

RESEARCH PAPER

Involvement of α_2 - and β_2 -adrenoceptors on breast cancer cell proliferation and tumour growth regulation

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BACKGROUND AND PURPOSE

β -Adrenoceptors are expressed in human and experimental animal breast cancer cells. However, the effect of the agonists and antagonists reported on cell proliferation and tumour growth was paradoxical, precluding their utilization as possible adjuvant therapy, mainly in the cases of refractory tumours.

EXPERIMENTAL APPROACH

β -Adrenoceptor expression was analysed by immunofluorescence and RT-PCR. Cell proliferation was assessed by [3 H]-thymidine incorporation, tumour growth by measuring with a calliper and ERK 1/2 phosphorylation by Western blotting.

KEY RESULTS

β_2 -Adrenoceptor expression was confirmed in the mouse and human cells tested. Cell proliferation was increased by adrenaline (by α_2 -adrenoceptor action) and decreased in every tested cell line by the β -adrenoceptor agonist isoprenaline and the β_2 -adrenoceptor agonist salbutamol. Isoprenaline and salbutamol reduced tumour growth in every tumour tested (mouse C4-HD and CC4-3-HI and human IBH-4, IBH-6 and MDA-MB-231 cell lines growing as xenografts in nude mice). These effects were reversed by the β -adrenoceptor antagonist propranolol. The α_2 -adrenoceptor antagonist rauwolscine and the β_2 -adrenoceptor agonist salbutamol were equally effective in diminishing tumour growth. ERK 1/2 activation analysed in IBH-4 tumours correlated with tumour growth, with the β -adrenoceptor agonists decreasing its activation. Inhibition of ERK 1/2 phosphorylation *in vitro* was mainly mediated by the PKA pathway.

CONCLUSIONS AND IMPLICATIONS

In our experimental models, the β -adrenoceptor agonists inhibited breast cancer cell proliferation and tumour growth, probably mediated by inhibition of ERK 1/2 phosphorylation. The β -adrenoceptor agonists were as effective as the α_2 -adrenoceptor antagonist rauwolscine, providing possible novel adjuvant treatments for breast cancer.

Abbreviations

6Bnz, N6-benzoyl-cAMP; 8CTP, 8-(4-chlorophenylthio)-2'-O-methyl-cAMP; DMEM, Dulbecco's modified Eagle's medium; EPAC, exchange protein directly activated by cAMP; F12, Ham's F12 medium; FCS, fetal calf serum

Introduction

Breast cancer is by far the most common cancer of women, comprising 23% of all female cancers (Ferlay *et al.*, 2010). In the United States, breast cancer was in 2010 the most important female cancer in incidence, with 28% of female neo-

plasms, although mortality was of 15%, second after lung and bronchus cancer (Jemal *et al.*, 2011). In the States, in 1987 lung cancer surpassed breast cancer as the leading cause of cancer death in women (Jemal *et al.*, 2011). In the United Kingdom, it has been suggested (Parkin, 2009) that the recent decline in breast cancer incidence observed since 1999

between ages 50 and 59 and since 2003 between ages 60 and 64 is a consequence of the reduced use of hormone replacement therapy.

Clinical studies have suggested that stress, chronic depression and social support might influence cancer onset and progression (Antoni *et al.*, 2009) and β -adrenoceptors could be involved in the mediation of these effects (Antoni *et al.*, 2006; Thaker *et al.*, 2006). The first studies of the adrenergic stimulation and adrenoceptors of the mammary gland involved the normal bovine mammary gland because of their effects on milk production (Roets *et al.*, 1984; Roets *et al.*, 1986; Hammon *et al.*, 1994; Inderwies *et al.*, 2003). β -Adrenoceptors were also characterized in the normal mammary gland (Clegg and Mullaney, 1985; Lavandero *et al.*, 1985) and in mammary tumours of experimental animals (Marchetti *et al.*, 1989; Marchetti *et al.*, 1990; Marchetti and Labrie, 1990; Marchetti *et al.*, 1991). β -Adrenoceptors have since been identified in human breast cancer MDA-MB-231, MCF-7, VHB-1, T47-D and BT-20 cell lines, comparing them with the HBL-100 cells (Vandewalle *et al.*, 1990). A weak correlation ($0.05 < P < 0.1$) was observed between β -adrenoceptors and progesterin receptors but no correlation found between β -adrenoceptors and oestrogen receptors (Draoui *et al.*, 1991).

The paradoxical nature of β -adrenoceptor action in breast cancer cells has recently been reviewed (Luthy *et al.*, 2009). On one hand, β -adrenoceptor agonists inhibited cell proliferation. Thus, MDA-MB-231 human breast cancer cells expressed high β -adrenoceptor levels, and receptor stimulation by an agonist evoked immediate and robust reductions in DNA synthesis (Slotkin *et al.*, 2000). Moreover, stimulation of β_2 -adrenoceptors induced significant tumour growth suppression and tumour regression in mice bearing established MDA-MB-231 human breast tumours into the mammary fat pads (Carie and Sebti, 2007). On the other hand, inhibition of cell proliferation has been induced by β -adrenoceptor antagonists in oestrogen-responsive (MCF-7, ZR-75-1, MDA-MB-361) and oestrogen non-responsive (MDA-MB-453, MDA-MB-435, MDA-MB-468) cell lines derived from human breast cancers (Cakir *et al.*, 2002). However, these authors did not assess the possibility that the antagonists behaved as partial agonists. Several clinically used β -adrenoceptor antagonists are agonists at the human β -adrenoceptor. Sometimes these agonist effects were small at the cAMP level, but substantial at the transcriptional level (Baker *et al.*, 2003). Therefore, the reported results may not be contradictory. However, there is a close correlation between progressing, static, and regressing tumours after ovariectomy and β -adrenoceptor concentration in membranes prepared from dimethylbenz(a)anthracene (DMBA)-induced tumours (Marchetti *et al.*, 1989). In the same model, the rapid reduction of the β -adrenoceptor population in the tumour following ovariectomy was accompanied by the well-known regression of tumour growth (Marchetti *et al.*, 1991). Also, some single nucleotide polymorphisms of the β_2 -adrenoceptor gene have been associated with increased risk of breast cancer (Feigelson *et al.*, 2008).

α_2 -Adrenoceptors have also been described in the bovine mammary gland, related to milk production (Roets *et al.*, 1986). The number of α -adrenoceptors decreased from the teat to the mammary ducts to the parenchyma (Hammon *et al.*, 1994; Inderwies *et al.*, 2003). Actions at α_2 -adrenoceptors in human breast cancer cells have been

described by our group (Vazquez *et al.*, 1999) and these receptors were characterized in several breast tumour and non-tumour cell lines by RT-PCR, immunocytochemistry and binding studies. These α_2 -adrenoceptors were associated with increased cell proliferation *in vitro* (Vazquez *et al.*, 2006) and with increased tumour growth *in vivo* (Bruzzone *et al.*, 2008).

The aim of the present work was to assess if compounds active at β -adrenoceptors were able to inhibit tumour growth in five tumours from two independent breast cancer models, and if the treatment with a β -adrenoceptor agonist and an α_2 -adrenoceptor antagonist was equally effective in inhibiting tumour growth in these models.

Methods

Cell culture

The mouse mammary epithelial MC4-L5 (Lanari *et al.*, 2001) cell line was routinely cultured in phenol red-free (Berthois *et al.*, 1986) Dulbecco's modified Eagle's medium (DMEM) : Ham's F12 medium (F12) (1:1) supplemented with heat-inactivated 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin and 15 mM HEPES. Cells were sub-cultured once weekly after trypsinization (0.25% trypsin – 0.2% EDTA) (Lanari *et al.*, 2001). The medium was changed twice weekly.

The human breast cancer MDA-MB-231 and the IBH-4 and IBH-6 cell lines, developed in our laboratory, were cultured as already described (Vazquez *et al.*, 2004). Briefly, cells were maintained in DMEM : F12 (1:1) medium supplemented with heat-inactivated 10% FCS, 2 mM glutamine, 2 μ g mL⁻¹ bovine insulin, 100 IU mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin and 15 mM HEPES. Cells were grown on plastic, incubated at 37°C in 5% CO₂ and sub-cultured at 80% confluence using 0.25% trypsin – 0.025% EDTA.

Preparation of cell suspension for injections

IBH-4, IBH-6 and MDA-MB-231 cells were dispersed with 0.25% trypsin – 0.025% EDTA, washed twice with PBS or medium without serum, concentrations adjusted to $1\text{--}2 \times 10^7$ viable cells and suspended in 200 μ L. The cells were inoculated s.c. in the flank, through a 21 gauge needle tunnelled 1–2 cm to prevent leakage of the cell inoculum (Bruzzone *et al.*, 2009).

Animals

All animal care and experimental procedures complied with institutional guidelines, the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, 1996), which has been updated and is available online and in print (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, 2011) and the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia (United Kingdom Co-ordinating Committee on Cancer Research U, 1998; Workman *et al.*, 2010). For human breast cell lines growing *in vivo*, congenitally athymic nude mice on a Swiss background (N : NIH(S)-nu) bred at *Bioterio de la Universidad de La Plata*, Argentina were used. Balb/c mice from the animal facility of the *Instituto de Biología y Medicina*

Experimental were used for transplantation of the C4-HD and CC4-3-HI mouse mammary tumours. All mice were 8 weeks old sexually mature females, and were housed in ventilated racks, pathogen-free conditions under a 12 h light–dark photoperiod with controlled humidity and temperature ($20 \pm 2^\circ\text{C}$). Boxes, bedding, food and water were sterilized. Sterility was maintained during the surgical procedures used for the inoculation of the cells to give rise to solid tumours and for subsequent removal and transplantation of tumours.

Tumours

IBH-4, IBH-6 and MDA-MB-231 human breast cancer cell lines were inoculated in nude mice as already described. Subsequent *in vivo* passages of the tumours were performed by trocar transplantation. C4-HD and CC4-3-HI tumours were transplanted by trocar in Balb/c mice (Lamb *et al.*, 2005). Tumour growth was monitored every second day. The two major diameters were measured with a calliper and the volume was calculated with the formula: $\text{volume} = (4 \times 3^{-1}) \pi \times \text{minor radius}^2 \times \text{major radius}$ (Bruzzone *et al.*, 2008). Mice were killed and the tumours extracted and frozen at -80°C . The animals were killed when ulceration, distension of covering tissues or cachexia were observed or when tumours attained near 10% body weight.

Treatments

One day after transplantation of the tumour, animals were given daily injections of the drugs. Three different experimental approaches were undertaken: in one, the β -adrenoceptor agonists were compared: the animals were separated in three groups of 10 animals each: (i) one s.c. injected with isoprenaline ($1.0 \text{ mg kg}^{-1} \text{ day}^{-1}$); (ii) another one s.c. injected with salbutamol ($1.2 \text{ mg kg}^{-1} \text{ day}^{-1}$); and (iii) the control group received physiological saline. The second approach was the reversal of the effects of β -adrenoceptor activation. For that purpose, the animals were separated in four groups of 10 animals each: (i) the β -adrenoceptor agonist group: s.c. injected salbutamol ($1.2 \text{ mg kg}^{-1} \text{ day}^{-1}$); (ii) the β -adrenoceptor antagonist group: s.c. injected with propranolol ($1.0 \text{ mg kg}^{-1} \text{ day}^{-1}$); (iii) the reversal group received salbutamol + propranolol at the stated doses; and (iv) the control group received physiological saline. For the third experimental approach the four groups of 10 animals each one, were: (i) the β -adrenoceptor agonist group: s.c. injected with salbutamol ($1.2 \text{ mg kg}^{-1} \text{ day}^{-1}$); (ii) the α_2 -adrenoceptor antagonist group s.c. injected with rauwolscine: $0.5 \text{ mg kg}^{-1} \text{ day}^{-1}$; (iii) the combined group: received salbutamol + rauwolscine at the stated doses; and (iv) the control group received physiological saline. The duration of the treatments varied, depending on the animal's condition. Every animal was treated beginning 1 day after the implantation of the tumour and continuing until the mice died. For C4-HD tumours, the duration of the experiments was of 25–27 days, for the CC4-3-HI tumours, the experiments lasted 22 days. Nude mice inoculated with the human breast cancer cell lines were treated for 25 days for IBH-4 cells, 30–35 days for IBH-6 cells and 35–40 days for MDA-MB-231 cells.

Proliferation assays

The MC4-L5 mouse mammary tumour cell line and the human IBH-4, IBH-6 and MDA-MB-231 cell lines were tested

for proliferation. Mouse cells were seeded at 7000 cells per well in 96 well plates. They were incubated in phenol red-free DMEM : F12 medium supplemented with 10% FCS, 2 mM glutamine, 100 IU mL^{-1} penicillin, $100 \mu\text{g mL}^{-1}$ streptomycin, 250 ng mL^{-1} amphotericin B and 15 mM HEPES. After 24 and 48 h, the medium was changed to a similar one but with FCS replaced by 1% charcoal-stripped FCS and the experiments lasted 48 h for the mouse cells. Human cells were seeded at 5000 cells per well in 2% charcoal-stripped FCS and the incubations lasted 72 h. Different adrenoceptor agonists were added. All solutions of these compounds were prepared in 10 mM ascorbic acid, frozen and diluted immediately before use. [^3H]-thymidine at $0.2 \mu\text{Ci}$ per well was added with the last change of medium. After 24 h, cells were harvested in a Nunc Cell Harvester 8 (Nunc, Rochester, NY, USA), and filters were counted in a liquid scintillation counter. The murine cells were trypsinized prior to harvesting.

Immunofluorescence

The cells were cultured in Lab-Tek Chamber Slide System (Nunc, Thermo Fisher Scientific, Rochester, NY, USA) with 10% FCS in phenol red-free DMEM : F12 medium; 1 day before immunocytochemistry the medium was changed to 1% (for mouse cells) or 2% (for human cells) charcoal-stripped FCS medium. The cells were treated with 10% FCS (without heat inactivation) and permeated with 0.1% Triton X-100 in 4% FCS. After overnight incubation at 4°C with the anti-human (which can also be used for the murine receptor) β_2 -adrenoceptor antibody at a concentration 1:50, and several PBS washes, cells were incubated with secondary donkey anti-rabbit IgG in 4% FCS, conjugated to fluorescein isothiocyanate (FITC). Primary antibody was omitted in the controls. Nuclei were stained with $0.1 \mu\text{g mL}^{-1}$ propidium iodine for 1 min, and the cells were mounted with Vectashield H-1000 (Vector Laboratories, Burlingame, CA, USA). Sections were analysed under a Nikon laser confocal microscope (Melville, NY, USA) (Plan Apo 40X/0.95 oil or Apo 60X/1.40 oil).

RT-PCR

When cells attained 80% confluence, they were incubated with medium without serum for additional 48 h. They were washed with PBS and total RNA was extracted by incubation with Tri Reagent (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature, according to the manufacturer's instructions. Chloroform (0.2 mL) was added per mL of initial extract. These samples were incubated at room temperature for 3 min and centrifuged at $10\,464 \times g$ for 15 min at 4°C . The aqueous phase was subsequently incubated with cold isopropanol (0.5 mL) for 10 min at room temperature with mild agitation and centrifuged again at $10\,464 \times g$ for 10 min. The nucleic acid pellet was washed with 75% ethanol, centrifuged at $3360 \times g$ for 5 min, resuspended in diethylpyrocarbonate (DEPC) water (Invitrogen, Life Technologies) and the concentration measured with a spectrophotometer. The RNA was reverse transcribed at 42°C for 15 min in $20 \mu\text{L}$ containing 200 units of Superscript II RNase HK Reverse Transcriptase, 4 mM oligo dT18, 50 mM Tris-HCl pH 8.3, 3 mM MgCl_2 , 75 mM KCl, 10 mM DTT, 500 mM dNTPs (dATP, dTTP, dCTP, dGTP), 40 units RNase OUT (Recombinant RNase Inhibitor) (Superscript First Strand Syn-

thesis System for RT-PCR, Invitrogen, Carlsbad, CA, USA). The reaction was terminated by heating at 99°C for 5 min and resting for 5 min at 5°C. Also, a parallel incubation with the extract in the absence of reverse transcriptase was performed in order to exclude any contamination with genomic DNA. This control is important in a gene without introns, such as that for the β_2 -adrenoceptor.

PCR amplification was carried out in the presence of 30 μM of each oligonucleotide primer, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2 mM MgCl_2 , 0.2 mM dNTPs (dATP, dTTP, dCTP, dGTP), and 0.05 units·mL⁻¹ Taq DNA polymerase (Taq DNA Polymerase, Invitrogen). Three microlitres of cDNA was amplified in the PCR mix made up to 25 μL with molecular quality water.

An initial denaturation step at 95°C for 2 min was followed by 35 cycles (or 30 cycles for murine β_2 -adrenoceptor) at 95°C for 35 s, 60°C for 35 s and 72°C for 1 min, followed by a single incubation at 72°C for 8 min. A reaction with water instead of cDNA was performed concurrently as a negative control for the PCR in each PCR to test the reactive purity. The primers used were the following: β_2 -adrenoceptor: for human cells: forward 5'-GGACTTCCATTGATGTGCTGT-3' and reverse 5'-GTCAGCAGGCTCTGGTACTTG-3' (112 bp amplicon), for murine cells: forward 5'-GCCATCCTCATGTCGGGTAT-3' and reverse 5'-AGCAGGCTCTGGTACTTGAA-3' (356 bp amplicon). For glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript detection, GAPDH primers forward: 5'-TTCGTCATGGGTGTGACC-3' and reverse 5'-AGTGAGCTTCCCGTTCAGC-3', giving a 300 bp PCR product, served as a control for RNA integrity and the RT-PCR process. The PCR products were analysed using electrophoresis on a 2% agarose gel stained with ethidium bromide. A 100 bp DNA ladder was used as a marker to determine the size of the PCR products.

Western blotting for ERK 1/2

For the *in vitro* experiments, IBH-4 cells were seeded in 6 well plates in 10% FCS supplemented medium. After 12 h the medium was changed to 0% FCS and the cells were arrested for 24 h. The medium was changed again to a medium without serum. After 1–2 h, the adrenoceptor agents or the mimics were added (adrenoceptor agents (1 μM): isoprenaline, propranolol, salbutamol, rauwolscine, dexmedetomidine), 25 μM of the PKA inhibitor H-89, the PKA stimulator 8Br-cAMP (100 μM), 100 μM of the PKA selective activator N6-benzoyladenosine-cAMP (6Bnz) or 100 μM of 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8CPT) the specific activator of exchange protein directly activated by cAMP (EPAC). The antagonists and inhibitors (rauwolscine, propranolol and H89) were added 20 min before the agonists. The control cells were incubated with medium without FCS. The incubation was performed for 15 min and then the protein extraction buffer (RIPA: 10 mM Tris, 150 mM NaCl, 2 mM sodium vanadate, 1% sodium deoxycholate, 0.1% SDS, 1% Igepal) was added after washing the cells with phosphate saline buffer.

Tumours were homogenized in a Polytron at a 1:4 ratio (tissue : buffer). The buffer was 20 mmol L⁻¹ Tris-HCl (pH 7.4), 1.5 mmol L⁻¹ EDTA, 0.25 mmol L⁻¹ dithiothreitol, 20 mmol L⁻¹ Na_2MoO_4 , and 10% glycerol. Protease inhibitors (0.5 mmol L⁻¹ phenylmethylsulfonyl fluoride,

0.025 mmol L⁻¹ N-Carbobenzyloxy-L-phenylalanyl chloromethyl ketone, 0.025 mmol L⁻¹ tosyl-lysylchloromethane, 0.025 mmol L⁻¹ tosylphenylalanylchloromethane, and 0.025 mmol L⁻¹ N α -p-Tosyl-L-arginine methyl ester hydrochloride) were added to the buffer immediately before use. The homogenate was sonicated and centrifuged for 20 min at 10 391 $\times$ g (4°C). The supernatant was stored at -20°C until later use in Western blot assays.

Tumour or cell extracts (50–100 μg total protein per lane) were separated on 10% discontinuous polyacrylamide gels (SDS-PAGE). The proteins were dissolved in sample buffer (6 mM Tris pH 6.8, 2% SDS, 0.002% bromophenol blue, 20% glycerol, 5% mercaptoethanol) and boiled for 5 min. After electrophoresis, they were blotted onto a 0.2 μm nitrocellulose membrane and blocked 1 h at room temperature in 5% skim milk in 0.1% TBST (10 mM Tris, 100 mM NaCl, 0.1% v/v⁻¹ Tween 20). The membranes were incubated 1 h at room temperature with antibody to ERK 1 or overnight at 4°C with antibody to phospho-ERK 1/2 (pERK), both at a 1:1000 dilution. Blots were probed with donkey anti-rabbit and sheep anti-mouse IgG horseradish peroxidase linked whole antibody (Amersham Life Science, Arlington Heights, IL, USA; 1:2000 dilutions in 5% skim milk 0.1% TTBS). The luminescent signal was generated with an ECL Western blotting detection solution [2.2 mg luminol (Sigma, St. Louis, MO, USA), 0.33 mg *p*-coumaric acid (Sigma), 15 μL hydrogen peroxide 30% (Merck, Darmstadt, Germany), 333 μL Tris-HCl 1.5 M pH 8.8, 4.6 mL distilled water], and the blots were exposed to an autoradiographic film (Curix RP1, Agfa, Buenos Aires, Argentina) for 10 s to 5 min. Band intensity was quantified only in unsaturated films. Quantification was performed with ImageJ software.

Statistical analysis

Statistical analysis for the *in vitro* effect of agonists was performed by ANOVA followed by Dunnett–Kramer test and the effect of the adrenergic compounds on tumour growth and the action of inhibitors or stimulators of ERK 1/2 phosphorylation by ANOVA followed by Tukey–Kramer tests (Dowdy and Wearden, 1983).

Materials

FCS, culture media, antibiotics, trypsin, Superscript First Strand Synthesis System for RT-PCR and Taq DNA Polymerase trypsin were purchased from Invitrogen Life Technologies. Methyl [³H]-thymidine (NET 027E; specific activity: 20 Ci·mmol⁻¹) was from Dupont-New England Nuclear (Boston, MA, USA). Liquid scintillation cocktail was Optiphase 'Hisafe' 3 (Wallac, Turku, Finland). Anti-human β_2 -adrenoceptor rabbit polyclonal antibody (sc-569), Phospho-ERK (pERK; sc-7383) and ERK (ERK 1; sc-94) antibodies were from Santa Cruz Biotechnology. Secondary antibodies were from Amersham (GE Healthcare Argentina S.A., Buenos Aires, Argentina). Vectashield H-1000 was from Vector Laboratories (Burlingame, CA, USA). The cAMP analogues, 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cAMP (8-CPT), 6Bnz and 8-bromoadenosine-3',5'-cAMP (8-Br-cAMP) were purchased from BioLog Life Science Institute (Bremen, Germany). All the other reagents, including isoprenaline, salbutamol, propranolol, rauwolscine-HCl, glutamine, DNase,

luminol, *p*-coumaric acid, forskolin, EGF and H-89 were from Sigma-Aldrich. Lab-Tek Chamber Slide System was from Nunc.

Drug and receptor nomenclature

Drug and receptor nomenclature conforms to the *Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Results

Expression of β -adrenoceptors in mammary cell lines of mouse and human origin

The present experiments were undertaken to confirm the expression of β_2 -adrenoceptors in a mouse mammary tumour cell line and three breast cancer cell lines of human origin. As shown in Figure 1A, mouse mammary tumour MC4-L5 cells express β_2 -adrenoceptors. These receptors are shown in green, while the nuclei are stained with propidium iodine in red. The control in the absence of primary antibody (Figure 1B) showed no staining. Figure 1C depicts the staining of IBH-4 cells compared with its control (Figure 1D). Figure 1E shows the staining of IBH-6 cells and Figure 1F its control. As a positive control, MDA-MB-231 human breast cancer cells were included (Figure 1G, H for the control), because β_2 -adrenoceptors are known to be expressed in these cells (Vandewalle *et al.*, 1990). Staining for β_2 -adrenoceptors was positive in both the plasma membrane and the cytoplasm.

The expression of these receptors was also confirmed by RT-PCR. As can be seen in Figure 1, all the human (Figure 1I) and murine (Figure 1J) breast cancer cell lines tested expressed β_2 -adrenoceptors, with IBH-4 cells showing a lighter band for receptor expression as compared with MDA-MB-231. The MC4-L5 murine cells showed a faint but positive band. As the primers are different between human and murine cells, the receptor concentrations cannot be compared.

Cell proliferation is inhibited by β -adrenoceptor agents

Once the expression of β_2 -adrenoceptors was confirmed in these cell lines, the next step was to analyse the action of the adrenoceptor agents on cell proliferation. Figure 2A shows the effect of adrenaline on the mouse mammary tumour cell line MC4-L5, Figure 2D on the human breast cancer cells IBH-4, Figure 2G on the human breast cancer cells IBH-6 and Figure 2J on the human breast cancer cells MDA-MB-231. In every case, adrenaline caused a significant increase on cell proliferation assessed by [3 H]-thymidine incorporation. However, as adrenaline binds to α_2 , α_1 and β -adrenoceptor, this effect could be due to the mitogenic effect of α_2 -adrenoceptors, already described in breast cancer cells (Vazquez *et al.*, 2006). The MC4-L5 and to a lesser extent, the IBH-4 and MDA-MB-231 cells exhibited biphasic curves, consistent with opposite actions mediated by the α_2 -adrenoceptor mitogenic effect and a β -adrenoceptor inhibitory effect at higher concentrations. To test this hypothesis, as shown in Figure 2 (Figure 2B for MC4-L5, Figure 2E for IBH-4, Figure 2H for IBH-6 and Figure 2K for MDA-MB-231), every cell line was tested in the presence of adrenaline at the stimulatory concentration in the presence or absence of an excess of the α_2 -adrenoceptor antagonist rauwolscine (left bars). In every case, significant stimulation of cell proliferation was reversed

by the α_2 -adrenoceptor antagonist. In MC4-L5 and IBH-4 cells, rauwolscine alone showed a significant inhibition of cell proliferation, behaving as an inverse agonist, as previously described (Bruzzone *et al.*, 2011). However, in IBH-6 cells, as previously reported (Bruzzone *et al.*, 2011) and in MDA-MB-231 cells, this antagonist alone exerted no effect in cell proliferation. The cells were also treated in the presence of higher adrenaline concentrations in the presence or absence of the β -adrenoceptor antagonist propranolol (middle bars). The adrenaline concentrations chosen for these incubations were those at which some reversal of the enhancement of cell proliferation was evident in the adrenaline curves. A very significant increase of cell proliferation was evident in every cell line tested, except in IBH-6 (the only showing a non-biphasic adrenaline curve) when the β -adrenoceptor action of adrenaline was reversed by simultaneous incubation with propranolol. The endogenous adrenoceptor agonist adrenaline was tested because of its pivotal role during stress. However, as it binds to several different adrenoceptors, the β_2 -adrenoceptor agonist salbutamol was also tested for competition with the antagonist propranolol (right bars). In every case significant inhibition of cell proliferation was reversed by propranolol, with no effect of this compound *per se*.

The effect of β -adrenoceptor agonists was further analysed. Figure 2C shows the effect of isoprenaline and salbutamol on the mouse mammary tumour cell line MC4-L5, on the human breast cancer cells IBH-4 (Figure 2F), on the human breast cancer cells IBH-6 (Figure 2I) and on the human breast cancer cells MDA-MB-231 (Figure 2L). In every case, the β -adrenoceptor agonists inhibited cell proliferation. The apparent EC₅₀ for isoprenaline on MC4-L5 cells was 1.37 pM, 0.852 nM for the human IBH-4 cells, 0.362 nM for IBH-6 cells and 1.996 nM for MDA-MB-231. The maximum for the Gaussian salbutamol curve for IBH-4 cells was 0.85 pM, and the apparent EC₅₀ for salbutamol on the mouse MC4-L5 cells was 0.165 nM, on the human IBH-6 cells was 23.32 pM and for MDA-MB-231 cells, 2.63 pM. The mouse MC4-L5 cells exhibited a very impressive sensitivity to isoprenaline, whereas in all the human cells the sensitivity was similar for both compounds or higher for salbutamol (MDA-MB-231).

β -adrenoceptor mediated inhibition of tumour growth

Figure 3 depicts the action of the β -adrenoceptor agonist isoprenaline and the specific β_2 -adrenoceptor agonist salbutamol on tumour growth. Figure 3A shows the action of both agonists on the mouse progestin-dependent tumour C4-HD (Lanari *et al.*, 2009) in the presence of medroxyprogesterone acetate (MPA), Figure 3C on the progestin-independent CC4-3-HI, both growing in Balb/c mice. C4-HD is the parent tumour from MC4-L5 cell line, HD being hormone-dependent, whereas HI signifies hormone-independent. As these tumours were induced by inoculation of MPA, the hormonal status refers to MPA (Lanari *et al.*, 2009). Panels on Figure 3 (Figure 3A, G and I) depict the comparison of isoprenaline and salbutamol in IBH-4, IBH-6 and MDA-MB-231 tumour growth. As a β -adrenoceptor mediated effect has been described for tumours derived from inoculation of MDA-MB-231 cells in nude mice (Carie and Sebt, 2007), these tumours were included as a positive control. As can be seen in Figure 3, in every case the β -adrenoceptor agonist significantly dimin-

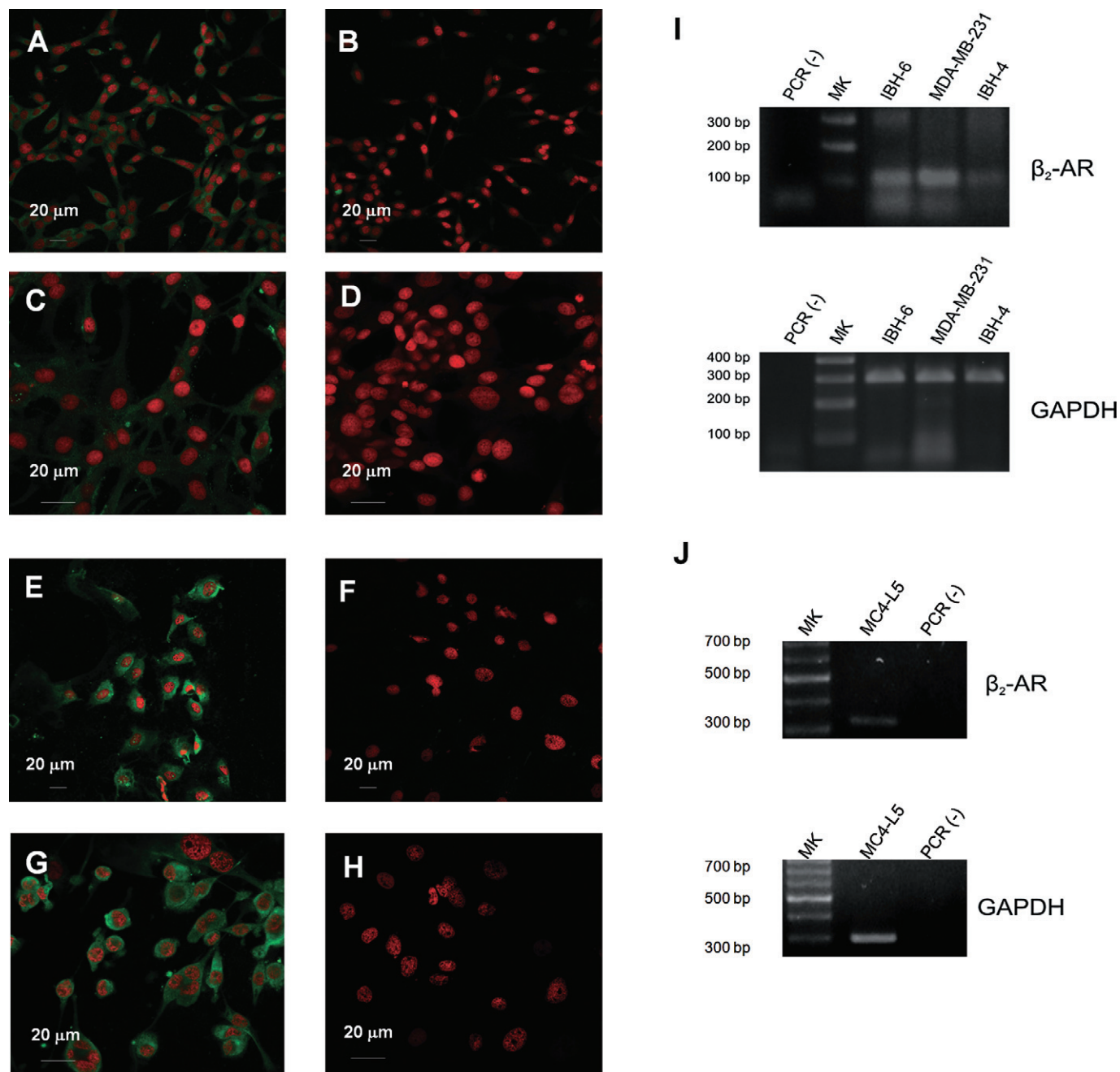


Figure 1

Expression of β_2 -adrenoceptors in mouse mammary tumour cell line MC4-L5 and the human IBH-4, IBH-6 and MDA-MB-231 cells by immunofluorescence and RT-PCR. (A,B) Mouse mammary tumour cells MC4-L5. (C,D) Human breast cancer cells IBH-4. (E,F) Human breast cancer cells IBH-6. (G,H) Human breast cancer MDA-MB-231 cells. (A,C,E,G) β_2 -AR. (B,D,F,H) Negative controls incubated without the primary antibody. (A,B,E,F) Photographs taken with a Plan Apo 40X/0.95 oil objective and (C,D,E,F) with an Apo 60X/1.40 oil one. (I) RT-PCR for β_2 -adrenoceptors (β_2 -AR) in human IBH-4, IBH-6 and MDA-MB-231 cells. (J) RT-PCR for β_2 -adrenoceptors in mouse MC4-L5 cells. In every case GAPDH was used as a control for RNA integrity and the RT-PCR process. The negative control was performed in the absence of cDNA.

ished tumour growth, both in mouse tumours growing in Balb/c mice and in human cells growing in nude mice. The reversal of the action by propranolol was also tested (Figure 3B for C4-HD, D for CC4-3-HI, Figure 3F for IBH-4, H for IBH-6 and Figure 3J for MDA-MB-231). Propranolol given alone in IBH-4 tumours was completely ineffective. However

in C4-HD, CC4-3-HI and IBH-6 tumour, this antagonist alone showed a curve intermediate between the control and the agonist. In the case of the MDA-MB-231 tumours, the antagonist alone produced an inhibition of growth similar to the agonist. The antagonist was able to reverse the agonist effect in the majority of the tumours.

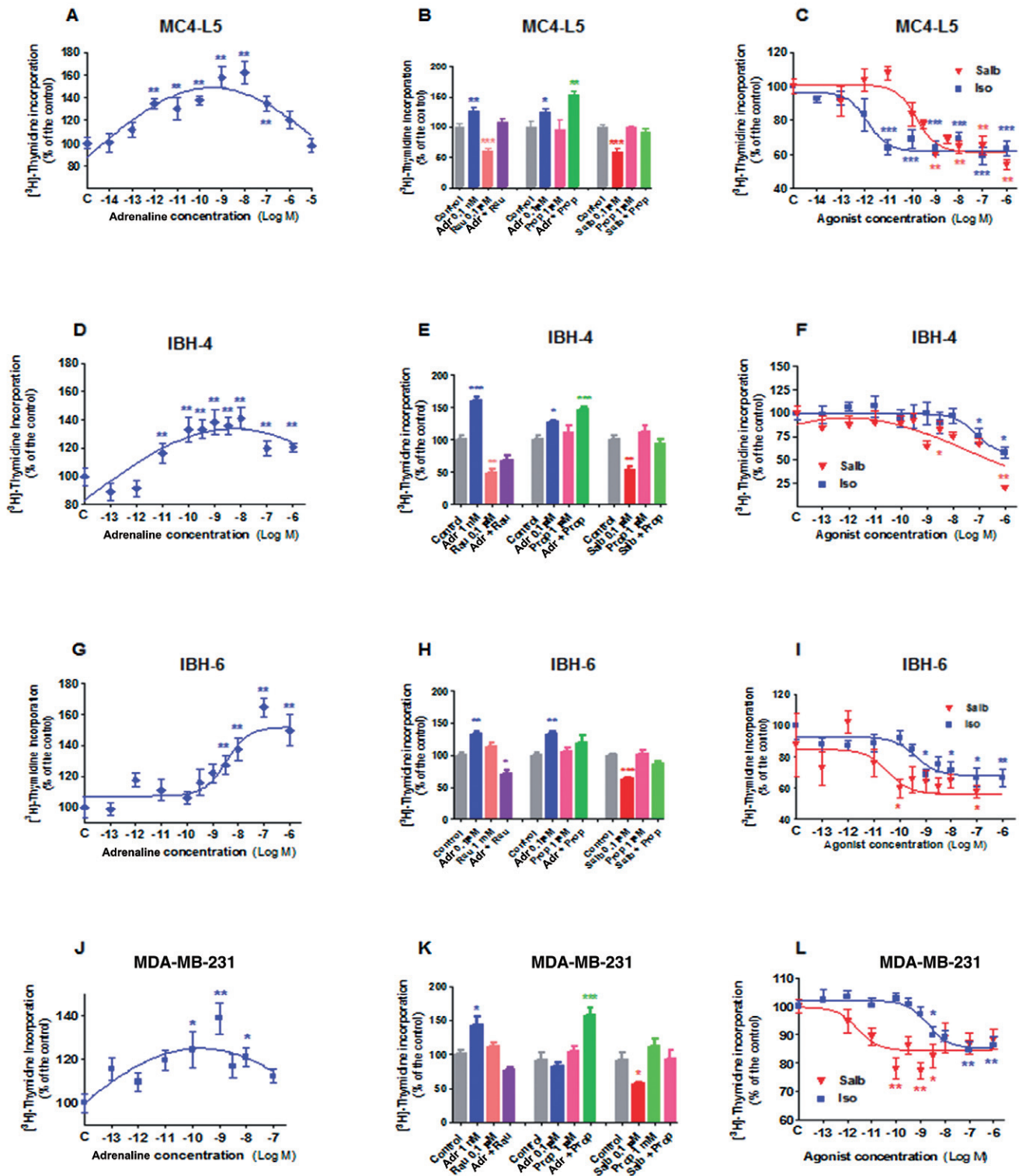


Figure 2

β -adrenoceptor agents and [³H]thymidine incorporation in mouse MC4-L5 and human IBH-4, IBH-6 and MDA-MB-231 cell lines. (A–C) MC4-L5 cells. (D–F) IBH-4 cells. (G–I) IBH-6 cells. (J–L) MDA-MB-231 cells. The cells were incubated with increasing concentrations of adrenaline (Adr; A,D,G,J), the β -adrenoceptor agonist isoproterenol (Iso) and the selective β_2 -adrenoceptor agonist salbutamol (Salb; C,F,I,L). (B,E,H,K) Cells were incubated with mitogenic concentrations of adrenaline in the presence or absence of the α_2 -adrenoceptor antagonist rauwolfscine (Rau; left bars); higher adrenaline concentrations in the presence or absence of propranolol (middle bars) and with inhibitory concentrations of salbutamol in the presence or absence of propranolol (right bars). The addition of the antagonist was performed 30 min before the agonist. * $P < 0.05$, ** $P < 0.01$ with respect to control; $n = 8$ replicates for treatments and 16 for controls; ANOVA followed by Dunnett–Kramer test. The experiments were repeated at least twice with similar results.

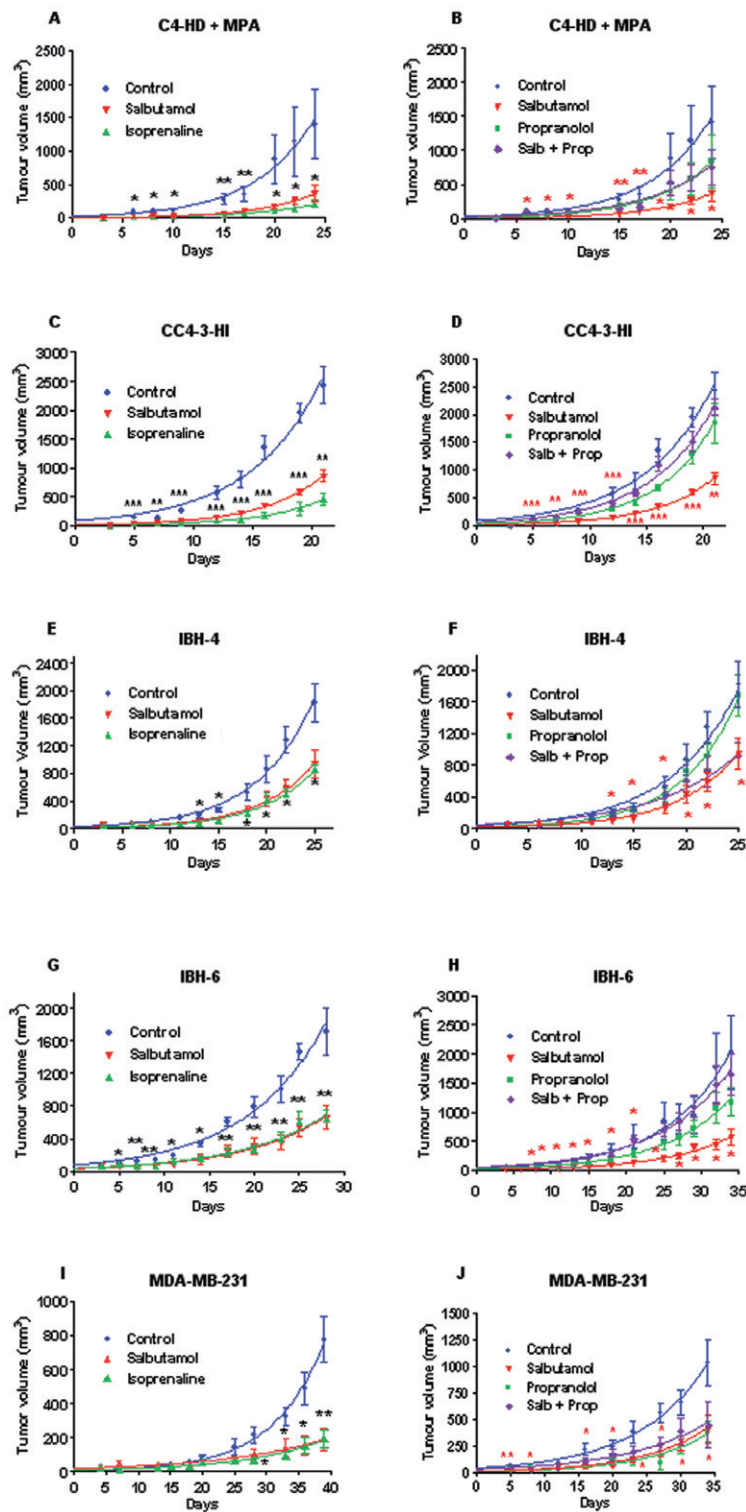


Figure 3

Effect of β -adrenoceptor agents on *in vivo* tumour growth of the mouse C4-HD and CC4-3-HI and human IBH-4, IBH-6 and MDA-MB-231 tumours. (A,B) Mouse progestin-dependent C4-HD tumour in the presence of MPA depot in Balb/c mice. (C,D) Mouse progestin independent CC4-3-HI in Balb/c mice. (E,F) Human IBH-4 tumour growing in nude mice. (G,H) Human IBH-6 tumour growing in nude mice. (I,J) MDA-MB-231 tumour growing in nude mice. A, C, E, G and I compare the action of isoprenaline and salbutamol. The control group received s.c. daily injections of physiological saline. (B,D,F,H,J) The animals were s.c. daily injected with the following compounds: salbutamol ($1.2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$); propranolol (Prop; $1.0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) or (salbutamol + propranolol; Salb + Prop) at the same doses. * $P < 0.05$, ** $P < 0.01$, with respect to control; $n = 10$ animals/group; ANOVA followed by Tukey–Kramer test. Each experiment was repeated twice with similar results.

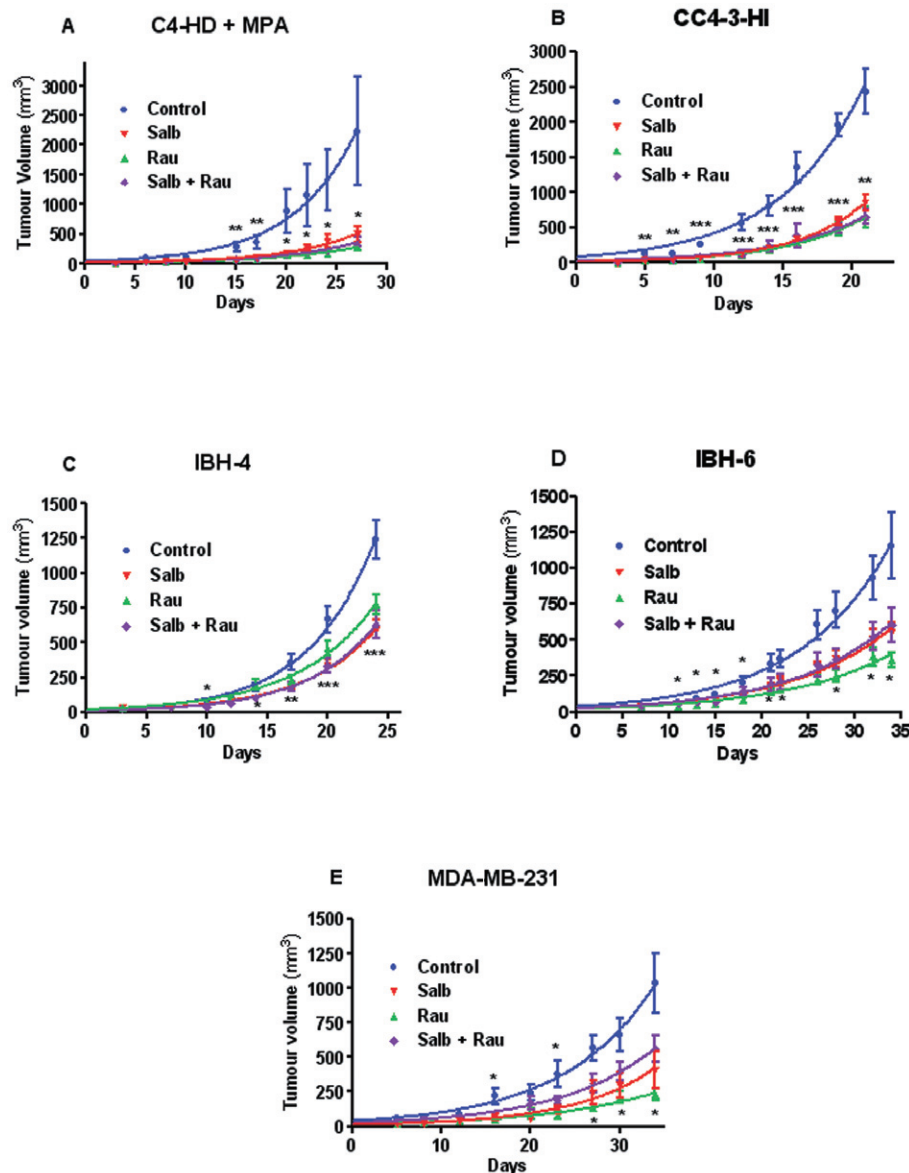


Figure 4

Mouse C4-HD and CC4-3-HI and human IBH-4, IBH-6 and MDA-MB-231 tumour growth *in vivo* comparing the α_2 -adrenoceptor antagonist rauwolscline (Rau) and the β_2 -adrenoceptor agonist salbutamol (Salb). (A) Mouse progestin-dependent C4-HD tumour in the presence of MPA depot in Balb/c mice. (B) Mouse progestin independent CC4-3-HI in Balb/c mice. (C) Human IBH-4 tumour growing in nude mice. (D) Human IBH-6 tumour growing in nude mice. (E) MDA-MB-231 tumour growing in nude mice. The animals were daily injected with the following compounds: salbutamol ($1.2 \text{ mg kg}^{-1} \text{ day}^{-1}$); rauwolscline: $0.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ or (salbutamol + rauwolscline) at the same doses. The control group received physiological saline. * $P < 0.05$, ** $P < 0.01$; with respect to control; $n = 10$ animals/group, ANOVA followed by Tukey–Kramer test. Each experiment was repeated twice with similar results.

Comparison of α_2 and β_2 -adrenoceptor mediated effects on tumour growth

The next set of experiments was undertaken in order to evaluate if the β_2 -adrenoceptor agonist salbutamol was as efficient as the α_2 -adrenoceptor antagonist rauwolscline, already described by our group as an inhibitor of tumour growth (Bruzzzone *et al.*, 2008; Bruzzzone *et al.*, 2011), in diminishing this parameter in the same tumours tested before. As can be seen in Figure 4, in every tumour tested both compounds

worked equally well to reduce tumour growth. However, combining them caused no further action on this parameter at the doses tested.

ERK phosphorylation in the tumours correlated with their effect on tumour growth

A complex signalling pathway for β_2 -adrenoceptors has been described (Johnson, 2006; Carie and Sebt, 2007) which includes ERK. As the activation of the ERK pathway is some-

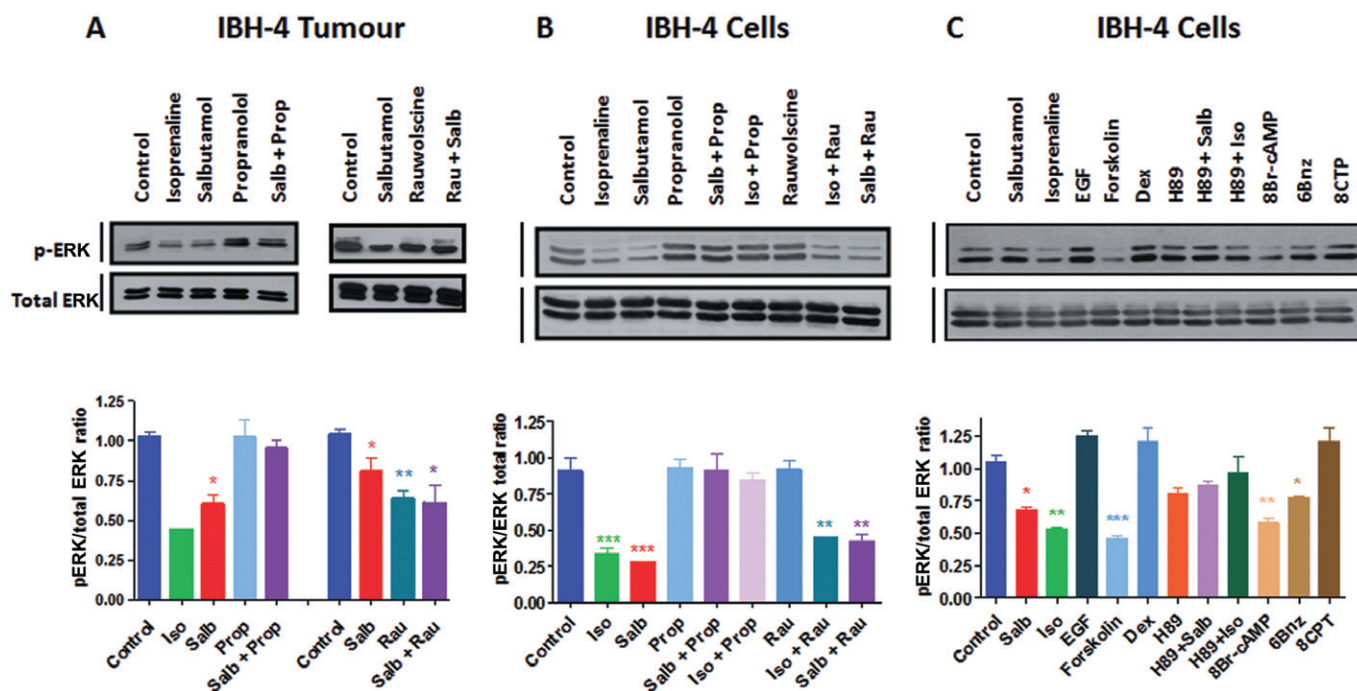


Figure 5

ERK 1/2 phosphorylation in protein extracts from IBH-4 tumours and cells. (A) Whole tumour extracts from IBH-4 tumours were tested for ERK 1/2 phosphorylation by Western blotting with the phospho-ERK (pERK; sc-7383) and ERK (ERK 1; sc-94) antibodies. The results are expressed as the mean $\pm$ SEM of three experiments, showing the phospho-ERK 1/2/total ERK 1/2 ratio; a representative sample is shown. (B,C) IBH-4 cells were tested for ERK 1/2 phosphorylation by Western blotting with the same antibodies. The results are expressed as the mean $\pm$ SEM of three experiments, stating the phospho-ERK 1/2/total ERK 1/2 ratio and a representative Western blot is shown. The cells were incubated during 15 min in the presence of the following compounds: 1 μ M salbutamol (Salb), 1 μ M isoprenaline (Iso), 1 μ M rauwolscline; 1 μ M propranolol, 20 ng mL⁻¹ EGF, 10 μ M forskolin, 1 μ M dexmedetomidine (Dex), 25 μ M of the PKA inhibitor H-89 (with and without salbutamol or isoprenaline), the PKA stimulator 8Br-cAMP (100 μ M), 100 μ M of the PKA selective activator 6Bnz or 100 μ M of the EPAC specific activator (8CPT). * P < 0.05, ** P < 0.01, with respect to control; ANOVA followed by Dunnett–Kramer test. The isoprenaline in the *in vivo* experiment (A) is a single experiment, so no statistics have been performed in this column.

times associated with cell proliferation and tumour growth, the phosphorylation of ERK 1/2 was analysed in extracts of IBH-4 tumours, treated with different adrenoceptor agents (Figure 3F and Figure 4C). Figure 5A (a representative Western blot and the mean of the quantification of different experiments) shows phosphorylated ERK 1/2 relative to total ERK in extracts of whole tumours derived from this human breast cancer cell, growing in nude mice. As can be seen in this figure, inhibition of ERK 1/2 phosphorylation *in vivo* exactly correlated with tumour growth decrease in every treatment.

Analysis of signalling pathways involved in the inhibition of ERK phosphorylation

To assess the effects of the adrenoceptor agents on the tumours *in vitro*, the IBH-4 human breast cancer cell were incubated with the same treatments. As can be seen in Figure 5B,C, incubation with β -adrenoceptor agonists for 15 min caused a significant inhibition of ERK 1/2 phosphorylation, which was reversed by incubation with propranolol. However, no effect on ERK 1/2 phosphorylation was evident *in vitro* following incubation with rauwolscline. These results suggest that the β -adrenoceptor agonists could exert this

effect in tumour ERK 1/2 phosphorylation acting directly on the tumour cells, whereas the effect of the α_2 -adrenoceptor antagonist rauwolscline could be indirect.

In order to analyse the signalling pathway involved in ERK 1/2 phosphorylation inhibition, IBH-4 cells were incubated *in vitro* in the presence of the agonists or treated with several mimics or antagonists. The *in vitro* inhibition of ERK 1/2 phosphorylation (Figure 5C) was similar to that provoked by the adenylyl cyclase activator forskolin or the cAMP analogue 8-Br-cAMP and was reversed by the simultaneous presence of propranolol. The two different pathways activated by cAMP were then studied. The PKA-activating analogue of cAMP (6Bnz) caused a clear inhibition of phosphorylation. The PKA inhibitor H-89 was able to reverse the β -adrenoceptor agonist effect, confirming the implication of PKA in the inhibition of ERK 1/2 phosphorylation. The cAMP pathway mediated by EPAC was also tested. As can be seen in Figure 5, the EPAC-activating analogue of cAMP 8CPT, showed consistent, though not significant, increases in ERK 1/2 phosphorylation in these cells, suggesting that this pathway is not implicated in the inhibition of ERK 1/2 phosphorylation mediated by the β -adrenoceptor agonists. The positive controls EGF and the α_2 -adrenoceptor agonist dexmedetomidine were also assessed. As these cells have high

spontaneous ERK 1/2 phosphorylation and proliferation, it is difficult to cause significant stimulation of this parameter. As a consequence of these signalling studies, the significant decrease of ERK 1/2 phosphorylation caused by β -adrenoceptor agonists seemed to be mediated by PKA stimulation.

Discussion

Expression of β -adrenoceptors in mammary cell lines of mouse and human origin

As stated in the introduction, binding to β -adrenoceptors in the mammary gland has been known since 1985 (Clegg and Mullaney, 1985; Lavandero *et al.*, 1985; Marchetti *et al.*, 1989; Vandewalle *et al.*, 1990; Draoui *et al.*, 1991; Marchetti *et al.*, 1991). The aim of the present investigation was to assess if β -adrenoceptor agonists could be potential adjuvant drugs for breast cancer. To analyse this possibility, the first step was to confirm β -adrenoceptor expression in the cells to be used for *in vivo* experiments. All cell lines tested showed expression of β_2 -adrenoceptors. However, this staining was not restricted to the cell membrane but widespread within the cell, and even in the nucleus. The expression of another GPCR, the leukotriene D₄ receptor, CysLT₁, is increased in colorectal adenocarcinomas and the nuclear localization of this receptor correlated with the proliferative marker Ki-67 (Nielsen *et al.*, 2005). Also, a functional population of intracellular GPCRs coexists with membrane associated receptors (Zhu *et al.*, 2006) and while immediate responses could be associated with the membrane receptors, more long-lasting effects could be caused by the intracellular receptors. Intracellular receptors have been described (Zhu *et al.*, 2006) for platelet-activating factor, prostaglandins and lysophosphatidic acid in different tissues.

Cell proliferation is inhibited by β -adrenoceptor agents

As there was debate over the activation or inhibition of cell proliferation by β -adrenoceptor agonists, we tested the effect of the natural agonist adrenaline and the β -adrenoceptor agonists, isoprenaline and salbutamol in different cell lines in order to analyse the effect on cell proliferation. The natural agonist adrenaline was included because it is released during both acute and chronic stress and its effects on breast cancer models are therefore relevant. Our data clearly show that in the mouse MC4-L5, and the human IBH-4, IBH-6 and MDA-MB-231 cell lines, the natural agonist adrenaline significantly enhanced cell proliferation, measured by [³H]-thymidine incorporation. However, as adrenaline binds to α_1 -, α_2 - and β -adrenoceptors, we hypothesized that this proliferative effect was probably due to α_2 -adrenoceptor binding. We have previously described in the MC4-L5 and the IBH-6 cell lines, a significant mitogenic effect associated with α_2 -adrenoceptor binding (Vazquez *et al.*, 2006; Bruzzzone *et al.*, 2008). Three cell lines exhibited biphasic curves, consistent with opposing actions mediated by the α_2 -adrenoceptor mitogenic effect and a β -adrenoceptor inhibitory effect at higher concentrations. The EC₅₀ for the mitogenic action of the α_2 -adrenoceptor agonists ranged from 20 fM to 80 pM (Vazquez *et al.*, 2006).

The incubation of all the cell lines with adrenaline and the specific antagonists confirmed that the stimulation was mediated by α_2 -adrenoceptors, whereas the decrease in the stimulation was mediated by β -adrenoceptors. The IBH-6 cell line was the only one to show a monophasic curve. The increase in cell proliferation in the presence of both adrenaline and propranolol suggests that when the β -adrenoceptor effect is blocked only the α_2 -adrenoceptor action is effective. Administration of the α_2 -adrenoceptor antagonist was always able to reverse adrenaline-induced stimulation of cell proliferation.

We tested then two β -adrenoceptor agonists, isoprenaline and salbutamol, in the same cells. In every case, the agonists inhibited cell proliferation, although the affinity of the effect was rather poor in the case of IBH-4 cells. The maximal inhibitory effect was similar for isoprenaline and for salbutamol in MC4-L5, IBH-4, IBH-6 and MDA-MB-231 cells. However, the sensitivity for these drugs was differed between mouse and human cells. Isoprenaline is an agonist for every β -adrenoceptor, and the expression of other subtypes of β -adrenoceptors have not been assessed in these cells. Then, the possibility of an action of β -adrenoceptors stimulated by this agonist cannot be excluded, mainly in the murine cells, which show an enhanced sensitivity to isoprenaline, compared with the human cells. On the other hand, the most important β -adrenoceptor in human cells is the β_2 -subtype (Draoui *et al.*, 1991; Badino *et al.*, 1996). Salbutamol and isoprenaline interact with different sites within the β_2 -adrenoceptor (Granier *et al.*, 2007; Katritch *et al.*, 2009) and polymorphic variants of human β_2 -adrenoceptors differ in several actions (Insel, 2011). The possibility of different variants in the diverse human cell lines could thus explain the differences in agonist sensitivities. An inhibition of cell proliferation has already been described in MDA-MB-231 human breast cancer cells in culture (Slotkin *et al.*, 2000). β -Adrenoceptor signalling is rather complex and the actions of the agonists comprise a wide range of effects. For example, in the rat skeletal muscle cell line L6, β -adrenoceptor agonists increase 2-deoxy-[³H]-D-glucose uptake via a β_2 -adrenoceptor-mediated pathway involving both cAMP and PI3K. PKA nevertheless appears to negatively regulate β_2 -adrenoceptor-mediated glucose uptake (Nevzorova *et al.*, 2006). These effects could affect cell proliferation by regulation of cell metabolism in addition to the other signalling pathways. However, an increase of glucose uptake should increase rather than decrease cell proliferation.

Inhibition of tumour growth mediated by β -adrenoceptors

The aim of these experiments was to analyse the action of β -adrenoceptor agonists on *in vivo* models of breast cancer. The first model used was a transplantable murine mammary tumour with different sensitivity to MPA (Lanari *et al.*, 2009). As can be seen in Figure 3, the progestin independent CC4-3-HI tumours responded as well as the progestin-sensitive C4-HD tumours to the treatment with β -adrenoceptor agonists. This model has several common features with luminal breast cancer: they are of ductal origin, invasive and metastatic, hormone-responsive, they express oestrogen and progestin receptors and respond to chemotherapy (Lanari *et al.*, 2009). The advantage of this model consists of the intact immune system. The second *in vivo* model consisted of

human breast cancer cell lines IBH-4 and IBH-6 growing in nude mice. These tumours derive from primary human ductal carcinomas. They are able to grow in nude mice without the addition of oestradiol, but they respond to tamoxifen. While IBH-4 metastasizes to the lung, IBH-6 invades the peritoneum (Bruzzone *et al.*, 2009). The advantage of this model is the human origin of the cells and the disadvantage, the need to make them grow in immunodeficient mice. MDA-MB-231 cells growing in nude mice were analysed as positive controls. The tumours of human origin were also sensitive to the inhibitory effects of the β -adrenoceptor agonist.

The doses chosen in the present paper for the *in vivo* treatment were much lower than the usual ones. For example, the doses of isoprenaline that have been used were 10 mg kg⁻¹ day⁻¹ (Landen *et al.*, 2007) or 200 mg kg⁻¹ day⁻¹ (Carie and Sebti, 2007). Propranolol was used in infants at an initial dose of 0.5 mg kg⁻¹ day⁻¹ and increased to an oral dose of 2 mg kg⁻¹ day⁻¹ (Jadhav and Tolat, 2010). A similar dose of 2 mg kg⁻¹ day⁻¹ has been used in mice (Wang *et al.*, 2008). However, considerably higher doses have also been used (Horinouchi *et al.*, 2007). In preliminary tests the effects on tumour growth of the doses chosen proved sufficient to cause an action, without significant side effects.

In the case of the tumours of human origin, the β -adrenoceptor agonist isoprenaline and the specific β_2 -adrenoceptor salbutamol similarly inhibited tumour growth. The latter is a drug currently used as a rapid and short acting treatment for asthma (Cazzola and Matera, 2008) and has been classified as a non-catechol partial agonist (Yao *et al.*, 2006). We have already described (Bruzzone *et al.*, 2009) that these cell lines growing in nude mice are insensitive to oestrogen or progestins, but sensitive to tamoxifen inhibition. In MDA-MB-231 cells inoculated into the fat pad of nude mice, the compound ARA-211 induced regression of tumours and potently inhibited tumour growth by acting through β -adrenoceptors (Carie and Sebti, 2007). These authors also stated that isoprenaline was able to inhibit tumour growth without causing regression of tumours (Carie and Sebti, 2007). However, a completely opposite action has been reported associating β -adrenoceptor activation with tumour growth. In tumours induced in rats by DMBA, intact animals with progressing tumours had significantly higher levels of β -adrenoceptors than intact animals with static tumours, and regressing tumours in ovariectomized animals showed a sharp decrease in β -adrenoceptors (Marchetti *et al.*, 1989). The results shown herein with β -adrenoceptor agonists are consistent with the inhibition of tumour growth described for the MDA-MB-231 tumours (Carie and Sebti, 2007). In the present investigation, propranolol in the majority of the cases completely reversed the agonist effect. However, in certain tumours, propranolol administered alone caused a diminution of tumour growth. Although effects on other receptors cannot be excluded, it is possible to ascribe this inhibition of tumour growth as a partial agonist effect, as has already been described mainly at the transcriptional level (Baker *et al.*, 2003). Another possibility is that the *in vivo* propranolol inhibition of tumour growth could be indirect, as suggested by the complete absence of effect of the drug given alone in the *in vitro* experiments. This effect could be mediated by the inhibition of macrophage infiltration and crosstalk, inhibiting the prometastatic cell signalling switch-

ing in the cancer cells (Powe and Entschladen, 2011). Propranolol is used to treat infantile haemangiomas (Tan *et al.*, 2011) and this compound inhibited *in vitro* human brain endothelial cells tubulogenesis (Annabi *et al.*, 2009). A similar effect cannot be discounted in this model. Moreover, a very promising effect of β -adrenoceptor antagonists, mainly propranolol, has recently been reported in breast cancer human patients (Powe *et al.*, 2010; Barron *et al.*, 2011; Ganz *et al.*, 2011). Interestingly, the administration of β -adrenoceptor antagonists was associated with improved relapse-free survival but not overall survival among patients with triple-negative breast cancer (Melhem-Bertrandt *et al.*, 2011).

Some important actions described for β -adrenoceptors in different cancer models must be considered in pre-clinical research before considering the possibility of testing adrenoceptor agents in clinical trials. The migration of breast, prostate and colon carcinoma cells is enhanced by the stress-related neurotransmitter noradrenaline *in vitro*, and this effect can be inhibited by propranolol (Masur *et al.*, 2001; Palm *et al.*, 2006). PC-3 prostate carcinoma cells growing in athymic BALB/c nude mice developed lumbar lymph node metastases at an increased rate with the application of noradrenaline, whereas propranolol inhibited this effect. The growth of the primary tumour was not affected by either treatment (Palm *et al.*, 2006). Nicotine can enhance colon tumour growth (HT-29 cells growing as xenograft in nude mice) partly by stimulation of β -adrenoceptor, preferentially the β_2 -adrenoceptor, with subsequent stimulation of COX-2, PGE₂, and VEGF expression (Wong *et al.*, 2007). Chronic behavioural stress caused higher levels of tissue catecholamines, greater tumour burden and more invasive growth of two different β -adrenoceptor-positive ovarian cancer cell lines (HeyA8 and SKOV3ip1) intraperitoneally inoculated (Thaker *et al.*, 2006). The MDA-MB-231 breast cancer cells injected into the mammary fat pad showed a similar increase of tumour growth. These effects were primarily mediated through activation of β -adrenoceptors and the cAMP-PKA signalling pathway. Ovarian tumours in stressed animals showed markedly increased vascularization and enhanced expression of VEGF, MMP2 and MMP9 (Thaker *et al.*, 2006). All these different effects of adrenaline, noradrenaline or stress, generally mediated by β_2 -adrenoceptors, must be carefully analysed in pre-clinical studies before considering clinical trials.

Comparison of α_2 and β -adrenoceptor action on tumour growth

We have previously demonstrated that the α_2 -adrenoceptor antagonist/inverse agonist rauwolscine was able to significantly inhibit tumour growth in a model of mouse mammary tumours (Bruzzone *et al.*, 2008) and human breast cancer cells growing in nude mice (Bruzzone *et al.*, 2011) in the absence of agonist treatment. We have carefully analysed the action of this compound elsewhere (Vazquez *et al.*, 2006; Bruzzone *et al.*, 2008; Bruzzone *et al.*, 2011). In summary, although a systemic effect of this compound reversing the stimulatory action of adrenaline or noradrenaline, released by stress in the animal, cannot be discarded as a mechanism of action, *in vitro* data indicated that at least part of this action could be due to an inverse agonist effect directly on the cells. In the present work, with the objective of finding a possible

adjuvant drug for breast cancer, we compared the effect of this α_2 -adrenoceptor inverse agonist and a β_2 -adrenoceptor agonist salbutamol in inhibiting tumour growth. Both compounds performed equally well in this parameter. As stated before for the action of the β -adrenoceptor compounds, it is still too soon to think in clinical trials. The action of both β -adrenoceptor agonists and the α_2 -adrenoceptor antagonist rauwolscine on cell proliferation, apoptosis, invasion, metastasis and differentiation should be carefully investigated before any clinical trial is pursued. At this stage of the investigation it is therefore not possible to describe either of them as more relevant than the other in inhibiting tumour growth.

Several interactions between α_2 and β -adrenoceptors have been described (Desai *et al.*, 2004; Desai *et al.*, 2005; Schwencke *et al.*, 2005). We have also previously found an increase of α_2 -adrenoceptor concentration when the cells were incubated in the presence of isoprenaline (Vazquez *et al.*, 2006). We therefore expected an interaction, to be shown in different growth curves in the simultaneous presence of the α_2 -adrenoceptor antagonist and the β_2 -adrenoceptor agonist. However, in the present work the curves of the α_2 -adrenoceptor antagonist, the β_2 -adrenoceptor agonist and both compounds together were superimposable, showing no evident interaction of these receptors on tumour growth, at least at the chosen doses.

Adrenoceptors are involved in complex and pleiotropic actions. The α_{2A} -adrenoceptor subtype has very clear hypotensive actions. Stimulation of α_{2B} -adrenoceptors in vascular smooth muscle leads to vasoconstriction, which causes hypertension (Philipp and Hein, 2004). β -Adrenoceptors mediate also the redistribution of blood flow to skeletal muscle during exercise (Figuroa *et al.*, 2009) and are involved in tumour angiogenesis (Chakroborty *et al.*, 2009). However, for the results presented herein, the anti-mitogenic effect seems more relevant than the metabolic and angiogenic actions, because the combined result is a significant reduction of tumour growth in every tumour tested.

ERK phosphorylation

β -Adrenoceptor signalling is complex. As reviewed (Johnson, 2006), although most of the actions of the β_2 -adrenoceptor seem mediated by G_s proteins and the cAMP-dependent PKA system, these receptors can also couple to G_i proteins, resulting in stimulation of the ERK and p38 MAPK pathways. Activation of this pathway by the β_2 -adrenoceptor requires receptor phosphorylation by PKA and is mediated by $\beta\gamma$ -subunits of the G protein acting as an assembly scaffold for a number of intracellular proteins, including non-receptor tyrosine kinases cSrc, Ras and Raf culminating in the stimulation of MAPKs (Johnson, 2006). Also, β_2 -adrenoceptor stimulation with an agonist results in a transient pulse of cAMP, GPCR kinase (GRK6)-mediated receptor phosphorylation leading to β -arrestin mediated receptor inactivation and cAMP-dependent kinase-mediated induction of cAMP metabolism by phosphodiesterases (Violin *et al.*, 2008). It has been reported (Houslay and Baillie, 2003) that phosphodiesterase PDE4 isoforms can interact with β -arrestin and thus be recruited to the β_2 -adrenoceptor in an agonist-dependent fashion. The recruited PDE4 can switch coupling of the β_2 -adrenoceptor to activation of G_i and hence to ERK. In

hepatocellular HepG2 and MHCC97H cancer cells, isoprenaline promoted cell proliferation and transiently ERK 1/2 phosphorylation by EGFR-independent mechanisms (Yuan *et al.*, 2010). Also (Plummer *et al.*, 2004) the expression of mRNA that encodes a G-protein coupled inwardly rectifying potassium channel (GIRK1) has been shown in several human breast cancer cell lines and tissue samples from approximately 40% of primary human breast cancers. The tobacco carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, which shows high affinity for β -adrenoceptors, stimulated activation of ERK 1/2 in MDA-MB-453 cells (Plummer *et al.*, 2004).

However, recently Carie and Sebti (Carie and Sebti, 2007) searched for inhibitors of p-ERK 1/2 in the human breast cancer cell line MDA-MB-231. They found a compound, ARA-211, which activated β_2 -adrenoceptors and suppressed the Raf-1/Mek-1/ERK 1/2 pathway by a cAMP-dependent activation of PKA, but not by the EPAC pathway. Similarly, isoprenaline blocks increases in ERK 1/2 phosphorylation in freshly dispersed rat parotid acinar cells (Soltoff and Hedden, 2010). The inhibitory action of isoprenaline was reproduced by forskolin and mimetics, including one selective for PKA, while EPAC selective activators did not mimic the blockade of ERK by isoprenaline, suggesting that inhibition involved PKA (Soltoff and Hedden, 2010). The stimulation of ERK by phosphorylation induces a series of different actions. As recently reviewed (Kim and Choi, 2010), the ERK signalling pathway plays a role in several steps of tumour progression. ERK phosphorylates several proteins promoting cancer cell migration. The ERK pathway also induces expression of matrix metalloproteinases promoting degradation of extracellular matrix proteins and consequent tumour invasion. ERK 1/2 signalling regulates the activities of pro-apoptotic and anti-apoptotic proteins promoting cancer cell survival. However, the most studied action of ERK 1/2 activation is related to cell proliferation, as recently reviewed (Mebratu and Tesfagzi, 2009). In the present paper we have analysed the activation of ERK 1/2 by phosphorylation in extracts of the IBH-4 tumours. In this experimental model, ERK inhibition of phosphorylation perfectly matched tumour growth decrease in every treatment.

There is controversy with respect to the effects of β -adrenoceptor agonists on ERK 1/2 phosphorylation. On the one hand, the mechanisms involving the $\beta\gamma$ -subunits of the G_i protein, β -arrestin, GIRK1 channel and EGFR transactivation were reported to stimulate ERK 1/2 phosphorylation and thus cell proliferation or tumour growth. On the other hand, PKA stimulation was directly involved in the inhibition of both ERK 1/2 phosphorylation and cell proliferation/tumour growth. A possible explanation for the reported discrepancies on ERK phosphorylation and cell proliferation is that the final result depends on the balance of the stimulating and the inhibiting pathways in each experimental model and conditions.

The results presented herein suggest that the inhibition of ERK 1/2 phosphorylation in this experimental model is mediated by PKA and not by EPAC. These results are consistent with the pathways described for β -adrenoceptor agonists in MDA-MB-231 cells (Carie and Sebti, 2007) and in freshly dispersed rat parotid acinar cells (Soltoff and Hedden, 2010).

In conclusion, our results shown here demonstrated in two independent experimental models of breast cancer that the β -adrenoceptor agonists inhibited breast cancer cell proliferation and tumour growth when growing as xenografts. This effect is probably mediated by ERK 1/2 inhibited phosphorylation. The possibility of inhibiting tumour growth both in hormone-dependent as well as in hormone-independent tumours is promising. The β -adrenoceptor agonist isoprenaline and the β_2 -adrenoceptor agonist salbutamol (currently in use in humans for the treatment of asthma) were as effective as the α_2 -adrenoceptor antagonist rauwolfscine, providing possible novel therapeutic adjuvant treatments for breast cancer.

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Conflicts of interest

None.

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