kg of the $F(ab)_2$ fragment of IGN311. Data are from 4 independent experiments, in which one control group was compared to at least two treatment groups that were injected in parallel. In order to correct for interassay differences of tumor weights, the mean tumor weight in each control group was set 100% and the individual treatment groups compared to that reference value. Data are means ± s.e.m., n= 21 in the control and 10 mg/kg IGN311-treated group; n= 9 in the 30 mg/kg IGN311-treated group; n= 6 in the 3 mg/kg IGN311 and 20 mg/kg $F(ab)_2$-treated groups, respectively. The differences between all treatment groups other than 3 mg/kg IGN311 and the control are statistically significant (ANOVA followed by Tukey's test).

Figure 4. Treatment with IGN311 or its $F(ab)_2$ fragment downregulates ErbB1 expression in the tumor xenograft model. Tumors were lysed as described in "Materials and Methods". Lysates were subjected to SDS-PAGE and subsequently immunoblotted against ErbB1. Lanes 1-3 and 10-12 are lysates from control mice, lanes 4-6 are lysates derived from mice treated with 10 mg/kg IGN311. Lanes 7-9 are lysates from mice treated with 30 mg/kg IGN311 and lanes 13-15 are lysates from mice treated with 20 mg/kg of the Fab fragment .Immunoblotting for cytokeratin 8 (bottom) was done as a loading control.

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Figure 1

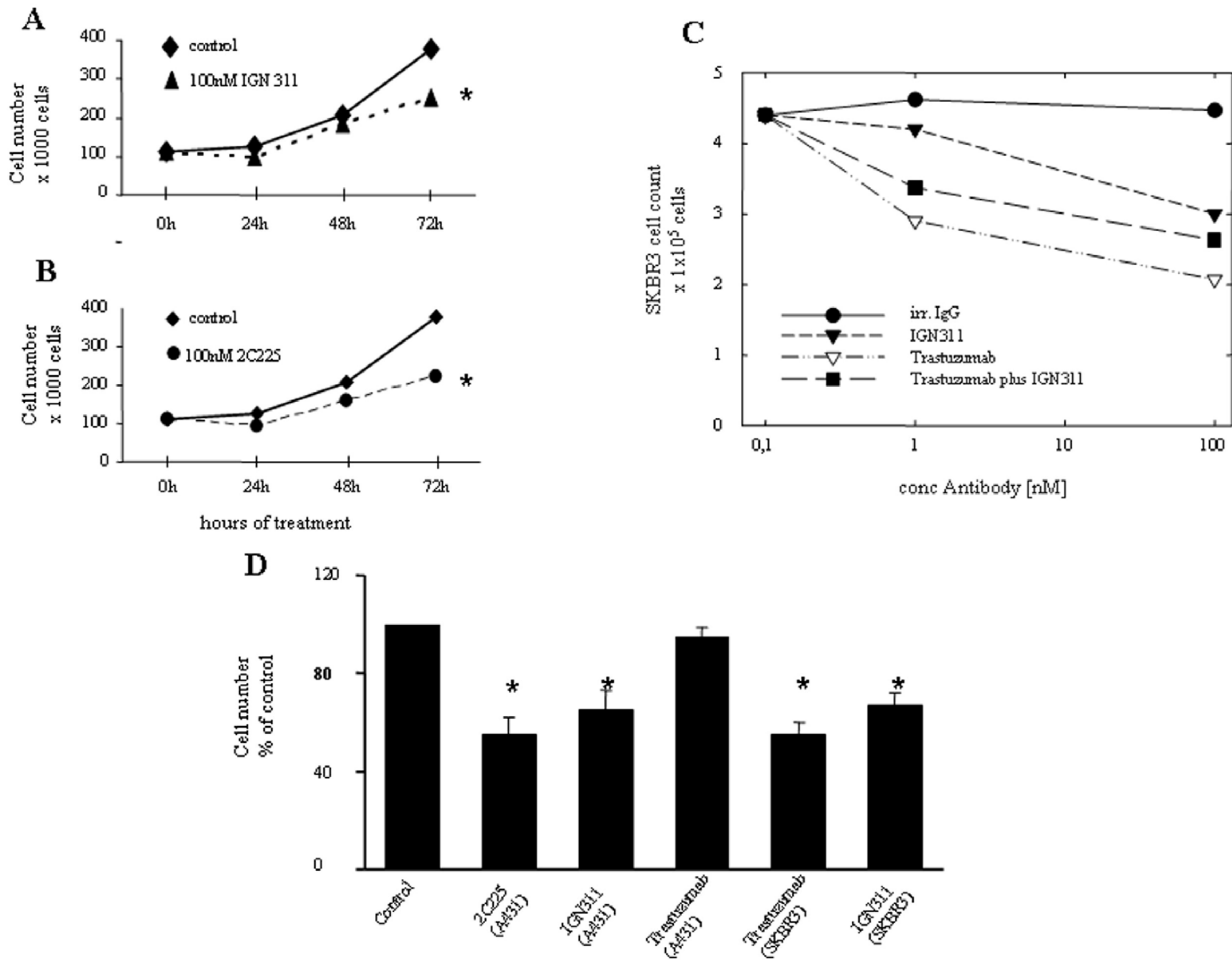

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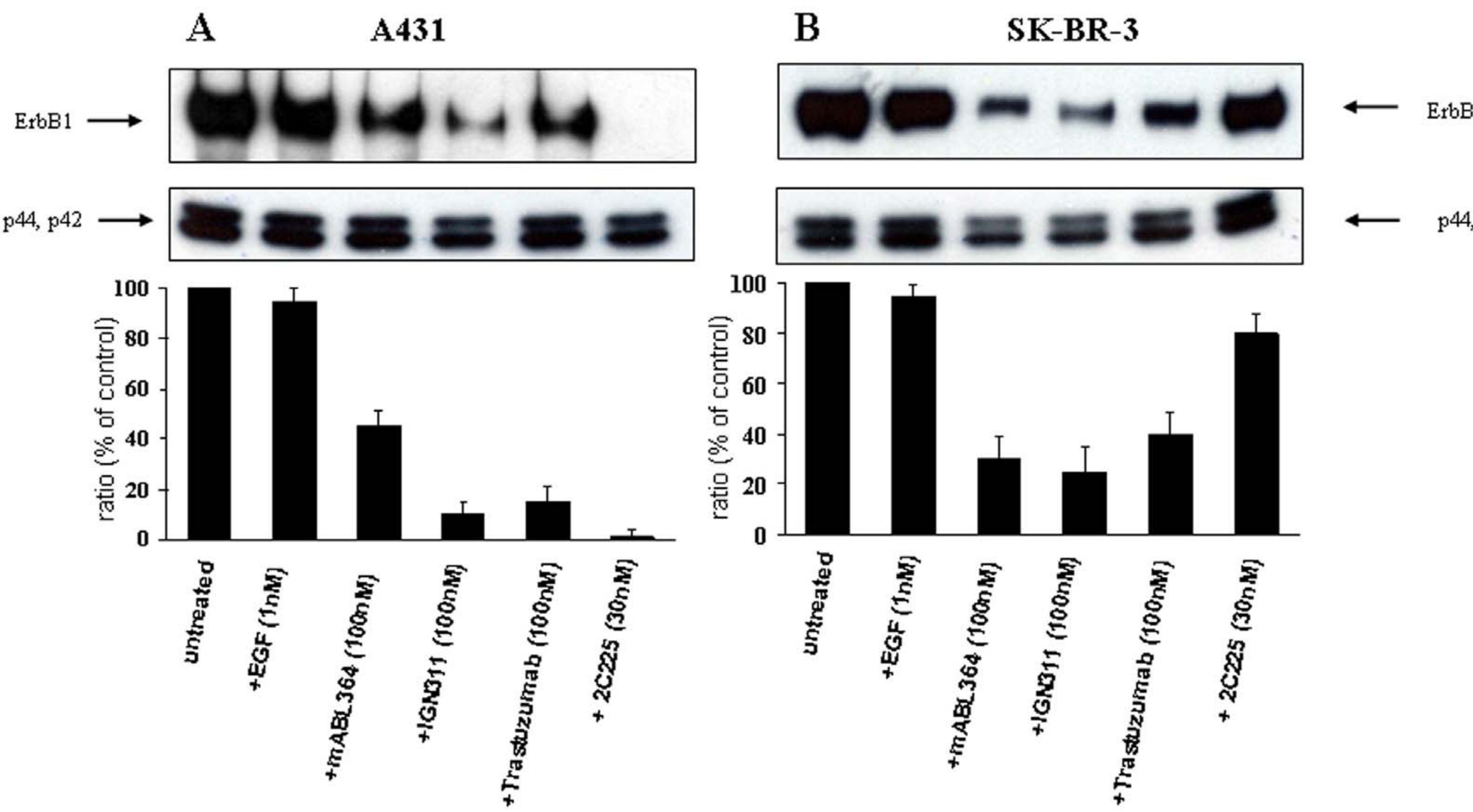
Figure 2



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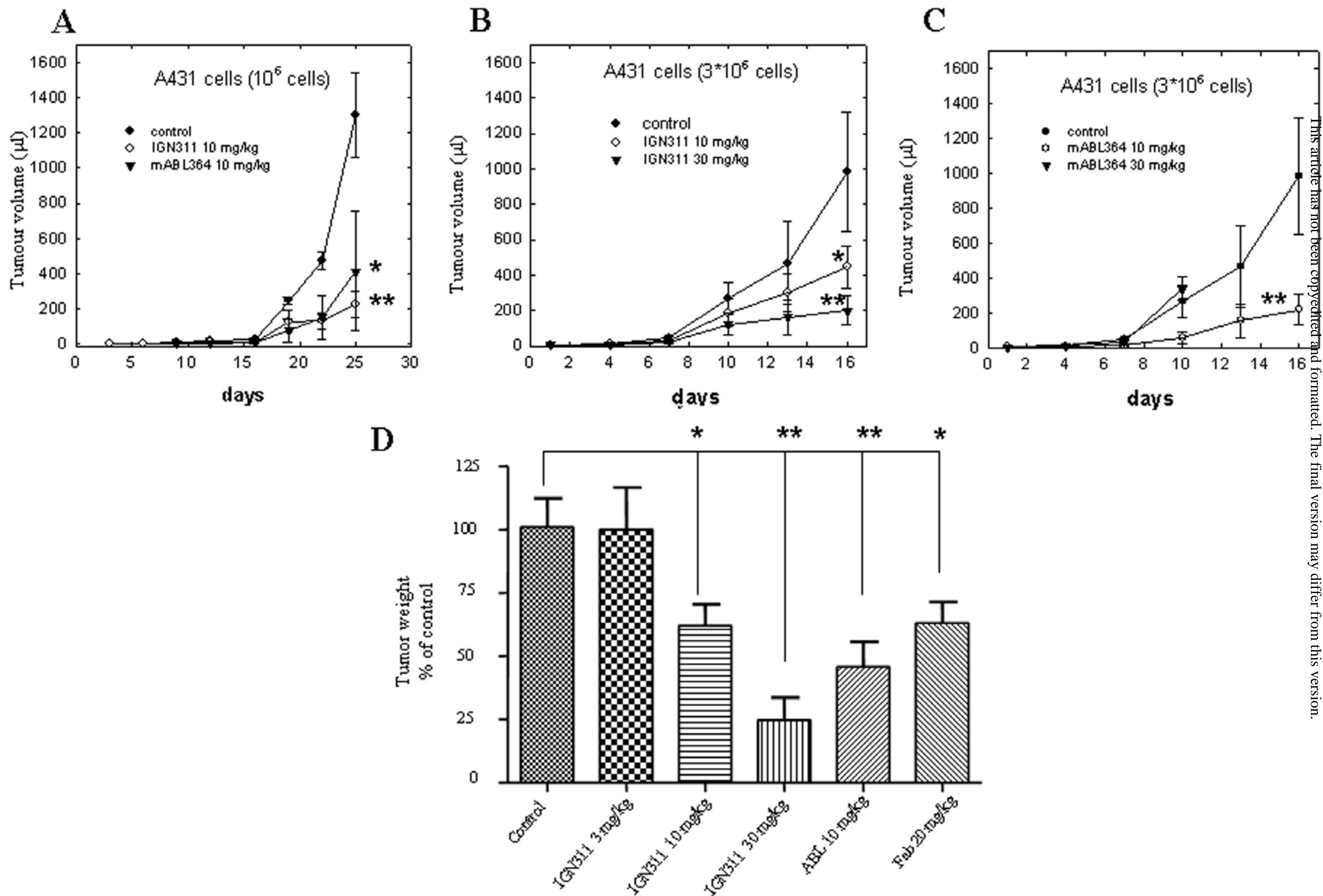

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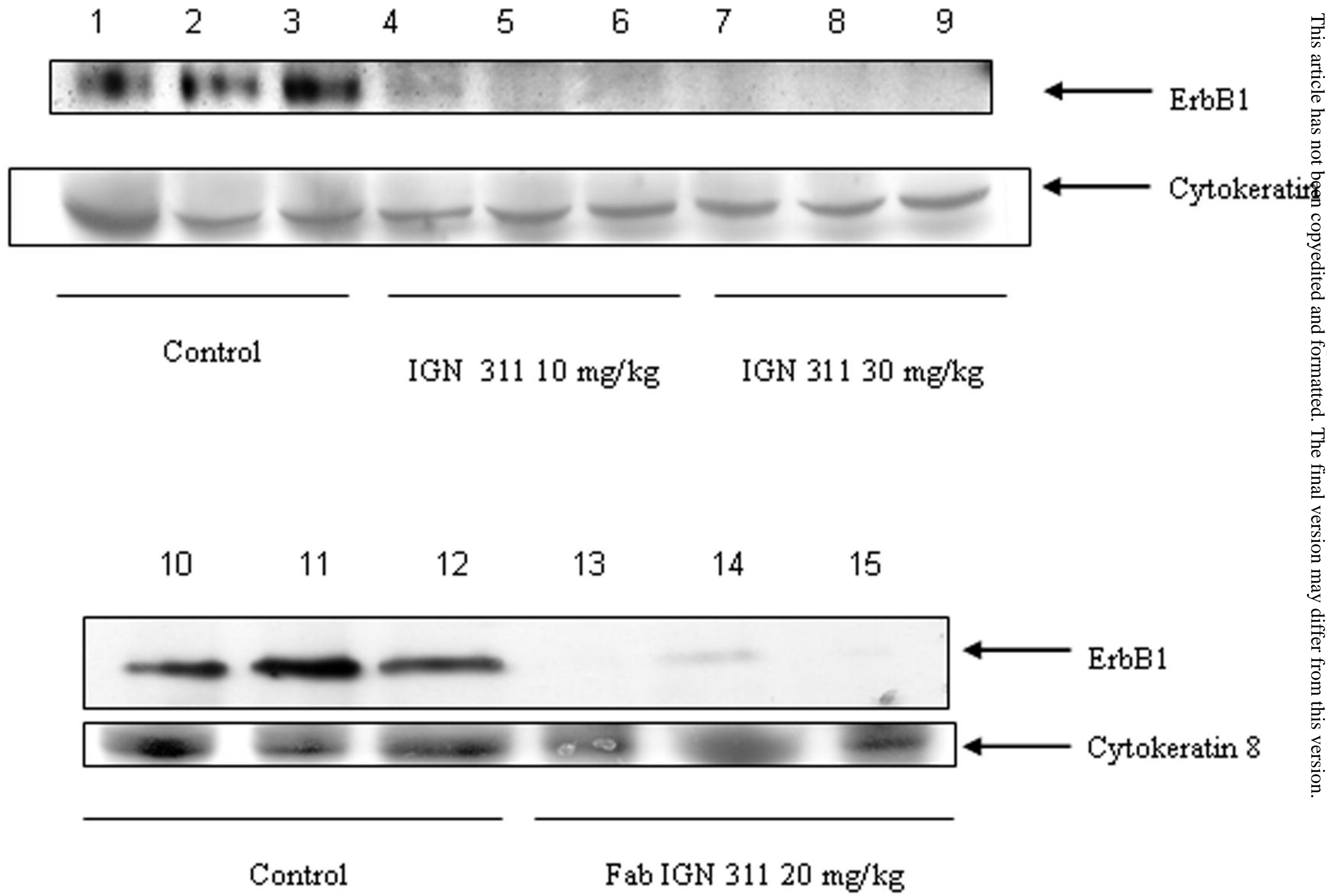
Figure 4



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