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), 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 *ad 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 Promochem, 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 L-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 (15×10^3 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, aprotinin 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 20 000 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 1:500 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 (10^4 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 $2.5\text{--}3.0 \times 10^6$ 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% Isoflurane). 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 midline in a cranial direction. The small incision was closed with metallic suture clips.

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% O₂, 5% CO₂ 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 $(L \times W^2)/2$, where L and W are the major and minor diameters respectively. Once tumour volumes reached 150–200 mm³, 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 $(W_t/W_i) \times 100$, where W_t is the body weight at any given time and W_i is the body weight at treatment initiation. Net tumour volume was calculated as $V_t - V_i$, 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 ~ 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

Figure 1 Immunoblotting for the angiotensin II receptor subtypes. AT1 receptor (A) and AT2 receptor (B) content was analysed in lysates of three breast cancer cell lines: (Lane B, T47D; Lane C, MDA-MB-231; Lane D, MCF-7), and smooth muscle cells (Lane A). Only lysates from the three breast cancer cell lines (Lanes B–D) contained the AT2 receptor, though MDA-MB-231 cells contained relatively little: there was no detectable AT2 receptor in RASMC (Lane A).

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 μ 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 μ 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 μ 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.

Figure 2 (A) Dose-dependent inhibition of cell survival by R6313/G2 in the presence of 100 nM angiotensin II was determined by XTT assay after 48 h. Threshold values (*) for the three cell lines ($P < 0.05$ or better) were 0.05 µM for MCF-7 and MDA-MB-231 cells, and 1.25 µM for T47D cells; IC50 values were: 2.8 µM, 153 nM and 30 nM for T47D, MDA-MB-231 and MCF-7 cells respectively. (B) Losartan alone produced a 43% inhibition of T47D cells at 25 µM, other cell types were unaffected at all concentrations used. Results are means of eight samples $\pm$ s.d.

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

Figure 3 (A and B) Effect of R6313/G2 on caspase-3/7 activity in T47D cells in the presence and absence of 100 nM angiotensin II after 12 and 48 h. After 12 h, angiotensin II inhibited caspase-3/7 activity compared with untreated control values, and this inhibition was blocked by R6313/G2. This blocking action of R6313/G2 was less marked at the higher doses used, but after 48 h R6313/G2 alone gave a dose-dependent increase of caspase-3/7 activity. (C) In MCF7 cells, R6313/G2 alone reduced caspase-3/7 activity, but in the presence of angiotensin II it dose dependently increased caspase-3/7 activity. Data are means of eight samples $\pm$ s.d. * $P < 0.05$, ** $P < 0.01$.

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.

Figure 4 (A) In the *in vitro* hollow fibre assay, R6313/G2 dose dependently inhibited survival of all three breast cell lines (T47D, MCF-7 and MDA-MB-231) in the presence of angiotensin II after 48 h. (B) In the *in vivo* hollow fibre assay at the s.c. site, R6313/G2 dose dependently inhibited cell survival only in MCF-7 cells, though MDA MB 231 cells were inhibited at the highest dose. (C) In the *in vivo* hollow fibre assay at the i.p. site, R6313/G2 inhibited MCF7 cell survival in animals treated with 0.07 and 0.7 mg/kg (2.5 and 25 nmol/kg) twice per day, and in T47D and MBA MB 231 cells at 0.7 mg/kg (25 nmol/kg) twice per day. Values are expressed as means $\pm$ S.D. * $P < 0.05$, ** $P < 0.001$. (D) Body weights of the experimental animals showed no changes during the period of treatment.

In vivo xenograft assay

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 after 7 days therapy giving 74 and 76% tumour regression compared with the untreated controls

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).

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 Paeppe *et al.* 2001). Although raised against a region of the receptor that is

Figure 5 *In vivo* R6313/G2 treatment in the animals with MCF7 cell xenograft implants gave no weight loss at doses of 0.4 mg/kg (13 nmol/kg; —■—) and 0.8 mg/kg (27 nmol/kg; —▲—); twice per day, and there was no mortality during the treatment period, but in the 0.8 mg/kg group, four died by day 8. Body weight was significantly reduced in animals receiving 1.36 mg/kg (45.3 nmol/kg; —●— * $P < 0.05$) and all died by day 8. Data are means $\pm$ S.E.M., $n = 8$, except where indicated otherwise (n numbers in brackets). Control (—◆—).

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, Decaillet *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 *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 *in vivo* assay for anti-cancer activity. For these reasons, hollow fibre methodology was adopted as the first choice for the *in vivo* study. In the first study (Fig. 4B and C), the same cell preparations were used in hollow fibres *in vivo* as for the *in vitro* data in Fig. 4A. Strikingly, the *in vivo* results, most clearly in the i.p. site, show a close parallel to the *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 *in vivo* than *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 *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,

Figure 6 *In vivo* actions of R6313/G2 on MCF-7 cell xenografts. (A) MCF-7 tumour volumes, means $\pm$ S.E.M. (B) Same data as for A, treated values expressed as percentages of the mean control values. * $P < 0.05$, ** $P < 0.001$.

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 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.

Figure 8 Blood pressures of rats treated with R6313/G2 (ScFv, 0.4 mg/kg per day for 3 days) compared with controls (Con) receiving PBS alone (see Materials and methods). * $P < 0.05$, comparison of Con and ScFv diastolic pressures.

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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References

Arrieta O, Guevara P, Escobar E, Garcia-Navarrete R, Pineda B & Sotelo J 2005 Blockage of angiotensin II type I receptor decreases the synthesis of growth factors and induces apoptosis in C6 cultured cells and C6 rat glioma. *British Journal of Cancer* **92** 1247–1252.

Barker S, Marchant W, Ho MM, Puddefoot JR, Hinson JP, Clark AJL & Vinson GP 1993 A monoclonal antibody to

a conserved sequence in the extracellular domain recognizes the angiotensin II AT1 receptor in mammalian tissues. *Journal of Molecular Endocrinology* **11** 241–245.

Barker S, Kapas S, Corder R & Clark AJ 1996 Adrenomedullin acts via stimulation of cyclic amp and not via calcium signalling in vascular cells in culture. *Journal of Human Hypertension* **10** 421–423.

Berry C, Touyz R, Dominiczak AF, Webb RC & Johns DG 2001 Angiotensin receptors: signaling, vascular pathophysiology, and interactions with ceramide. *American Journal of Physiology. Heart and Circulatory Physiology* **281** H2337–H2365.

Boutinaud M, Guinard-Flament J & Jammes H 2004 The number and activity of mammary epithelial cells, determining factors for milk production. *Reproduction, Nutrition, Development* **44** 499–508.

Bridges EM, Bibby MC & Burchill SA 2006 The hollow fiber assay for drug responsiveness in the Ewing's sarcoma family of tumors. *Journal of Pediatrics* **149** 103–111.

Decailiot FM, Befort K, Filliol D, Yue S, Walker P & Kieffer BL 2003 Opioid receptor random mutagenesis reveals a mechanism for G protein-coupled receptor activation. *Nature Structural Biology* **10** 629–636.

Diep QN, Li JS & Schiffrin EL 1999 *In vivo* study of AT(1) and AT(2) angiotensin receptors in apoptosis in rat blood vessels. *Hypertension* **34** 617–624.

Egami K, Murohara T, Shimada T, Sasaki K, Shintani S, Sugaya T, Ishii M, Akagi T, Ikeda H, Matsuishi T *et al.* 2003 Role of host angiotensin II type 1 receptor in tumor angiogenesis and growth. *Journal of Clinical Investigation* **112** 67–75.

Fryzek JP, Poulsen AH, Lipworth L, Pedersen L, Norgaard M, McLaughlin JK & Friis S 2006 A cohort study of antihypertensive medication use and breast cancer among Danish women. *Breast Cancer Research and Treatment* **97** 231–236.

Fujimoto Y, Sasaki T, Tsuchida A & Chayama K 2001 Angiotensin II type 1 receptor expression in human pancreatic cancer and growth inhibition by angiotensin II type 1 receptor antagonist. *FEBS Letters* **495** 197–200.

Fujita M, Hayashi I, Yamashina S, Itoman M & Majima M 2002 Blockade of angiotensin AT1a receptor signaling reduces tumor growth, angiogenesis, and metastasis. *Biochemical and Biophysical Research Communications* **294** 441–447.

Fujita M, Hayashi I, Yamashina S, Fukamizu A, Itoman M & Majima M 2005 Angiotensin type 1a receptor signaling-dependent induction of vascular endothelial growth factor in stroma is relevant to tumor-associated angiogenesis and tumor growth. *Carcinogenesis* **26** 271–279.

de Gasparo M 2002 AT(1) and AT(2) angiotensin II receptors: key features. *Drugs* **62** 1–10.

de Gasparo M, Catt KJ, Inagami T, Wright JW & Unger T 2000 International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacological Reviews* **52** 415–472.

- Gonzalez-Perez A, Ronquist G & Rodriguez LAG 2004 Breast cancer incidence and use of antihypertensive medication in women. *Pharmacoepidemiology and Drug Safety* **13** 581–585.
- Greco S, Elia MG, Muscella A, Storelli C & Marsigliante S 2002a AT1 angiotensin II receptor mediates intracellular calcium mobilization in normal and cancerous breast cells in primary culture. *Cell Calcium* **32** 1–10.
- Greco S, Muscella A, Elia MG, Salvatore P, Storelli C & Marsigliante S 2002b Activation of angiotensin II type I receptor promotes protein kinase C translocation and cell proliferation in human cultured breast epithelial cells. *Journal of Endocrinology* **174** 205–214.
- Greco S, Muscella A, Elia MG, Salvatore P, Storelli C, Mazzotta A, Manca C & Marsigliante S 2003 Angiotensin II activates extracellular signal regulated kinases via protein kinase C and epidermal growth factor receptor in breast cancer cells. *Journal of Cellular Physiology* **196** 370–377.
- Green KA & Streuli CH 2004 Apoptosis regulation in the mammary gland. *Cellular and Molecular Life Sciences* **61** 1867–1883.
- Gupta A, Decaillet FM, Gomes I, Tkalych O, Heimann AS, Ferro ES & Devi LA 2007 Conformation state-sensitive antibodies to G-protein-coupled receptors. *Journal of Biological Chemistry* **282** 5116–5124.
- Hii SI, Nicol DL, Gotley DC, Thompson LC, Green MK & Jonsson JR 1998 Captopril inhibits tumour growth in a xenograft model of human renal cell carcinoma. *British Journal of Cancer* **77** 880–883.
- Hollingshead MG, Alley MC, Camalier RF, Abbott BJ, Mayo JG, Malpais L & Grever MR 1995 *In vivo* cultivation of tumor cells in hollow fibers. *Life Sciences* **57** 131–141.
- Inwang ER, Puddefoot JR, Brown CL, Goode AW, Marsigliante S, Ho MM, Payne JG & Vinson GP 1997 Angiotensin II type I receptor expression in human breast tissues. *British Journal of Cancer* **75** 1279–1283.
- Kapas S, Hinson JP, Puddefoot JR, Ho MM & Vinson GP 1994 Internalization of the type-1 angiotensin-II receptor (AT1) is required for protein-kinase-C activation but not for inositol trisphosphate release in the angiotensin-II stimulated rat adrenal zona glomerulosa cell. *Biochemical and Biophysical Research Communications* **204** 1292–1298.
- Kaschina E & Unger T 2003 Angiotensin AT1/AT2 receptors: regulation, signalling and function. *Blood Pressure* **12** 70–88.
- Kasukabe T, Okabe-Kado J, Kato N, Sassa T & Honma Y 2005 Effects of combined treatment with rapamycin and cotylenin a, a novel differentiation-inducing agent, on human breast carcinoma MCF-7 cells and xenografts. *Breast Cancer Research* **7** R1097–R1110.
- Kivinen K, Kallajoki M & Taimen P 2005 Caspase-3 is required in the apoptotic disintegration of the nuclear matrix. *Experimental Cell Research* **311** 62–73.
- Lecat S, Bucher B, Mely Y & Galzi JL 2002 Mutations in the extracellular amino-terminal domain of the NK2 neurokinin receptor abolish cAMP signaling but preserve intracellular calcium responses. *Journal of Biological Chemistry* **277** 42034–42048.
- Lever AF, Hole DJ, Gillis CR, McCallum IR, McInnes GT, MacKinnon PL, Meredith PA, Murray LS, Reid JL & Robertson JWK 1998 Do inhibitors of angiotensin-I-converting enzyme protect against risk of cancer? *Lancet* **352** 179–184.
- Li CI, Malone KE, Weiss NS, Boudreau DM, Cushing-Haugen KL & Daling JR 2003 Relation between use of anti hypertensive medications and risk of breast carcinoma among women ages 65–79 years. *Cancer* **98** 1504–1513.
- Meier CR, Derby LE, Jick SS & Jick H 2000 Angiotensin-converting enzyme inhibitors, calcium channel blockers, and breast cancer. *Archives of Internal Medicine* **160** 349–353.
- Mi Q, Lantvit D, Reyes-Lim E, Chai H, Zhao W, Lee IS, Peraza-Sanchez S, Ngassapa O, Kardono LB, Riswan S *et al.* 2002 Evaluation of the potential cancer chemotherapeutic efficacy of natural product isolates employing *in vivo* hollow fiber tests. *Journal of Natural Products* **65** 842–850.
- Miyajima A, Asano T & Hayakawa M 2001 Captopril restores transforming growth factor-beta type II receptor and sensitivity to transforming growth factor-beta in murine renal cell cancer cells. *Journal of Urology* **165** 616–620.
- Miyajima A, Kosaka T, Asano T, Seta K, Kawai T & Hayakawa M 2002 Angiotensin II type I antagonist prevents pulmonary metastasis of murine renal cancer by inhibiting tumor angiogenesis. *Cancer Research* **62** 4176–4179.
- Mulrow PJ 1999 Angiotensin II and aldosterone regulation. *Regulatory Peptides* **80** 27–32.
- Muscella A, Greco S, Elia MG, Storelli C & Marsigliante S 2002 Angiotensin II stimulation of NA⁺/K⁺ ATPase activity and cell growth by calcium-independent pathway in MCF-7 breast cancer cells. *Journal of Endocrinology* **173** 315–323.
- Nouet S, Amzallag N, Li JM, Louis S, Seitz I, Cui TX, Alleaume AM, Di Benedetto M, Boden C, Masson M *et al.* 2004 Trans-inactivation of receptor tyrosine kinases by novel angiotensin II AT2 receptor-interacting protein, ATIP. *Journal of Biological Chemistry* **279** 28989–28997.
- Ohashi H, Takagi H, Oh H, Suzuma K, Suzuma I, Miyamoto N, Uemura A, Watanabe D, Murakami T, Sugaya T *et al.* 2004 Phosphatidylinositol 3-kinase/Akt regulates angiotensin II-induced inhibition of apoptosis in microvascular endothelial cells by governing survivin expression and suppression of caspase-3 activity. *Circulation Research* **94** 785–793.
- De Paepe B, Verstraeten VL, De Potter CR, Vakaet LA & Bullock GR 2001 Growth stimulatory angiotensin II type-1 receptor is upregulated in breast hyperplasia and *in situ* carcinoma but not in invasive carcinoma. *Histochemistry and Cell Biology* **116** 247–254.

- De Paepe B, Verstraeten VM, De Potter CR & Bullock GR 2002 Increased angiotensin II type-2 receptor density in hyperplasia, dcis and invasive carcinoma of the breast is paralleled with increased inos expression. *Histochemistry and Cell Biology* **117** 13–19.
- Prunet C, Lemaire-Ewing S, Menetrier F, Neel D & Lizard G 2005 Activation of caspase-3-dependent and -independent pathways during 7-ketocholesterol- and 7 β -hydroxy-cholesterol-induced cell death: a morphological and biochemical study. *Journal of Biochemical and Molecular Toxicology* **19** 311–326.
- Puddefoot JR, Udeozo UKI, Barker S & Vinson GP 2006 The role of angiotensin II in the regulation of breast cancer cell adhesion and invasion. *Endocrine-Related Cancer* **13** 895–903.
- Puett D, Li Y, Angelova K, Demars G, Meehan TP, Fanelli F & Narayan P 2005 Structure–function relationships of the luteinizing hormone receptor. *Annals of the New York Academy of Sciences* **1061** 41–54.
- Rivera E, Arrieta O, Guevara P, Duarte-Rojo A & Sotelo J 2001 AT1 receptor is present in glioma cells; its blockage reduces the growth of rat glioma. *British Journal of Cancer* **85** 1396–1399.
- Ronquist G, Rodriguez LA, Ruigomez A, Johansson S, Wallander MA, Frithz G & Svardsudd K 2004 Association between captopril, other antihypertensive drugs and risk of prostate cancer. *Prostate* **58** 50–56.
- Stoll M & Unger T 2001 Angiotensin and its AT2 receptor: new insights into an old system. *Regulatory Peptides* **99** 175–182.
- Suganuma T, Ino K, Shibata K, Kajiyama H, Nagasaka T, Mizutani S & Kikkawa F 2005 Functional expression of the angiotensin II type 1 receptor in human ovarian carcinoma cells and its blockade therapy resulting in suppression of tumor invasion, angiogenesis, and peritoneal dissemination. *Clinical Cancer Research* **11** 2686–2694.
- Tahmasebi M, Puddefoot JR, Inwang ER, Goode AW, Carpenter R & Vinson GP 1998 Transcription of the prorenin gene in normal and diseased breast. *European Journal of Cancer* **34** 1777–1782.
- Tahmasebi M, Barker S, Puddefoot JR & Vinson GP 2006 Localisation of renin-angiotensin system (RAS) components in breast. *British Journal of Cancer* **95** 67–74.
- Takeda H & Kondo S 2001 Differences between squamous cell carcinoma and keratoacanthoma in angiotensin type-1 receptor expression. *American Journal of Pathology* **158** 1633–1637.
- Thomas MA, Fleissner G, Stohr M, Hauptfleisch S & Lemmer B 2004 Localization of components of the renin–angiotensin system in the suprachiasmatic nucleus of normotensive sprague–dawley rats: Part a. Angiotensin I/II, a light and electron microscopic study. *Brain Research* **1008** 212–223.
- Uemura H, Ishiguro H, Nakaigawa N, Nagashima Y, Miyoshi Y, Fujinami K, Sakaguchi A & Kubota Y 2003 Angiotensin II receptor blocker shows antiproliferative activity in prostate cancer cells: a possibility of tyrosine kinase inhibitor of growth factor. *Molecular Cancer Therapeutics* **2** 1139–1147.
- Uemura H, Nakaigawa N, Ishiguro H & Kubota Y 2005 Antiproliferative efficacy of angiotensin II receptor blockers in prostate cancer. *Current Cancer Drug Targets* **5** 307–323.
- Vinson GP, Ho MM, Puddefoot JR, Teja R & Barker S 1994 Internalisation of the type I angiotensin II receptor (AT1) and angiotensin II function in the rat adrenal zona glomerulosa cell. *Journal of Endocrinology* **141** R5–R9.
- Vinson GP, Barker S, Puddefoot JR & Tahmasebi M 2007 The renin–angiotensin system in the breast. In *Frontiers in Research of the Renin–Angiotensin System on Human Disease*, Ed PS Leung. Springer Verlag: Berlin.
- Volpert OV, Ward WF, Lingen MW, Chesler L, Solt DB, Johnson MD, Molteni A, Polverini PJ & Bouck NP 1996 Captopril inhibits angiogenesis and slows the growth of experimental tumors in rats. *Journal of Clinical Investigation* **98** 671–679.
- Weidekamm C, Hauser P, Hansmann C, Schwarz C, Klingler H, Mayer G & Oberbauer R 2002 Effects of AT1 and AT2 receptor blockade on angiotensin II induced apoptosis of human renal proximal tubular epithelial cells. *Wiener Klinische Wochenschrift* **114** 725–729.
- Wiseman BS & Werb Z 2002 Stromal effects on mammary gland development and breast cancer. *Science* **296** 1046–1049.
- Yang X, Zhu MJ, Sreejayan N, Ren J & Du M 2005 Angiotensin II promotes smooth muscle cell proliferation and migration through release of heparin-binding epidermal growth factor and activation of EGF-receptor pathway. *Molecules and Cells* **20** 263–270.
- Yoshiji H, Yoshii J, Ikenaka Y, Noguchi R, Tsujinoue H, Nakatani T, Imazu H, Yanase K, Kuriyama S & Fukui H 2002 Inhibition of renin–angiotensin system attenuates liver enzyme-altered preneoplastic lesions and fibrosis development in rats. *Journal of Hepatology* **37** 22–30.