Anti-cancer actions of a recombinant antibody (R6313/G2) against the angiotensin II AT1 receptor

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Abstract

Although several tumour types express both AT1 and AT2 angiotensin II receptors, and angiotensin II stimulates cell proliferation, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are not effective anti-cancer agents. Development of a biologically active monoclonal antibody (6313/G2) against the AT1 receptor prompted the testing of a recombinant short-chain variable fragment form (R6313/G2) against breast cancer cells in vitro and in vivo. Cell lines MCF-7, MDA-MB-231 and T47D all expressed both receptor subtypes. In vitro, R6313/G2 suppressed cell proliferation in the presence of 100 nM angiotensin II, with IC50s of 30 nM, 153 nM and 2.8 μM for the three cell types respectively; in contrast, the AT1 receptor blocker losartan was effective only in T47D cells, at 25 μM. Studies on MCF-7 and T47D cells showed R6313/G2 also opposed the angiotensin II-induced inhibition of caspase-3/7 activity. In vivo, hollow fibres containing the cell lines were implanted in nu/nu balb-c mice at two sites, s.c. and i.p. Treatments of R6313/G2 at 2.5 nmol/kg and 25 nmol/kg twice per day for 7 days dose dependently reduced cell numbers for all three cell lines, but here MCF-7 cells responded most sensitively and MDA-MB-231 cells least. Although T47D cells were refractory at the s.c. site, growth was inhibited at the i.p. location, and otherwise results were similar at the two sites. In xenografts, MCF-7 cell tumours were dose dependently reduced by R6313/G2, and 13 and 27 nmol/kg R6313/G2 twice/day gave means of 74 and 76% tumour regression after 7 days. The data suggest that the anti-cancer action of R6313/G2 is considerably more effective than AT1 antagonists.

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Introduction

Angiotensin II is the primary effector of the renin–angiotensin system (RAS), and in the circulation it regulates blood pressure, salt and water homeostasis and the secretion of aldosterone (Mulrow 1999, Kaschina & Unger 2003). Its actions are more widespread than this, however, reflecting the existence of tissue RAS that are located in close proximity to target tissues. The breast appears to contain such a system (Tahmasebi et al. 1998, 2006) that probably directly supplies angiotensin II to the epithelium lining the secretory ducts, which is rich in angiotensin receptors (Inwang et al. 1997, Tahmasebi et al. 1998, De Paepe et al. 2001).

Among its many functions, angiotensin II can stimulate both cell proliferation and apoptosis, acting through its two receptor subtypes, designated AT1 and AT2 (Stoll & Unger 2001, de Gasparo 2002, Weidekamm et al. 2002).

It may be that angiotensin II is involved in both of these processes during the normal breast cycle, in which the ductal epithelium undergoes periodic proliferation during puberty, pregnancy and lactation, and then apoptotic involution when lactation ceases (Wiseman & Werb 2002, Boutinaud et al. 2004, Green & Streuli 2004, Vinson et al. 2007).

Both receptors are also present in breast tumours (Inwang et al. 1997, Tahmasebi et al. 1998, De Paepe et al. 2001).
et al. 2001), and expression varies with tumour stage (De Paepe et al. 2001, 2002). The actions of angiotensin II on breast cancer cells through AT1-mediated signalling pathways include stimulation of cell proliferation (Greco et al. 2002a,b, 2003), and in addition, angiotensin II inhibits MCF-7 cell binding to, and migrating through, extracellular matrix proteins, including collagen and laminin (Puddefoot et al. 2006).

Because of these clear implications for cancer, studies have been carried out in animal cancer models, and it has been shown that angiotensin II blockers and angiotensin-converting enzyme (ACE) inhibitors can reduce tumour size, angiogenesis and metastasis (Fujita et al. 2002, 2005, Uemura et al. 2003, 2005). Despite this, the promise that long-term use of ACE inhibitors or angiotensin II blockers may limit the development of cancer in humans (Lever et al. 1998) has not been fulfilled (Meier et al. 2000, Li et al. 2003, Gonzalez-Perez et al. 2004, Ronquist et al. 2004, Fryzek et al. 2006).

Though the interaction of the N-terminal domain of G-protein coupled receptors (GPCR) with ligand has long been known for the luteinizing hormone receptor (Puett et al. 2005), a growing body of evidence now suggests that the N-terminus in other GPCR may undergo activation-dependent conformational changes and influence receptor activity (Lecat et al. 2002, Decaillot et al. 2003, Gupta et al. 2007). However, relatively little is known about the N-terminus-mediated signalling pathways.

We previously showed that a monoclonal antibody (6313/G2) to a conserved sequence in the extracellular domain of the AT1 receptor stimulates inositol trisphosphate and aldosterone production, while non-competitively inhibiting angiotensin II-induced protein kinase C (PKC) activation. At that time these properties were attributed to the antibody-blocking receptor internalisation (Barker et al. 1993, Kapas et al. 1994, Vinson et al. 1994). Such discrimination between different intracellular signalling pathways is an unusual consequence of binding to receptors, and offers the possibility of alternative ways of modulating the AT1 receptor response. Accordingly, a short-chain variable fragment (ScFv) form of the antibody has been developed, and this paper describes its actions on three breast cancer cell lines that express AT1 receptors, namely T47D, MCF-7 and MDA-MB-231 in vitro and in vivo. Since a wide variety of cancers express AT1 receptors or are susceptible to AT1 receptor blockade (Volpert et al. 1996, Hii et al. 1998, Fujimoto et al. 2001, Miyajima et al. 2001, 2002, Takeda & Kondo 2001, Fujita et al. 2002, Yoshiji et al. 2002, Egami et al. 2003), the findings may have significance for cancer in general.

Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma (Sigma–Aldrich). Rabbit polyclonal antibodies for AT1 receptor (sc-1173) and AT2 receptor (sc-9040) were purchased from Santa Cruz Biotechnology (Insight Biotechnology Ltd, Wembley, UK).

Animals

All mice used in this study were 6- to 8-week-old female balb/c (nu/nu) athymic mice (B&K Universal Ltd, Grimston, UK). Mice were housed in box ventilated racks, with Hepa filters in an air-conditioned room with alternating cycles of light and dark, and allowed to access standard laboratory food and water ab libitum. For blood pressure experiments, female Wistar rats were used whose body weights were ~220 g.

All animal experimentation was performed according to UK Home Office regulations and the UK Coordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (UKCCCR Guidelines, 1998).

Generation of recombinant ScFv from 6313/G2 hybridoma RNA

The 6313/G2 mouse monoclonal antibody hybridoma was grown as previously described (Barker et al. 1993). A pool of cDNA derived from mRNA was used to obtain heavy and light chains by PCR. A linker fragment encoding (Gly4Ser)3 was used to assemble the ScFv library of inserts, and a phage display library was created by directionally cloning these inserts into the pCANTAB 5 E phagemid vector (Amersham Pharmacia). An E-tag for the expressed sequence GAPVPYPDPLEPR was included in this vector and used in subsequent panning and purification steps. The phagemid library was then used to transform TG1 Escherichia coli, and phagemid rescue was carried out using the M13KO7 helper phage followed by several rounds of panning. Positive expressing clones were identified by ELISA using 96-well plates coated with the original antigenic peptide (EDGIKRIQDD) and an anti-E-tag antibody (Amersham Pharmacia) detected using an HRP-linked secondary antibody. One particular clone was taken forward for expression and purification as well as functional assessment on the basis of giving the highest signal in the antigen ELISA.

The 6313/G2 ScFv (R6313/G2, clone 12D) was purified using HiTrap E-tag columns (Amersham
Pharmacia) followed by purification using a Protein L column (BD Clontech), which binds immunoglobulins, including ScFv. For *in vitro* and *in vivo* experiments, it was necessary to carry out medium-scale purification followed by overnight dialysis against PBS and concentration using 30 kDa cut-off concentrators (Millipore, Watford, UK). The final antibody stock was routinely reconstituted on PBS at a concentration of 10 mg/ml.

**Cell culture procedures**

MCF-7, T47D and MDA-MB-231 breast cancer cells were obtained from The American Tissue Culture Collection (LGC Promocem, Teddington, UK). Rat aortic smooth muscle cells (RASMC) were developed from primary culture (Barker et al. 1996). MCF-7 cells were maintained in minimal essential medium (MEM), T47D and MDA-MB-231 cells in RPMI 1640 medium, and RASMC were maintained in Dulbecco’s modified Eagle’s medium (DMEM).

All media were supplemented with 2 mM 1-glutamine, 10% fetal bovine serum (FBS), 50 U/ml penicillin and 0.05 mg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere (95% oxygen, 5% CO₂).

**Cell viability assay**

Confluent cell monolayers were removed from tissue culture flasks using trypsin/EDTA. Cells (15 × 10⁵ per well) were seeded into 96-well tissue culture plates containing the appropriate medium for each cell line. After 24 h, cells were treated with angiotensin II (100 nM) and the recombinant monoclonal antibody (R6313/G2) at a range of concentrations from 0.005 to 25 μM, or with losartan at a similar concentration range, and incubated for a further 48 h. Cell viability was assessed by the ability of metabolically active cells to reduce 2, 3-bis[2-Methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) to the coloured formazan product. Absorbance was measured using a Multiskan Ascent microplate reader (Thermo Labsystem, Helsinki, Finland) at a wavelength of 450 nm and a reference wavelength of 630 nm. Each measurement was performed in triplicate. IC50s were calculated using a non-linear regression formula using GraphPad Prism v4.0 software (GraphPad Software Inc., San Diego CA, USA).

**Protein extraction and western blotting**

Cells were grown in the presence or absence of angiotensin II (100 nM) for 24 h then washed thrice in sterile PBS (pH 7.4), incubated for 5 min in lysis buffer (PBS (pH 7.4), 1% Nonidet P-40/Triton X-100, 0.1% SDS and 0.5% sodium deoxycholate, with the protease inhibitors leupeptin 10 μg/ml, aproatin 30 μg/ml and 0.1 mM phenylmethylsulphonylfluoride) and harvested. Cell lysates were homogenised using an ultrasonicator (2 × 5 s cycles; Bandelin Sonoplus, SLS, Hessle UK). After homogenisation, the samples were centrifuged at 20000 g for 10 min at 4°C. The supernatants were removed and stored at −80°C. Protein concentrations were estimated using the Bio-Rad protein assay (Bio-Rad Laboratories). For western blotting, samples containing 50 μg total cell lysates were loaded on to a 10% SDS-polyacrylamide gel and subjected to electrophoresis. Proteins were transferred to Hybond-C membranes (Amersham Biosciences Ltd) in transfer buffer (39 mM glycine, 48 mM Tris-Base, 20% methanol and 0.037% SDS), using a transBlot transfer apparatus (Bio-Rad Laboratories) at 120 mA for 1.5 h at 4°C. Membranes were washed and then incubated in blocking buffer (1 × Tris-buffered saline (TBS), 0.1% Tween 20 and 5% dried milk) for 1 h at room temperature and subsequently washed thrice for 10 min in washing buffer (1 × TBS and 0.1% Tween 20). The membranes were incubated with polyclonal rabbit anti-AT1 or anti-AT2 receptor antibodies at a dilution of 1:2000 in blocking buffer. After overnight incubation at 4°C, the membranes were washed as described above and incubated with anti-rabbit IgG secondary antibody (Amersham Biosciences; 1:2000) for 1 h at room temperature. Additional washes were carried out and immune detection was performed by incubating the membranes for 1 min in ECL western blotting detection reagent (Amersham Biosciences) and exposed to a Biomax chemiluminescence detection film (Kodak).

**Apoptosis – caspase-3/7 activity**

Activation of caspases during apoptosis was determined using the Apo-ONE homogenous caspase-3/7 assay (Promega Corp.), according to the manufacturer’s instructions. Briefly, cells were grown to 90% confluence and washed thrice with sterile PBS. Cells were harvested using trypsin/EDTA and counted. Cells (10⁴ per well) were seeded into a 96-well plate and incubated with R6313/G2 at concentrations from 0.1 to 3 μM in the presence or absence of 100 nM angiotensin II, total volume 150 μl, for 24 and 48 h. After incubation, caspase-3/7 Z-DEVD-R110 substrate (100 μl) was added to each well. Blank wells contained reagent alone, and controls omitted the antibody and/or angiotensin II. Fluorescence was measured every 2 h over an 8-h period using a Fluostar Optima.
spectrofluorimeter (BMG Laboratories, Offenburg Germany), with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

**Hollow fibre assay**

Hollow fibre procedures followed the method of Hollingshead et al. (1995).

**Preparation of hollow fibres**

Polyvinylidene difluoride (PVDF) hollow fibres (500 kDa cut-off, 1 mm inner diameter; Spectrum Europe BV, Breda, Netherlands) were flushed through with 70% ethanol using a blunt 21 gauge needle and 10 ml syringe. Fibres were then immersed in 70% ethanol for 72 h, flushed through again with 70% ethanol, then distilled water and then autoclaved at 131 °C. Finally, before loading cell suspensions, fibres were flushed through with RPMI 1640 culture medium. Cells (MCF-7, T47D and MDA-MB-231) were introduced into fibres at densities 2.5–3.0 × 10^6 cells/ml. Fibres were then heat sealed at 2 cm intervals and placed in Petri dishes containing 3 ml of the cell appropriate medium.

To test the efficacy of the method before use in vivo, cell-loaded fibres were incubated in vitro for 48 h in the presence or absence of antibody at a range of concentrations from 0.33 to 33 µM and angiotensin II (100 nM).

**In vivo hollow fibre assay**

Cell-loaded hollow fibre segments were incubated at 37 °C in culture medium overnight before implantation into pure strain 5- to 6-week old female balb/c nu/nu mice. Fibres were implanted into the animals under anaesthesia (2% Isofluorane). Three fibres (2 cm), each containing one of the cell lines MCF-7, T47D or MDA-MB-231, were implanted at both s.c. and i.p. sites into each animal. For i.p. implants, a small incision was made through the skin and musculature of the ventral abdominal wall. Fibres were placed into the peritoneal cavity and both incisions were closed with metallic suture clips (Harvard Instruments, Edenbridge, UK). For s.c. implants, a small incision was made dorsally. The fibres were implanted to the left of the dorsal support, thereafter mice received weekly s.c. injections of 17B-oestradiol valerate (0.1 mg/kg body weight), in sesame oil (Kasukabe et al. 2005) for a further 8 weeks. Animals were monitored daily for general health, and body weights were measured twice weekly. Tumour size was measured thrice per week with slide callipers, and volumes were calculated as (L × W^2)/2, where L and W are the major and minor diameters respectively. Once tumour volumes reached 150–200 mm^3, mice were randomised to treatment and control groups of 8–10 per group. Mice were treated by the s.c. injections of R6313/G2 in sterile PBS (0.1 ml) at doses of 0.4 mg/kg (13 nmol/kg), 0.8 mg/kg (27 nmol/kg) and 1.36 mg/kg (45.3 nmol/kg) body weight, twice per day for 7 days. Control mice received sterile PBS. At the termination of the study, animals were killed by cervical dislocation. Relative body weights (%) were calculated as (W/W_i) × 100, where W is the body weight at any given time and W_i is the body weight at treatment initiation. Net tumour volume was calculated as V_i − V_t, where V_t is the tumour

**Antibody treatment**

Mice (n = 5/group) with hollow fibre implants were treated with R6313/G2 (0.07 mg/kg (2.5 nmol/kg) and 0.7 mg/kg (25 nmol/kg) in 0.1 ml PBS, subcutaneously) twice per day for 6 days. Control animals (n = 5) received vehicle alone. After 24 h of the last injection, animals were killed by cervical dislocation and the fibres were recovered and transferred to pre-warmed RPMI 1640 medium containing 20% FBS for 30 min.

**Assessment of tumour cell growth within hollow fibres**

Cell viability was measured using a modified methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Fibres were incubated in RPMI 1640 with 20% FBS containing 1 mg/ml MTT, and incubated at 37 °C under 95% O_2, 5% CO_2 for 4 h. The reagent was aspirated and 2 ml sterile filtered 2.5% protamine sulphate (0.9 g NaCl, 2.5 g protamine sulphate in 100 ml water) was added. Specimens were stored at 4 °C for a minimum of 24 h in the dark to fix the formazan product. Fresh protamine sulphate (2.5%) was added and fibres were stored for a further 2–4 h at 4 °C. Each fibre was transferred to a well in a 24-well plate, cut in half and air dried overnight, protected from light. Dimethyl sulphoxide (DMSO: 300 µl) was added to each well and the formazan product extracted. An aliquot (190 µl) from each well was transferred to a 96-well plate and absorbance read at 540 nm using a Multiskan Ascent photometric microplate reader (Thermo labsystem, Staines, UK). Treated values were calculated as a percentage of the controls.

**In vivo xenograft assay**

Mice were injected s.c. on the right flank, with 150 µl sterile PBS containing 7.5 × 10^6 MCF-7 cells. Tumour cells were allowed to grow for 4 weeks without hormone support, thereafter mice received weekly s.c. injections of 17β-oestradiol valerate (0.1 mg/kg body weight), in sesame oil (Kasukabe et al. 2005) for a further 8 weeks. Animals were monitored daily for general health, and body weights were measured twice weekly. Tumour size was measured thrice per week with slide callipers, and volumes were calculated as (L × W^2)/2, where L and W are the major and minor diameters respectively. Once tumour volumes reached 150–200 mm^3, mice were randomised to treatment and control groups of 8–10 per group. Mice were treated by the s.c. injections of R6313/G2 in sterile PBS (0.1 ml) at doses of 0.4 mg/kg (13 nmol/kg), 0.8 mg/kg (27 nmol/kg) and 1.36 mg/kg (45.3 nmol/kg) body weight, twice per day for 7 days. Control mice received sterile PBS. At the termination of the study, animals were killed by cervical dislocation. Relative body weights (%) were calculated as (W/W_i) × 100, where W is the body weight at any given time and W_i is the body weight at treatment initiation. Net tumour volume was calculated as V_i − V_t, where V_t is the tumour
volume at any given time and \( V_i \) is the tumour volume at the start of treatment, and expressed as a percentage of \( V_i \).

**Rat blood pressure**

Rats were chosen for this part of the study in view of their greater tractability in blood pressure assays. Female Wistar rats were first acclimatised to handle the blood pressure equipment for 4–5 days before experimentation. Blood pressure in the conscious animals was determined using a Kent Scientific Corporation (Torrington, CT, USA) Coda 6+ tail cuff system in which the animals were held in warmed restrainers while blood pressures were assessed. Animals were first stabilised and their basal blood pressure taken before treatment. They were then briefly removed from the restrainers for the s.c. injection of R6313/G2, 0.4 mg/kg in sterile PBS (0.1 ml). Controls received PBS alone. Blood pressures were then taken at intervals over a period of 1 h, before restoring to cages. The procedures were repeated daily for 3 days.

**Statistical analysis**

All data were presented as means \( \pm \) S.E.M. Statistical analysis was performed using one-way ANOVA. In the case of a significant result in the ANOVA, Student’s \( t \)-test was used for the dose-response curves. A \( P \) value < 0.05 was considered statistically significant.

**Results**

**Angiotensin II receptor subtypes in breast cancer cells**

Immunoblotting analysis of AT1 and AT2 receptor expression was used in breast cancer cell lines in order to select potential targets for R6313/G2 actions. Anti-AT1 and anti-AT2 receptor antibodies specifically detected immunoreactive bands of 43 kDa and \( \sim 50 \) kDa proteins respectively, consistent with previous studies (Nouet et al. 2004, Thomas et al. 2004). The three breast cancer cell lines studied (MCF-7, T47D and MDA-MB-231) and RASMC cells all expressed the AT1 receptor (Fig. 1A). The breast cancer cells also expressed the AT2 receptor, though only at a low level in MDA-MB-231 cells, but RASMC did not (Fig. 1B).

**Differential sensitivity of breast cancer cells to R6313/G2**

The effects of R6313/G2 on cell proliferation and survival in the presence of angiotensin II are shown in Fig. 2. The concentrations of R6313/G2 which reduced cell survival by 50% (IC50s) were determined from cell survival curves (Fig. 2). The IC50 values for the 48-h incubation period were 30 nM, 153 nM and 2.8 \( \mu \)M for MCF-7, MDA-MB-231 and T47D cells respectively. No inhibition was observed in any of the cells at the lower concentrations of antibody (<12.5 nM), whereas concentrations of R6313/G2 above 5 \( \mu \)M reduced the number of surviving cells to almost zero (\( P < 0.001 \)) in each case (Fig. 2). In marked contrast, losartan had no inhibitory effect on cell growth at any concentration in MCF7 and MDA-MB-231 cells, and only at 25 \( \mu \)M in T47D cells.

**Caspase-3/7 activation by R6313/G2 in breast cancer cells**

Caspase-3/7 activation was assayed 12 and 48 h after R6313/G2 treatment in T47D cells and after 48 h in MCF-7 cells, in the presence or absence of 100 nM angiotensin II. Angiotensin II added alone significantly reduced caspase-3/7 activity in both cell types, at 12 h in T47D and after 48 h in MCF-7 cells (Fig. 3A–C). In T47D cells, R6313/G2 alone had no effect at 12 h, but produced a dose-dependent activation after 48 h. However, in the presence of angiotensin II, R6313/G2 significantly increased caspase-3/7 activity at both time points, compared with angiotensin II alone. In MCF-7 cells, R6313/G2 alone reduced caspase activity after 48 h, but it produced a dose-dependent activation of caspase-3/7 in the presence of angiotensin II.
Responses of breast cancer cells in hollow fibres

in vitro

The hollow fibre system was first tested in vitro to ensure compatibility of the fibres with basal cell viability and the access of test substances. The data in Fig. 4A show that increasing concentrations of R6313/G2 decreased the number of viable cells in a dose-dependent manner for all the three cell lines used, although in this case MDA-MB-231 cells were less sensitive than T47D and MCF-7 cells. Comparison with Fig. 2 shows that the hollow fibre results for this cell line (MDA-MB-231) seemingly gave reduced sensitivity to R6313/G2. However, it is apparent that all of the cells survived well under basal conditions in the fibres, and that R6313/G2 adequately penetrated the hollow fibre micropores.

Effects of R6313/G2 on breast carcinoma cells

in hollow fibres

in vivo

The pattern of responsiveness of breast cancer cells in hollow fibres in vivo to R6313/G2 administration was impressively similar to that seen in vitro, and dose-related inhibition of cell growth was clear. Though T47D cells implanted in the i.p. site responded, whereas those at the s.c. site did not, the relative sensitivity of the other cell lines was similar. In each case, MCF-7 cells responded the most sensitively, whereas MDA-MB-231 cells only responded at the highest dose (Fig. 4B and C). Body weights, as an index of toxicity, showed no change in these groups (Fig. 4D), and there was no mortality.
Confirmation of the hollow fibre cell data was sought in xenograft experiments, and because MCF-7 cells responded most sensitively in the hollow fibre assays, this cell line was chosen for further studies in xenografts, using a standard s.c. preparation. R6313/G2 reduced tumour growth significantly in groups treated with 0.4 and 0.8 mg/kg (13 and 27 nmol/kg) twice per day, giving 74 and 76% tumour regression compared with the untreated controls (Figs 6A, B and 7). Although as judged by body weight, R6313/G2 did not cause loss in body weight in the xenograft group treated with 0.4 mg/ml or 0.8 mg/kg (13 and 27 nmol/kg) twice per day, some animals treated with 0.8 mg/kg died during treatment, and those treated with 1.36 mg/kg (46 nmol/kg) showed significant weight loss and none survived beyond 8 days (Fig. 5).

**Effect of R6313/G2 on blood pressure**

Blood pressure data through the 3 days of treatment are illustrated in Fig. 8. It will be noted that removal from the restrainers and s.c. injection caused a transient increase in systolic blood pressure in the control animals (despite previous habituation), though this was only partially reflected in diastolic pressures. However, such a response to moderate stress was not observed in the R6313/G2-treated animals. Outside the immediate period of moderate stress, there were no consistent differences between R6313/G2-treated animals and controls.

**Discussion**

The development and application of the highly specific AT1 receptor monoclonal antibody designated 6313/G2 (Barker et al. 1993), which recognises a sequence in the extracellular N-terminal domain, extended knowledge of AT1 receptor distribution in normal and primary human breast cancer (Inwang et al. 1997, Tahmasebi et al. 1998, De Paepe et al. 2001). Although raised against a region of the receptor that is respectively (Figs 6A, B and 7). Although as judged by body weight, R6313/G2 did not cause loss in body weight in the xenograft group treated with 0.4 mg/ml or 0.8 mg/kg (13 and 27 nmol/kg) twice per day, some animals treated with 0.8 mg/kg died during treatment, and those treated with 1.36 mg/kg (46 nmol/kg) showed significant weight loss and none survived beyond 8 days (Fig. 5).
apparently involved in neither hormone binding nor signal transduction, 6313/G2 was also shown to have a distinct biological activity that replicated the action of angiotensin II on aldosterone production by rat adrenal glomerulosa cells, but blocked angiotensin II-stimulated PKC activity (Kapas et al. 1994, Vinson et al. 1994). It has recently become clear that the extracellular domains of GPCR generally may be more involved in cell signalling than previously thought (Lecat et al. 2002, Decaillot et al. 2003, Gupta et al. 2007).

Angiotensin II, which may be provided to breast duct epithelia by a local tissue RAS (Tahmasebi et al. 1998, 2006), potentially has both damaging and beneficial actions in breast cancer. On one hand it stimulates cell proliferation (De Paepe et al. 2001, Greco et al. 2002a, b, 2003), but on the other hand it inhibits cell adhesion and migration through membranes coated with extracellular matrix proteins, processes that are associated with metastasis (Puddefoot et al. 2006). Because of this duality, conventional AT1 receptor blockers, such as losartan, may be expected to inhibit both the beneficial as well as the damaging aspects of angiotensin II actions. For this reason, it seemed appropriate to develop and test the actions of a 6313/G2-derived recombinant ScFv that binds to the same region of the receptor as the parent antibody, and which could be expected to have actions of interest. Accordingly, the first studies reported here were designed to confirm that the cancer cell lines chosen for study contain angiotensin receptors. Indeed, the three cell lines MCF-7, T47D and MDA MB-231 all expressed AT1 receptors (Fig. 1) consistent with previous results (Inwang et al. 1997, Muscella et al. 2002). All three also expressed AT2 receptor, albeit only in relatively low abundance in MDA MB-231 cells. The T47D and MCF-7 data are consistent with some previous findings (Inwang et al. 1997, Muscella et al. 2002) but not all. De Paepe et al. (2001) reported the absence of AT2 receptor from T47D cells.

Initial functional tests were carried out in vitro. Because it seems likely that angiotensin II promotes apoptosis through the AT2 receptor (de Gasparo et al. 2000, Berry et al. 2001, Stoll & Unger 2001), opposed to its proliferative actions via the AT1 receptor, it was postulated that specific blockade of the AT1 receptor in the presence of angiotensin II should lead to decreased proliferation and increased apoptosis, already shown for losartan in cancer cells, cf. Rivera et al. (2001) and Suganuma et al. (2005). This essentially proved to be the case (Figs 2–4) and R6313/G2 effectively blocked both proliferation and enhanced apoptosis. However, direct comparison with losartan using cells shows that 6313/G2 is significantly more potent in all three cell lines (Fig. 2). The importance of the presence of angiotensin II is shown in Fig. 3. Although alone R6313/G2 apparently reduced caspase activity in MCF7 cells, it reversed the inhibition of caspase-3/7 activity evoked by angiotensin II in both T47D and MCF7 cell types (Fig. 3). It should be noted that MCF-7 cells specifically lack caspase-3 (cf. e.g. Kivinen et al. 2005, Prunet et al. 2005), and this may account for the difference in magnitude of the effects of R6313/G2 on caspase-3/7 in T47D and MCF-7 cells.

It is likely that the responses are not attributable solely to the balance between AT1 and AT2 receptors as previously suggested (Rivera et al. 2001), because the observed IC50s (Fig. 2) show that, under these conditions, MDA-MB-231 cells with relatively low AT2 responded more sensitively to R6313/G2 than the T47D cells that express both subtypes similarly. However, different conditions can produce somewhat different results, cf Figs 2 and 4A. In these two data sets, the relative sensitivities of MDA-MB-231 and T47D cells were reversed, and it is possible that in Fig. 4A, in which the cells were contained within the hollow fibres, there were differences in the cell responses to the materials used. In any case, it also seems possible that the anti-proliferative, pro-apoptotic effects in Figs 2–4 are affected by cell context, beyond the presence of the AT1 receptor, and the relationship between AT1 receptor signalling and other hormones, especially growth factor signalling pathways and apoptotic events may play a crucial role in cancer (Greco et al. 2003, Arrieta et al. 2005, Yang et al. 2005). It has also been shown in vascular tissue that apoptosis may also be affected through AT1 receptors (Diep et al. 1999, Ohashi et al. 2004).

Recent studies have shown that AT1 receptor antagonists may have anti-tumour effects in various malignant tumour tissues in vivo. Specifically, candesartan inhibited ovarian tumour growth in mice by ~50% at doses of 10–100 mg/kg per day (~15–150 μmol/kg per day) and losartan inhibited glioma tumour cells in vivo by 39–79% at doses of 40–80 mg/kg per day (~100 μmol to 1 mmol/kg per day; Rivera et al. 2001, Suganuma et al. 2005). These doses are extremely high compared with the more usual doses used for adult hypertensive patients of perhaps 0.2 mg/kg per day (~0.3 μmol/kg per day) for candesartan, and 0.3–1.4 mg/kg per day (up to 3 μmol/kg per day) for losartan, but generally consistent with the relative ineffectiveness of losartan in suppressing growth in breast cancer cells shown in Fig. 2. At the normal dosage for hypertensives, losartan has been shown not to
have any effect on breast cancer (Fryzek et al. 2006), nor do ACE inhibitors (Meier et al. 2000, Li et al. 2003, Gonzalez-Perez et al. 2004), and in a study of the actions of candesartan in prostate cancer patients, infusion of up to 8 mg/day for at least 4 months gave PSA responses in only 8 out of 24 patients (Uemura et al. 2005). Though these different types of cancers may not be all comparable with breast cancer, there is a huge difference between the molar sensitivity of the cancer cell lines studied here in their responses to R6313/G2 and to losartan of perhaps more than a 1000-fold in the case of the MCF7 cells at least (Fig. 3). The antibody was therefore tested for its anti-cancer actions \textit{in vivo}.

Several papers have examined the efficacy of hollow fibre assays and find a high degree of correlation between hollow fibre and xenograft assays (e.g. Mi et al. 2002, Bridges et al. 2006), with the added advantages that more than one cell type can be tested at the same time, and thus comparison between different cell types is better controlled. Hollow fibre methodology has been adopted by the National Cancer Institute of the US as a wholly reliable primary \textit{in vivo} assay for anti-cancer activity. For these reasons, hollow fibre methodology was adopted as the first choice for the \textit{in vivo} study. In the first study (Fig. 4B and C), the same cell preparations were used in hollow fibres \textit{in vivo} as for the \textit{in vitro} data in Fig. 4A. Strikingly, the \textit{in vivo} results, most clearly in the i.p. site, show a close parallel to the \textit{in vitro} data and the relative sensitivities of the different cells were identical: MCF-7 cells responded most sensitively, T47D were intermediate and MDA-MB-231 least sensitive. For this reason, MCF-7 cells were chosen for the xenograft study. The specific effect on tumour size is quite striking, and R6313/G2 treatment for 7 days at 0.4–0.8 mg/kg (13–27 nmol/kg) twice per day reduced tumour volume by up to 90% (Figs 6A, B and 7). On a molar basis, this too is at least a thousand times more sensitive than reported by other authors for the AT1 receptor blockers in different cancer cell types. It should also be noted from comparison of Fig. 4A and B that the response to R6313/G2 appears to be much more sensitive \textit{in vivo} than \textit{in vitro}, perhaps as much as 100-fold. There is no immediate explanation for this unexpected effect, but it may be speculated that interactions between AT1 receptor and other systems \textit{in vivo} may give rise to this remarkable amplification.

Partly reflecting this great sensitivity of response, R6313/G2 also showed toxicity at the higher doses, though this varied considerably between the different experiments. In the hollow fibre study, no toxicity was seen at the doses illustrated, as judged by body weights (Fig. 4D) and all the animals survived. In contrast, in the xenograft study, body weights were significantly reduced by R6313/G2 at the highest doses illustrated, and at 1.36 mg/kg this resulted in mortality after 6 days, but at 0.4 mg/kg the animals appeared normal, fully active and with body weight not different from controls (in fact all the animals showed some weight loss, including the untreated controls; Fig. 5). The contrast with the hollow fibre group may possibly be attributed to the stress of the longer treatments of the

![Figure 6](https://example.com/figure6.png) **Figure 6** \textit{In vivo} actions of R6313/G2 on MCF-7 cell xenografts. (A) MCF-7 tumour volumes, means ± S.E.M. (B) Same data as for A, treated values expressed as percentages of the mean control values. *\(P<0.05\), **\(P<0.001\).

![Figure 7](https://example.com/figure7.png) **Figure 7** Sample MCF7 cell xenografts in control (upper) and (lower) after treatment for 7 days with 0.4 mg/kg R6313/G2 twice per day for 7 days, as for Fig. 6.
xenograft group, which were treated with oestrogen for several weeks before antibody treatment. The reasons for this variation in sensitivity are currently obscure, but on the basis of the data shown in Fig. 8, it appears generally unlikely to be attributable to an acute effect on the cardiovascular system. In these rats, R6313/G2 appeared to have the effect of moderating the slight stress induced by handling and treatment, but otherwise the blood pressures were not markedly different from those of the controls. The animals were in good health, and their body weights were unchanged by treatment (data not shown).

Taken together, the data confirm that R6313/G2 has unique biological properties that are quite unlike those of the hormone angiotensin II or the AT1 receptor antagonists. It strongly supports the view that the AT1 receptor is a highly promising target for anti-cancer therapy, and that the antibody’s properties can be developed to this end.

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