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RESEARCH ARTICLE

α-Adrenoceptor stimulation attenuates melanoma growth in mice

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Background and Purpose: Recently, β-adrenoceptor blockade has emerged as a potential strategy to inhibit melanoma growth. It remains to be ascertained whether β-adrenoceptor stimulation by circulating catecholamines increases melanoma growth in mice.

Experimental Approach: B16F10 melanoma-bearing mice were used to evaluate effects of adrenaline and specific adrenoceptor (AR) ligands on tumour volume. AR expression and effects of AR ligands on cell viability, production of mitochondrial reactive oxygen species (mROS), and proliferation activity in B16F10 cells, were determined by biochemical analyses.

Key Results: Real-time polymerase chain reaction (qPCR) analyses revealed that B16F10 cells express α1B-, α2A-, α2B- and β2-ARs. We found that treatment with the α- and β-AR agonist adrenaline or with the synthetic catecholamine isoprenaline, which selectively stimulates β-ARs, did not affect melanoma growth. Conversely, adrenaline reduced tumour growth in mice cotreated with propranolol, a β1β2-AR antagonist. Adrenaline had no effect in tumour-bearing β1β2-AR knockout mice, in which β1- and β2-ARs are lacking, but it reduced tumour growth when co-administered with propranolol suggesting that tumour β2-ARs negatively regulate adrenaline antitumour activity. Additionally, we found that α1-AR stimulation with cirazoline yielded a decrease in B16F10 melanoma size. These effects on melanoma growth were paralleled by reduced cell viability and proliferation activity as well as increased mROS production in α1-AR-stimulated B16F10 cells. Decreased viability, proliferation and mitochondrial function in B16F10 cells also occurred after α2-AR stimulation by α2-AR agonist ST91.

Conclusions and Implications: In the B16F10 melanoma model, stimulation of α-AR subtypes yields *in vivo* and *in vitro* anticancer activity.

KEYWORDS
adrenoceptors, catecholamines, melanoma, α-adrenoceptor agonists

Abbreviations: ADR, adrenaline; CIRA, cirazoline; PRA, prazosin; PRO, propranolol; ST91, 2-[2,6-diethylphenylamino]-2-imidazoline; YOH, yohimbine.

Sonia Maccari and Maria Buoncervello equally contributed to this work. Lucia Gabriele and Giuseppe Marano contributed to this work as senior authors.

1 | INTRODUCTION

Adrenergic antagonists such as α- and β-adrenoceptor blockers are pharmacological agents used alone or in combination with other drugs to treat a variety of clinical conditions such as myocardial ischemia, heart failure, capillary haemangioma, supraventricular arrhythmias, migraine and hypertension and to relieve symptoms associated with benign prostatic hyperplasia. Such therapeutic effects are explained by the ability of adrenergic antagonists to bind **adrenoceptors** (ARs), thus inhibiting the effects of endogenous catecholamines such as **adrenaline** (ADR) and **noradrenaline** (NA). There are three subclasses of ARs, α1, α2 and β, with nine adrenoceptor subtypes in total. The α-ARs include six subtypes (**α1A-AR**, **α1B-AR**, **α1D-AR** and **α2A-AR**, **α2B-AR**, **α2C-AR**) whereas the β-ARs have three subtypes (**β1-AR**, **β2-AR** and **β3-AR**). α1-ARs and α2-ARs are virtually ubiquitous in human tissues. β1-ARs are primarily expressed in the heart, β3-ARs in several tissues such as ovaries, gall bladder, placenta and urinary bladder, whereas β2-ARs are distributed in many organs and tissues, including the central nervous system. In recent years, a link among AR stimulation and cancer progression has been found, and the blockade of β-AR signalling has emerged as a potential therapeutic strategy for treatment of various cancer types including melanoma, the most aggressive and life-threatening skin cancer, and recently was tested in cancer patients, showing marked improvement in tumour biomarker and long term cancer outcomes (de Giorgi et al., 2018; Haldar et al., 2020; Ricon et al., 2019; Shaashua et al., 2017). In particular, treatment with **propranolol** (PRO), a β1- and β2-AR antagonist, attenuates cancer growth both in preclinical models of melanoma and in patients with melanoma (Barbieri et al., 2012; Bucsek et al., 2017; Calvani et al., 2019; de Giorgi et al., 2018; Glasner et al., 2010; Hasegawa & Saiki, 2002; Maccari et al., 2017; Wrobel & Le Gal, 2015). Moreover, in addition to the beneficial effect of PRO, some of these preclinical studies showed that there is a relationship between the levels of circulating catecholamines and melanoma growth suggesting that β-AR stimulation may enhance melanoma growth (Barbieri et al., 2012; Bucsek et al., 2017; Glasner et al., 2010). However, whether β-AR stimulation induced by circulating catecholamines is responsible for the effect that is inhibited by PRO remains to be determined.

Activation of sympathetic nervous system in response to diverse stimuli such as physical activity and psychological stress causes the release of NA and ADR from postganglionic neurons and the adrenal medulla, respectively. Once released, these two catecholamines bind both α- and β-ARs on peripheral tissues and promote the physiological and metabolic responses that follow stimulation of the sympathetic nervous system. ARs belong to the guanine nucleotide-binding G-protein coupled receptor (**GPCR**) superfamily and are membrane receptors that activate heterotrimeric G-proteins following the binding of a ligand. β-ARs activate **adenylyl cyclase** through Gs-protein, α2-ARs inhibit adenylyl cyclase through Gi-protein, and α1-ARs increase intracellular Ca^{2+} through Gq-protein. In addition to activating classical G-protein signalling, recent findings in the molecular biology of GPCRs show that these receptors can interact with other transduction proteins such as β-arrestins. These are inhibitors of G-protein signalling promoting receptor desensitization and internalization and also function to activate **mitogen-activated protein kinases**, especially **ERK1/2**, via G-protein-independent signalling pathways (Smith et al., 2018).

NA and ADR are mixed adrenergic agonists, that is, they are able to activate all of these AR subtypes, which are often co-expressed in the same cell. Thus, the response to NA or ADR is often the result of activation of multiple AR subtypes which could have opposite biological effects on the same system. The net effect will depend on catecholamine affinities for AR subtypes, as well as the AR density. For example, therapeutic doses of ADR increase blood flow to skeletal muscles via dominant stimulation of β-ARs which produces vascular smooth muscle relaxation and a decrease in vascular peripheral resistance. However, after the administration of PRO which is a β1- and β2-AR antagonist, only vasoconstriction due to α-AR stimulation occurs (Hoffman, 2001; Shank, 1967). Therefore, we also evaluated whether PRO, by blocking β1- and β2-ARs, unmasks the antitumour effect of stimulation of α-AR subtypes induced by catecholamines.

The objective of this study was threefold. First, determining whether β-AR stimulation by the catecholamine ADR enhances the growth of melanoma. Second, because ADR is both α- and β-AR agonist, we tested whether PRO, by blocking β1- and β2-ARs, unmasks the antitumour effect of stimulation of α-AR subtypes by ADR. Third, because both host cells and B16F10 tumour cells express β-ARs, mice

What is already known

- Melanoma is the most aggressive and life-threatening skin cancer.
- Blockade of β-adrenoceptors (β-ARs) has emerged as a potential therapeutic strategy for treatment of melanoma.

What does this study add

- B16F10 melanoma cells express α-adrenoceptors (α1B-, α2A- and α2B-AR) and β-adrenoceptors (β2-AR).
- β-AR blockade unmasks the antimelanoma activity of adrenaline. α1-AR stimulation inhibits B16F10 melanoma growth.

What is the clinical significance

- The results of the present study could help identify new therapeutic strategies for melanoma treatment.

deficient in both β1- and β2-ARs (β1β2-AR knockout [KO]) were used to evaluate the importance of host cells versus tumour cells as targets of β1- and β2-AR stimulation by ADR. To this aim, the B16F10 murine melanoma model was used (Maccari et al., 2017). The results of the present study suggest that stimulation of α-AR subtypes negatively affects the growth of B16F10 melanoma, but β2-AR stimulation extinguishes this effect.

2 | METHODS

2.1 | Animal welfare and ethics statement

Animal studies were performed in compliance with the ARRIVE and BJP guidelines (McGrath & Lilley, 2015) and the standards required by the UKCCCR guidelines on the welfare and use of animals in cancer research (Workman et al., 2010). Procedures involving animals were also carried out in compliance with the Italian guidelines for animal care (DL 116/92) and the European directives (2010/63/EU) and were approved by the Italian Ministry of Health (code n.744/2016-PR). Animals were deeply anaesthetized with **isoflurane** before being killed by cervical dislocation. All efforts were made to minimize animal suffering. Fifty-nine C57BL/6 male mice weighing 22.7 ± 0.9 g were used in this study and were purchased from Charles River Laboratories (Calco, Como, Italy). Additionally, 18 β1β2-AR double KO male mice weighing 28.8 ± 4.3 g were used. β1β2-AR KO, originally generated by Rohrer et al. (1999), were purchased from the Jackson Laboratory (*Adrb1*^{tm1Bkk}*Adrb2*^{tm1Bkk}/J, Stock No. 003810, Bar Harbor, ME, USA) and interbred to generate subsequent generations. Genotyping was done on tail DNA by polymerase chain reaction (PCR) as described (Maccari et al., 2020). Mice were housed in groups of three adults per cage and maintained in standardized conditions in our animal facility at 20°C ± 2°C room temperature, 40% ± 5% relative humidity and a 12-h light/dark cycle with dawn/dusk effect, water and standard pathogen-free chow diet provided ad libitum. We did not use female mice which is a limitation of our study.

2.2 | Cell cultures and reagents

B16F10 cell line (RRID:CVCL_0159) was cultured at 37°C in humidified atmosphere containing 5% CO_2. Low passage B16F10 murine melanoma cell line, obtained from ATCC, was maintained in DMEM high glucose medium (EuroClone, West York, UK) supplemented with 10% FBS (EuroClone, West York, UK) in the presence of penicillin and streptomycin.

2.3 | Melanoma model and treatment

The B16F10 melanoma model, the most frequently used syngeneic murine melanoma, was established as described (Maccari et al., 2017). B16F10 mouse melanoma cells (8×10^5 cells) in 200-μl PBS were injected s.c. into the back of C57BL/6 male mice.

Treatments were started immediately after the injection of the tumour cells and continued until Day 16, when mice were sacrificed. Tumour length (L) and width (W) were measured using a calliper, and the tumour volume was calculated as $L \times W^2 \times 0.5$.

Mice were randomly allocated to groups of six animals each. The control group was treated with vehicle (sterile saline) whereas the other groups received ADR, ADR plus PRO, **isoprenaline** (ISO) or **cirazoline** (CIRA), a selective α1-AR agonist. ADR, ISO and CIRA were dissolved in sterile saline and administered intraperitoneally (i.p.) at the dose volume of 5 ml·kg^{-1} and at the doses of 0.5, 1.0 and 0.3 mg·kg^{-1}·day^{-1} for 16 consecutive days, respectively. PRO was dissolved in tap water at the concentration of 0.5 g·L^{-1}, which corresponds to a dose of about 60 mg·kg^{-1}·day^{-1}. β1β2-AR KO mice were treated with vehicle (sterile saline), ADR or ADR plus PRO at the above dosages. Doses for systemic administration of β-AR ligands were selected on the basis of a detailed literature search. ISO at the dose of 1 mg·kg^{-1} increases heart rate by approximately 100 bpm (Wang et al., 2017) and is apparently well tolerated in mice following repeated administration (Pérez Piñero et al., 2012). ADR at the dose of 0.5 mg·kg^{-1} mobilizes NK cells in mice, an effect that is blocked by PRO (Pedersen et al., 2016). Furthermore, this dose appears well tolerated following repeated administration in mice (Pedersen et al., 2016). To minimize animal stress, PRO was administered in drinking water at a dose of about 60 mg·kg^{-1}·day^{-1}. It was used at the dose that produces plasma concentrations in mice ranging from 59 to 75 ng·ml^{-1} which are within therapeutic range (Fabritz et al., 2010; Musumeci et al., 2011). With regard to CIRA, the dose of 0.3 mg·kg^{-1} was greater than the dose of 0.2 mg·kg^{-1} which abolishes pentylenetetrazole (PTZ)-induced seizures in **dopamine β-hydroxylase** KO mice, an effect that is blocked by the administration of **prazosin** ((PRA), an α1-AR antagonist (Weinshenker et al., 2001), but lower than the dose of 0.6 mg·kg^{-1}·day^{-1} that causes an approximately 15% increase in cardiac mass when administered for 14 days in mice (Papay et al., 2013).

2.4 | RNA isolation and quantification

Total RNA was extracted from tissue or cell samples by using TRIzol (Invitrogen, Monza, Italy) and purified by using RNA purelink mini kit (Invitrogen, Monza, Italy). The concentration and purity of the RNA solution were determined by using a NanoDrop spectrophotometer (Fisher Scientific, Monza, Italy), whereas its overall quality was analysed using the Agilent 2100 bioanalyser with an RNA LabChip (RNA 6000 Nano kit, Agilent, Milan, Italy). cDNA was obtained by using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). mRNA expression levels of α-AR subtypes (α1A, α1B, α1D and α2A, α2B, α2C) and β-ARs (β1-AR and β2-AR) were determined using TaqMan gene expression assays (gene ADRA1a cod. Mm 00442668_m1, gene ADRA1b cod. Mm 00431685_m1, gene ADRA1d cod. Mm 01328600_m1, gene ADRA2a cod. Mm 00845383_s1, gene ADRA2b

cod. Mm 00477390_s1 and gene ADRA2c cod. Mm 00431686_s1 for α-AR subtypes; gene ADRB1 cod. Mm 00431701_s1 and gene ADRB2 cod. Mm 02524224_s1, for β-AR subtypes; Applied Biosystems, Foster City, CA, USA). Real-time, quantitative PCR (qPCR) analysis was performed using 7500 RealTime PCR system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (cod. Mm 99999915_g1, Applied Biosystems, Foster City, CA, USA) was used as reference gene and relative expression levels were reported as ΔCt. The ΔCt, that is, the difference between Ct (cycle threshold) of interest gene and Ct of reference gene, was used for statistical analysis (Schmittgen & Livak, 2008).

2.5 | Colour power Doppler

B16F10 mouse melanoma cells were injected as described above. Mice were treated with CIRA at the dose of 0.3 $mg \cdot kg^{-1}$. Colour power Doppler imaging was performed to quantitatively assess tumour blood flow, using an 18-MHz linear array probe attached to a Hitachi Aloka ultrasound scanner (Milan, Italy). Care was taken to minimize motion artefact during image acquisition by positioning the transducer parallel to the long axis of the tumour. Post-processing of tumour images with specific software allowed for the calculations of colour pixel density (CPD = numbers of colour pixels/total number of pixels) and vascularity index (VI = sum of colour pixel intensities/total number of pixels) for regions of interest.

Measurements were acquired before and after CIRA administration, approximately 10 min after the drug injection. Mean values of CPD and VI before and after treatment were calculated. CPD is an indicator of in vivo tumour vascular density, whereas VI is more indicative of blood flow through the tumour.

2.6 | Cell culture and treatments

Mouse melanoma B16F10 cells were cultured as described above and treated for 48 h with 1 μM of the AR agonist ADR, the β-AR agonist ISO, the β1β2-AR antagonist PRO, the α1-AR agonist CIRA, the α2-AR agonist ST91 (2-[2,6-diethylphenylamino]-2-imidazoline) (Jasper et al., 1998), the α1-AR antagonist PRA or the α2-AR antagonist yohimbine (YOH), alone or in combination. Cells untreated were considered as control.

2.7 | Cell death assay

Dead cells were quantified by calcein-AM (Thermo Fisher Scientific, Milan, Italy), a cell-permeant dye used to determine cell viability in eukaryotic cells. In metabolically active cells only, the nonfluorescent calcein-AM is converted to green-fluorescent calcein. At the end of treatments, cells were stained with 2-μM calcein-AM for 30 min in the dark at 37°C and immediately analysed on a cytometer. Results were expressed as percentage of cells negative for calcein-AM.

2.8 | Mitochondrial membrane potential

The mitochondrial membrane potential of both control and treated cells was studied by using tetramethylrhodamine ester 1 μM (TMRM; Thermo Fisher Scientific, Milan, Italy). JC-1 (Molecular Probes, Eugene, OR, USA) was also used as described previously (Matarrese et al., 2005). After staining cells were immediately analysed on a cytometer. Acquisition of the samples was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 argon laser and with a 635 red diode laser and at least 10,000 events per sample were run. Data were analysed using the Cell Quest Pro software (BD Biosciences, San Jose, CA, USA). Results were reported as median fluorescence intensity (MFI).

2.9 | Mitochondrial reactive oxygen species

To detect mitochondrial reactive oxygen species (mROS), B16F10 cells (5×10^4) were incubated with 5-μM MitoSOX (red mitochondrial superoxide indicator, Thermo Fisher Scientific, Milan, Italy) in complete medium, for 30 min at 37°C. After staining, cells were immediately analysed on a cytometer. Acquisition of the samples was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 argon laser and with a 635 red diode laser and at least 10,000 events per sample were run. Data were analysed using the Cell Quest Pro software (BD Biosciences, San Jose, CA, USA). Results were reported as MFI.

2.10 | Proliferation assay

Proliferation activity was analysed by Ki-67 nuclear antigen expression using the phycoerythrin (PE)-mouse anti-human Ki-67 Set according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA). All samples were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488 argon laser and with a 635 red diode laser and at least 10,000 events per sample were run. Data were analysed using the Cell Quest Pro software (BD Biosciences, San Jose, CA, USA). Results were reported as MFI.

2.11 | Data and statistical analysis

The manuscript complies with BJP's recommendations and requirements on experimental design and analysis (Curtis et al., 2018). Data are expressed as mean ± SD and analysed using GraphPad Prism version 5.03 software program (GraphPad Software Inc., San Diego, CA, USA) (RRID:SCR_002798). Studies were designed to generate groups of equal size, using randomization and blinded analysis. Mice were randomly allocated to the experimental groups using the Excel Random function. The person performing tumour size or power Doppler measurements was blinded with regard to group allocation of animals. All available data were included in the

analysis and presentation without of removal potential outliers. The null hypothesis is that stimulation of α-AR subtypes does not affect growth and function of B16F10 melanoma cells. Descriptive analysis has been applied to all other outcomes of the study. Unpaired *t* test or ANOVA were performed to accept or reject the null hypothesis. Differences with $P < 0.05$ were considered significant.

The size of animal groups was selected based on previous studies with adrenergic drugs in the same model (Maccari et al., 2017). Furthermore, taking into account the results from our preliminary experiments on B16F10 cell survival with adrenergic drugs, for a *t* test with Bonferroni correction for multiple comparison, a fivefold difference between means (8% control group vs. 40% treated group), a SD of 3 and a Bonferroni-adjusted alpha level of 0.005 (0.05/10), five biochemical assays per group guaranteed a power of 80% using G*Power 3.1 (RRID:SCR_013726).

Group size is the number of independent values, and statistical analysis was done using these independent values. Statistical analysis was undertaken only for studies where each group size was at least $n = 5$.

In multigroup studies with parametric variables, post hoc tests were conducted only if *F* in ANOVA achieved the necessary level of statistical significance and there was no significant variance inhomogeneity as assessed by Bartlett's test. Gene expression and cell viability measurements were normalized to correct the fluctuations in target gene expression levels caused by technical variations in the quantity of total RNA or in the cDNA synthesis or to correct inter-well variability introduced by cell number, respectively. All gene expression data have been shown both with and without correction for the reference gene.

2.12 | Materials

ADR, ISO, PRO, CIRA, PRA and YOH were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas ST91 (2-[2,6-diethylphenylamino]-2-imidazoline) was from R&D Systems (R&D Systems, Minneapolis, MN, USA). DMEM high glucose medium and fetal bovine serum (FBS) (EuroClone, West York, UK), phosphate buffered saline (PBS), penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA), calcein-AM, tetramethylrhodamine ester (TMRM) and red mitochondrial superoxide indicator (MitoSOX) (Thermo Fisher Scientific, Milan, Italy), JC-1 (Molecular Probes, Eugene, OR, USA), phycoerythrin (PE)-mouse anti-human Ki-67 (BD Biosciences, San Jose, CA, USA).

2.13 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3 | RESULTS

3.1 | B16F10 cells express both α- and β-ARs

Given that PRO blocks both β1- and β2-ARs and that catecholamines such as ADR and NA are mixed α- and β-AR agonists, we evaluated whether cultured B16F10 tumour cells express β1- and β2-ARs as well as α-ARs. Heart tissue samples obtained from C57BL/6 mice were used as positive controls. As shown in Figure 1, qPCR analysis revealed that under basal conditions B16F10 cells express β2-ARs, which is in line with the results from previous studies (Calvani et al., 2019; Dal Monte et al., 2013), as well as α1B-, α2A- and α2B-AR subtypes (Figure 1a,b). Thus, B16F10 cells express multiple ARs that are target of catecholamines.

3.2 | ADR reduces tumour growth in mice cotreated with PRO

Because previous studies showed that PRO, a β1- and β2-AR antagonist, markedly reduces the growth of B16F10 melanoma, we evaluated whether treatment with catecholamines, which induce β-AR stimulation, enhances B16F10 melanoma growth. We found that B16F10 melanoma volume slightly decreased in mice treated with ADR (3.29 ± 0.48 cm^3, $P > 0.05$) or ISO, a selective β-AR agonist (3.38 ± 0.41 cm^3, $P > 0.05$) compared with control mice (3.49 ± 0.50 cm^3). These results suggest that β-AR stimulation by circulating catecholamines is not enough to promote B16F10 melanoma growth (Figure 2a).

Because both host cells and B16F10 cancer cells have α1-, α2- and β-ARs and it has been shown that the response to catecholamines is determined according to which AR is predominant, we then determined effects of stimulation of α-AR subtypes on B16F10 melanoma growth. Because ADR is both α- and β-AR agonist, we evaluated whether PRO, by blocking β1- and β2-ARs, unmasks the antitumour effect of stimulation of α-AR subtypes by ADR. We found a significant difference between the control group and the group cotreated with ADR plus PRO as determined by one-way ANOVA ($F(3,19) = 44.3$, $P < 0.05$). A Bonferroni post hoc test revealed that tumour volume reduced by 2.85 cm^3 between control and ADR plus PRO groups ($P < 0.05$), by 2.65 cm^3 between ADR and ADR plus PRO groups ($P < 0.05$) and by 2.78 cm^3 between PRO and ADR plus PRO groups ($P < 0.05$) (Figure 2b). Minimal differences in tumour volume were observed between the control (3.49 ± 0.50 cm^3) and PRO groups (3.42 ± 0.17 cm^3, $P > 0.05$) or the PRO (3.42 ± 0.17 cm^3) and ADR (3.29 ± 0.48 cm^3, $P > 0.05$) groups (Figure 2b). Collectively, these results suggest that stimulation of α-AR subtypes negatively affects B16F10 melanoma growth whereas stimulation of β1- and β2-ARs opposes this effect.

3.3 | Tumour cell β2-ARs are involved in ADR antitumour effects

Because β2-ARs are present on both host and tumour cells, β1β2-AR KO mice, in which both β1- and β2-AR subtypes are

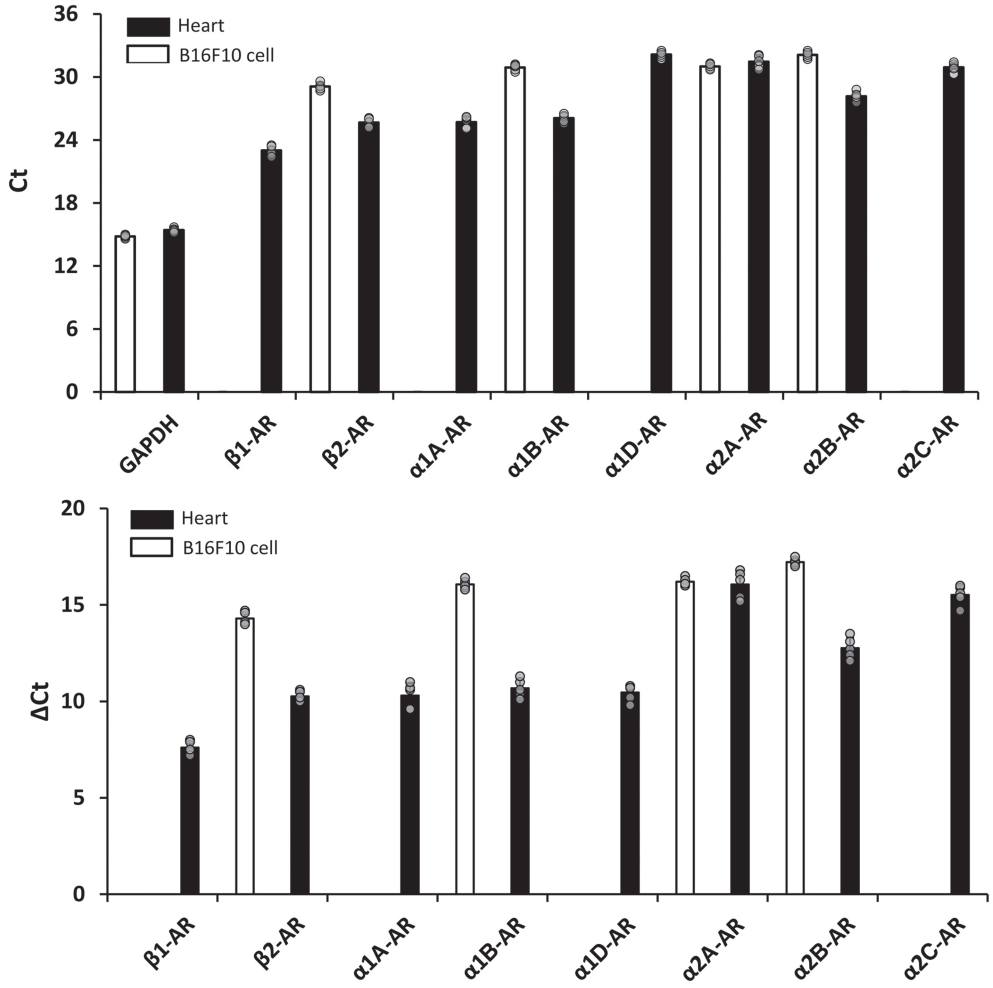

FIGURE 1 Gene expression of β1-ARs, β2-ARs and α-ARs. mRNA levels of tested genes in cultured cancer B16F10 cells were assessed by qPCR. Gene expression data are reported (a) without (Ct) and (b) with (ΔCt) correction for GAPDH (reference gene). Heart tissue samples from C57BL/6 male mice were used as positive controls. Results are from five independent experiments (mean ± SD) from untreated, cultured B16F10 cells. ΔCt = difference between Ct of interest gene and Ct of reference gene; Ct = cycle threshold

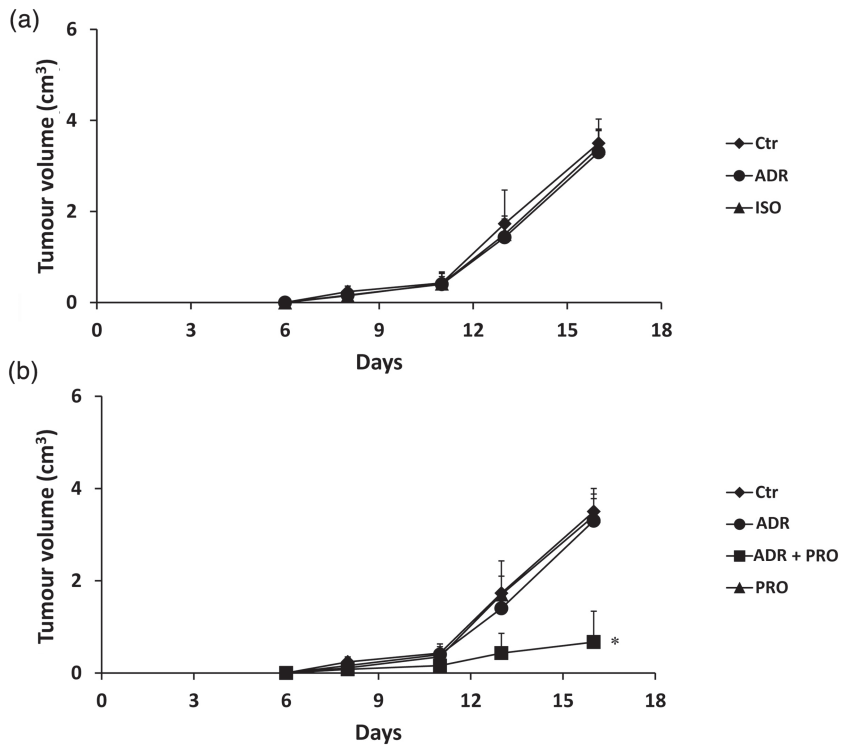

FIGURE 2 ADR reduces tumour growth in mice cotreated with PRO. (a) Treatment with natural or synthetic catecholamines had no effect on melanoma growth. B16F10 tumour-bearing mice were treated with vehicle (Ctr), adrenaline (ADR) (0.5 mg·kg^{-1}·day^{-1}) or isoprenaline (ISO) (1 mg·kg^{-1}·day^{-1}). Catecholamines were administered via i.p. route once daily for 16 days. Data represent mean tumour volume ± SD ($n = 6$ per group). (b) ADR reduced tumour growth in mice cotreated with propranolol (PRO), a β1β2-AR antagonist. ADR was administered via i.p. at the dose of 0.5 mg·kg^{-1}·day^{-1} whereas PRO in drinking water at the dose of about 60 mg·kg^{-1}·day^{-1}. ($n = 6$ per group; $^{*}P < 0.05$ compared with the Ctr group or ADR group or PRO group)

lacking, were used to evaluate the relative importance of host cells versus tumour cells as targets of ADR. To this aim, wild-type (WT) and β1β2-AR KO mice were treated with saline or ADR. We found that there was no significant interaction between the effects of genotype and ADR treatment on B16F10 melanoma growth as assessed by two-way ANOVA ($F(1,20) = 1.25$, $P > 0.05$). There were slight differences in tumour growth between WT and KO mice (3.22 ± 0.55 and 3.19 ± 0.17 cm^3 respectively, $P > 0.05$), which is in line with results from a previous study (Sereni et al., 2015), or between ADR-treated WT and ADR-treated KO mice (3.34 ± 0.58 and 2.92 ± 0.31 cm^3, respectively, $P > 0.05$) (Figure 3a). In a subsequent experiment, β1β2-AR KO mice were treated with saline, ADR or ADR plus PRO, a β1β2-AR antagonist. We found that there was a significant difference between groups as determined by one-way ANOVA ($F(2,15) = 91.5$, $P < 0.05$). A Bonferroni post hoc test revealed that tumour volume reduced by 2.48 cm^3 between untreated KO group and ADR plus PRO-treated KO group ($P < 0.05$) and by 2.21 cm^3 between ADR-treated KO group and ADR plus PRO-treated KO group ($P < 0.05$) (Figure 3b). Slight differences in tumour volume were observed between KO and ADR-treated KO groups (3.19 ± 0.17 and 2.92 ± 0.31 cm^3, respectively, $P > 0.05$) (Figure 3b). Collectively, these results suggest that β2-ARs located on B16F10 melanoma cells negatively regulate ADR antitumour activity.

3.4 | α1-AR stimulation inhibits tumour growth in mice

To ascertain whether antitumour effects of ADR are related to stimulation of α-AR subtypes, we administered CIRA, a selective α1-AR agonist, at the dose of 0.3 mg·kg^{-1}·day^{-1}. The results showed that mice treated with CIRA had significantly lower tumour volume (0.93 ± 0.54 cm^3, $t(10) = 8.2$, $P < 0.05$) at the end of the experiment compared with untreated mice (3.44 ± 0.52 cm^3) (Figure 3c).

Because CIRA is an α1-AR agonist and therefore a potential vasoconstrictor, we studied its effects on tumour perfusion by colour power Doppler. After 10 days of tumour growth, Doppler signal was evaluated before and after CIRA treatment. As shown in Figure 3c, CIRA did not affect tumour perfusion as evidenced by perfusion indices (Figure 3d,e). Together, these results imply that α1-AR stimulation affects tumour growth without altering tumour perfusion.

3.5 | Stimulation of α-AR subtypes affects cell viability, mitochondrial function and proliferation activity of B16F10 cells

Next, we tested the effects of several AR ligands or their combinations on some cell functions of cultured B16F10 cells. Multiple *t* test

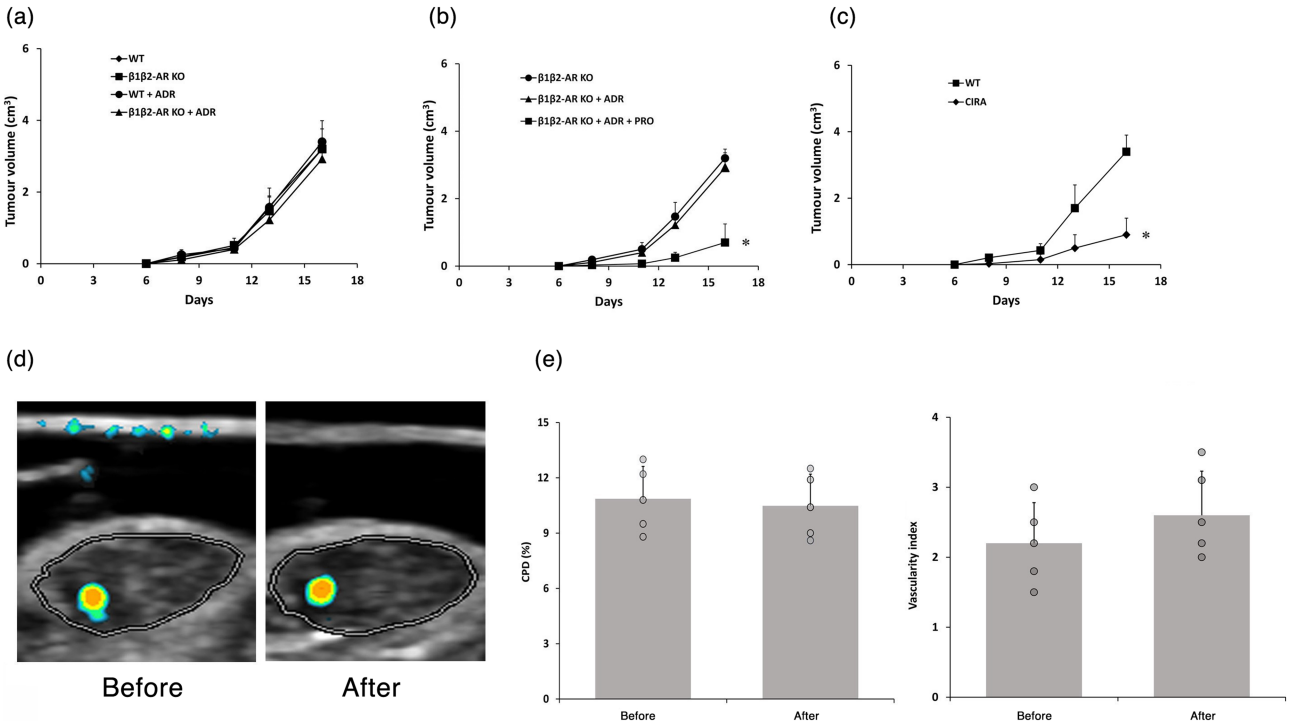

FIGURE 3 Stimulation of α-AR subtypes inhibits tumour growth in mice. (a) Adrenaline (ADR) does not affect tumour growth in β1β2-AR knockout (KO) mice, in which both β1- and β2-AR subtypes are lacking ($n = 6$ per group). WT, wild-type mice. (b) ADR reduced tumour growth in KO mice cotreated with propranolol (PRO) ($n = 6$ per group; $^*P < 0.05$ vs. KO or ADR-treated KO groups). (c) Cirazoline (CIRA), a selective α1-AR agonist, inhibits melanoma growth ($n = 6$ per group; $^*P < 0.05$ vs. WT group). (d) Representative images of power Doppler assessment of blood flow in melanoma tumours before and after CIRA treatment. (e) Left panel: colour pixel density (CPD) per cent (CPD = number of colour pixels/total number of pixels). Right panel: vascularity index (VI = sum of colour pixel intensities/total number of pixels). Five mice underwent non-invasive colour Doppler imaging to quantitatively assess tumour blood flow, using an 18-MHz linear array. No differences in vascularity indices were seen after CIRA treatment

comparisons were carried out and tested against a Bonferroni-adjusted alpha level of 0.005 (0.05/10). First, we found that cell death, as assessed by calcein-AM staining, significantly increased after treatment for 48 h with α1-AR agonist CIRA (49.4% ± 14.8%, $t(8) = 6.5$, $P < 0.05$), α2-AR agonist ST91 (53.4% ± 7.0%, $t(8) = 14.9$, $P < 0.05$) or ADR plus PRO (45.1% ± 5.5%, $t(8) = 15.5$, $P < 0.05$) compared with control (5.7% ± 1.3%) (Figure 4a). Moreover, the α1-AR antagonist PRA significantly inhibited CIRA effects (7.8% ± 0.8%, $t(8) = 6.3$, $P < 0.05$), whereas the α2-AR antagonist YOH inhibited ST91 effects (8.2% ± 1.6%, $t(8) = 14.02$, $P < 0.05$). Minimal changes in cell death were observed in B16F10 cells treated with ADR (6.0% ± 1.5%, $P > 0.05$), ISO (8.7% ± 1.5%, $P > 0.05$), PRA (6.3% ± 1.3%, $P > 0.05$), YOH (7.9% ± 1.0%, $P < 0.05$) or PRO (7.8% ± 1.4%, $P < 0.05$) compared with control (5.7% ± 1.3%) (Figure 4a). Second, because mitochondria have been attributed a central role in cell death and are considered both a source and an important target of ROS damage (Galadari et al., 2017; Moloney & Cotter, 2018), we have determined the effects of AR ligands on mitochondrial ROS production in cultured B16F10 cells. On this cell type, we found that mitochondrial ROS production, as assessed by MitoSOX staining, significantly increased after treatment for 48 h with α1-AR agonist CIRA (220.3 ± 9.9 MFI, $t(8) = 32.2$, $P < 0.05$), α2-AR agonist ST91 (263.2 ± 6.6 MFI, $t(8) = 53.9$, $P < 0.05$) or ADR plus PRO (306.1 ± 5.1 MFI, $t(8) = 75.3$, $P < 0.05$) compared with control (59.2 ± 5.2 MFI) (Figure 4b). Moreover, the α1-AR antagonist PRA significantly inhibited CIRA effects (80.3 ± 1.6 MFI, $t(8) = 33.3$, $P < 0.05$), whereas the α2-AR antagonist YOH inhibited ST91 effects (67.3 ± 4.9 MFI, $t(8) = 52.9$, $P < 0.05$). Slight or moderate changes of ROS production were observed in B16F10 cells treated with ADR (61.9 ± 5.4 MFI, $P > 0.05$), ISO (61.4 ± 2.5 MFI, $P > 0.05$), PRA (65.8 ± 3.5 MFI, $P < 0.05$), YOH (71.6 ± 1.6 MFI, $P < 0.05$) or PRO (71.3 ± 5.3 MFI, $P < 0.05$) compared with control (59.2 ± 5.2 MFI) (Figure 4b). Third, these alterations were paralleled by an increased mitochondrial membrane potential (ΔΨm), that is, hyperpolarization, considered as a mitochondrial alteration typical of

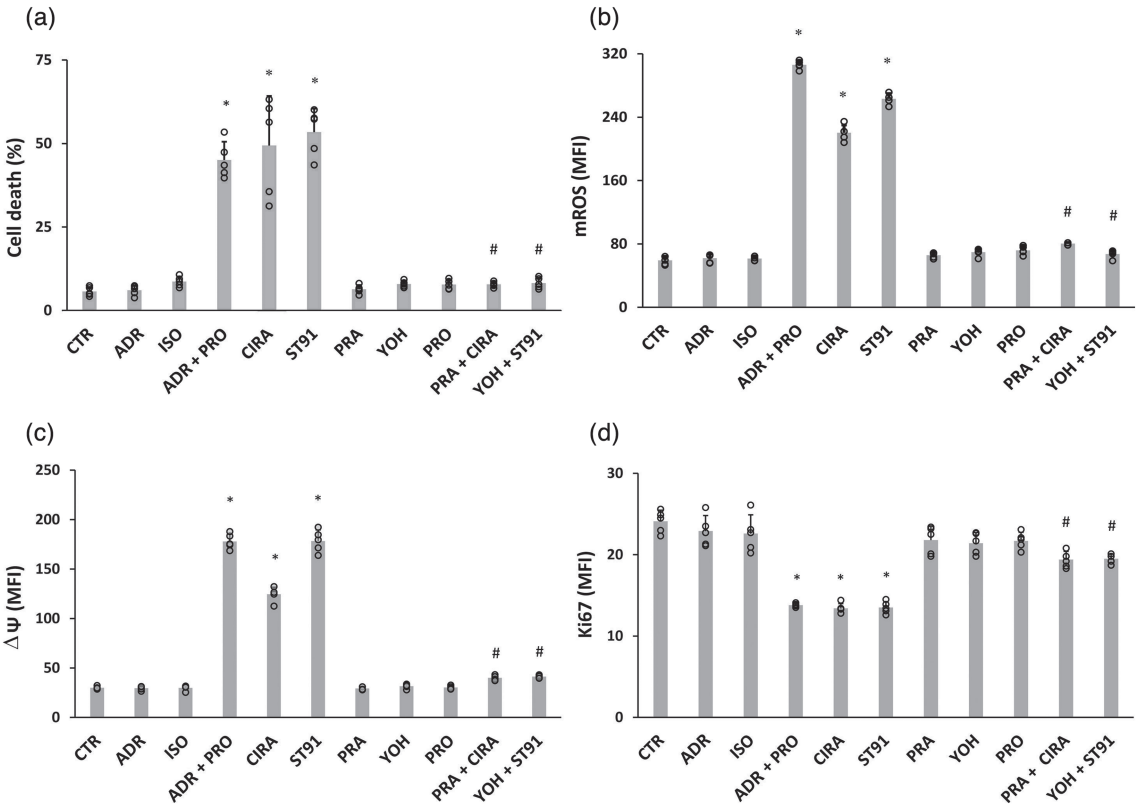

FIGURE 4 Decreased cell viability, mitochondrial function and proliferation activity in B16F10 cells after stimulation of α-AR subtypes. (a) Bar graph showing flow cytometric analysis after cell staining with calcein-AM (which is retained in the cytoplasm of live cells) of B16F10 cells treated for 48 h with different adrenoceptor ligands. Data represent the percentage of calcein-negative cells ($n = 5$). MFI, median fluorescence intensity; $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus the corresponding α-agonist treatment. (b) Bar graph showing flow cytometric evaluation, performed by using MitoSOX-red, of mitochondrial ROS production. Data represent the MFI in FL-2 fluorescence channel ($n = 5$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus the corresponding α-agonist treatment. (c) Bar graph showing the analysis of mitochondrial membrane potential (ΔΨm) obtained by cytofluorimetric analysis performed by using TMRM. Data represent the MFI in FL-2 fluorescence channel ($n = 5$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus the corresponding α-agonist treatment. (d) Bar graph showing the analysis of proliferation activity obtained by cytofluorimetric analysis performed by using Ki67. Data represent the MFI in FL-2 fluorescence channel ($n = 5$). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus the corresponding α-agonist treatment. All drugs were used at the concentration of 1 μM. Control (CTR), adrenaline (ADR), isoprenaline (ISO), propranolol (PRO), cirazoline (CIRA), ST91, prazosin (PRA), yohimbine (YOH)

apoptosis preceding mitochondrial depolarization and cytochrome C release in the cytoplasm (Mahapatra et al., 2017; Matarrese et al., 2005). In fact, we found that ΔΨm, as assessed by TMRM staining, significantly increased after treatment for 48 h with α1-AR agonist CIRA (124.4 ± 7.2 MFI, $t(8) = 28.6$, $P < 0.05$), α2-AR agonist ST91 (178.3 ± 11.1 MFI, $t(8) = 29.6$, $P < 0.05$) or ADR plus PRO (177.9 ± 7.6 MFI, $t(8) = 42.5$, $P < 0.05$) compared with control (30.0 ± 1.4 MFI) (Figure 4c). Again, the α1-AR antagonist PRA significantly inhibited CIRA effects (40.0 ± 2.3 MFI, $t(8) = 24.7$, $P < 0.05$), whereas the α2-AR antagonist YOH inhibited ST91 effects (41.2 ± 1.3 MFI, $t(8) = 27.3$, $P < 0.05$). Minimal changes of ΔΨm were observed in B16F10 cells treated with ADR (29.5 ± 1.9 MFI, $P > 0.05$), ISO (29.9 ± 2.5 MFI, $P > 0.05$), PRA (29.3 ± 1.6 MFI, $P > 0.05$), YOH (31.5 ± 2.2 MFI, $P > 0.05$) or PRO (30.2 ± 1.6 MFI, $P > 0.05$) compared with control (30.0 ± 1.4 MFI) (Figure 4c). Fourth, we found that proliferation activity, as assessed by Ki67 staining, significantly decreased after treatment for 48 h with α1-AR agonist CIRA (13.4 ± 0.6 MFI, $t(8) = 15.9$, $P < 0.05$), α2-AR agonist ST91 (13.5 ± 0.7 MFI, $t(8) = 15.2$, $P < 0.05$) or ADR plus PRO (13.8 ± 0.2 MFI, $t(8) = 16.5$, $P < 0.05$) compared with control (24.1 ± 1.4 MFI) (Figure 4d). Moreover, the α1-AR antagonist PRA significantly inhibited CIRA effects (19.3 ± 1.0 MFI, $t(8) = 11.4$, $P < 0.05$), whereas the α2-AR antagonist YOH inhibited ST91 effects (19.5 ± 0.6 MFI, $t(8) = 14.6$, $P < 0.05$). Slight changes in Ki67 were found in B16F10 cells treated with ADR (22.9 ± 1.9 MFI, $P > 0.05$), ISO (22.6 ± 2.3 MFI, $P > 0.05$), PRA (21.8 ± 1.7 MFI, $P > 0.05$), YOH (21.4 ± 1.3 MFI, $P < 0.05$) or PRO (21.7 ± 1.0 MFI, $P < 0.05$) compared with control (24.1 ± 1.4 MFI) (Figure 4d). Collectively, these results indicate that stimulation of α1- and α2-AR subtypes affects both survival rate and mitochondrial function as well as proliferation activity of cultured B16F10 melanoma cells.

4 | DISCUSSION

In recent years, inhibition of β-AR signalling has emerged as a potential strategy to inhibit melanoma growth. However, whether or not β-AR stimulation by circulating catecholamines enhances the growth of melanoma remains to be ascertained. In this study, we show that both ADR, an endogenous catecholamine, and ISO, a synthetic catecholamine that selectively binds β-ARs, do not enhance B16F10 melanoma growth in mice. Because catecholamines do not cross the blood–brain barrier, these results collectively suggest that stimulation of β-ARs in both tumour and peripheral tissues does not promote B16F10 melanoma growth.

A second, equally important result of this study is that stimulation of the α-adrenergic system negatively influences the growth of B16F10 melanoma, but β-AR stimulation extinguishes this effect. In fact, we found that ADR alone does not affect tumour growth whereas combined treatment with ADR plus PRO, a β1β2-AR antagonist that binds β-ARs and blocks their activation, markedly reduces tumour growth as well as survival, proliferation activity and mitochondrial function of B16F10 cultured cells. Also, we found that α1-AR stimulation with CIRA, an α1-AR agonist, inhibits melanoma growth by about 70% and that stimulation of α1- and α2-AR subtypes reduces survival, proliferation activity and mitochondrial function of B16F10 cultured cells.

Our study extends the results of previous research showing that the adrenergic system is involved in B16F10 melanoma growth. In particular, chronic stress induced by cold or immobilization is associated with an increase in the levels of circulating catecholamines, that is, ADR and NA, and an enhanced growth of melanoma (Barbieri et al., 2012; Bucsek et al., 2017; Glasner et al., 2010; Hasegawa & Saiki, 2002). Under these conditions, treatment with the β-blocker PRO reduced the deleterious effects of chronic stress on tumour growth suggesting that stimulation of β1- and/or β2-ARs located in the central nervous system and/or in peripheral tissues promotes melanoma growth. However, considering that catecholamines are mixed adrenergic agonists, that is, α1-, α2- and β-AR agonists, and are unable to cross the blood–brain barrier, another possibility is that treatment with PRO may have unmasked the antitumour effects of peripheral α-adrenergic system stimulation by circulating catecholamines. This hypothesis is supported by the results of the present study. Indeed, we found that chronic administration of ADR or ISO for 16 consecutive days does not affect tumour growth whereas the co-administration of ADR with PRO causes a relevant reduction in B16F10 melanoma growth suggesting that stimulation of β1- and β2-ARs located in peripheral tissues negatively regulates ADR antitumour activity.

Because α1- and α2-AR subtypes are present in both B16F10 melanoma cells, as evidenced by qPCR results, and the host, where they participate in the regulation of numerous functional processes including the regulation of blood flow, antitumour effects of ADR could be attributed to stimulation of host α-ARs, tumour α-ARs or both. The results of our study suggest that the antitumour effects of ADR are due to the stimulation of tumour α-AR subtypes. In fact, we found that ADR markedly reduced tumour growth in mice cotreated with PRO, a β1β2-AR antagonist, which binds both host and tumour β-ARs and blocks their activation. Conversely, ADR had no effect on melanoma growth when it was administered in β1β2-AR KO mice in which the host lacks both β1- and β2-ARs, but it again reduced tumour growth when co-administered with PRO suggesting that tumour β2-ARs negatively regulate ADR antitumour activity. These results agree with the results obtained in the in vitro study on B16F10 melanoma cells. Indeed, we observed that decreased survival, proliferation activity and mitochondrial function in B16F10 cultured cells also occurred after treatment with ADR plus PRO, CIRA, an α1-AR agonist, or ST91, an α2-AR agonist.

The present study also extends the current knowledge of AR crosstalk by demonstrating that β-AR activation negatively regulates effects mediated by stimulation of α1- and α2-AR subtypes on B16F10 melanoma cells. Crosstalk involving ARs has already been reported. For example, in rat neonatal cardiomyocytes, stimulation of α1-AR is able to inhibit β-AR-mediated cAMP accumulation (Barrett et al., 1993). Using transgenic mouse models, it has been found that overexpression of α1-AR causes β-AR desensitization (Rorabaugh et al., 2005). Also, in previous research, we found that stimulation of

β-ARs with ISO, a β-AR agonist, inhibits the fetal gene expression induced by α1-AR agonist phenylephrine and more generally that cAMP-mediated β-adrenergic signalling negatively regulates Gq cascade activation-induced fetal gene expression in cultured cardiomyocytes (Patrizio et al., 2008). Whether this mechanism also occurs in B16F10 melanoma cells with the activation of Gq which negatively regulates the growth of cancer cells, it remains to be determined.

Crosstalk also occurs between Gs- and Gi-protein pathways. In fact, we found that ADR has no effect on survival, proliferation activity and mitochondrial function of cancer cells whereas ST91 does.

ST91 is an α2-AR agonist that activates the Gi-protein pathway. This interaction is well known. In fact, the β2-ARs are coupled with the Gs-protein, whereas the α2-ARs are coupled to the Gi-protein. The Gs-protein coupled pathway stimulates adenylyl cyclase, which catalyses the formation of cAMP, but stimulation of the Gi-protein coupled pathway causes a reduction in cAMP.

α1-AR agonists are substances that cause vasoconstriction and, through this mechanism, can alter the blood flow to organs and tissues including the tumour. However, because the intratumour vasculature is structurally and functionally abnormal with vessels lacking anatomic elements required for normal vasoactive responses, a direct effect on tumour blood flow is extremely unlikely. An indirect effect is more likely and can be generated by vasoconstriction in healthy peritumoural tissues, which increases tumour blood flow. In fact, several studies indicate that the tumour behaves as a passive resistance, that is, tumour flow is inversely related to that in the host periphery (Isenberg et al., 2008; Zlotecki et al., 1995). This means that vasoconstriction of healthy peritumoural tissues could increase tumour flow through inverse steal effects. Given that the abnormal tumour environment is an important contributor to tumour growth, the increase in flow and the consequent reduction of intratumoural hypoxia could influence B16F10 melanoma growth. Therefore, we evaluated whether administration of the α1-AR agonist CIRA is capable of altering the tumour blood flow. The analysis of the blood flow using the power Doppler technique shows that the α1-agonist at the dose used in this study does not affect vascularity index suggesting that the anticancer effect is not attributable to alterations in the tumour blood flow.

A few additional features of our experiments are worth commenting on. First, agonists of α1- and α2-AR subtypes can increase vascular tone and, in turn, arterial blood pressure. However, both CIRA and ST91 appear to be well tolerated after oral administration. For example, ST91 does not increase blood pressure when administered orally to humans (Saunamäki, 1974). In mice, CIRA was administered in drinking water at the concentration of 10 mg·L^{-1} for 2 to 9 months, during which no adverse effects were noted with this treatment (Papay et al., 2013). Second, ADR can activate all nine AR subtypes and, in the presence of PRO, a β1- and β2-AR antagonist, is likely to activate not only subtypes of α-ARs but also β3-ARs. Because β3-AR stimulation may produce effects opposite to those of β1- and β2-AR stimulation (Gauthier et al., 1996), we cannot exclude that the stimulation of the β3-AR in the presence of β1- and β2-AR blockade may contribute to the anticancer activity of the combined treatment with PRO plus ADR. The extent of this potential contribution remains to be determined. Third, in the present study, we show that β2-AR stimulation counteracts the effects of α1-ARs but has no effect in its own. These results are in agreement with data from previous studies showing that ISO does not promote the in vitro growth of B16F10 melanoma cells (Maccari et al., 2017) and that treatment with **terbutaline**, a β2-AR agonist, does not affect B16F10 tumour growth in mice (Calvani et al., 2019). In addition, it has been found in neonatal cardiomyocytes that β-AR stimulation with ISO inhibits **atrial natriuretic peptide** and β-myosin heavy chain gene expression induced by the stimulation of

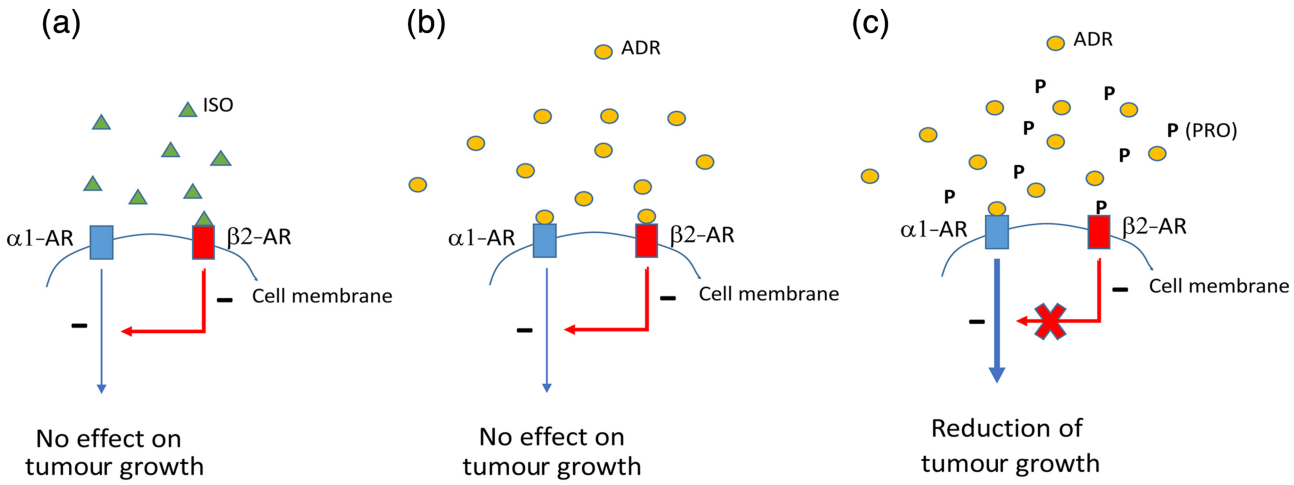

FIGURE 5 Adrenergic regulation of B16F10 melanoma growth. The proposed mechanism is that α1-AR stimulation reduces tumour growth whereas β2-AR stimulation indirectly regulates tumour growth by inhibiting α1-AR signalling. (a) β2-AR stimulation with isoproterenol (ISO) does not affect tumour growth when α1-AR stimulation is lacking. (b) The net effect of the simultaneous stimulation of both α1-ARs and β2-ARs with the mixed adrenoceptor agonist adrenaline (ADR) on tumour growth is negligible. (c) Adding the β-blocker propranolol (PRO) to ADR unmasks antimelanoma activity of the α1-AR stimulation by removing the inhibitory brake of the β2-AR stimulation

α1-ARs but ISO alone does not affect their expression (Patrizio et al., 2008). A possible interpretation of our results is that β2-AR stimulation indirectly regulates B16F10 tumour growth by inhibiting the antitumour activity of α1-AR stimulation. The proposed mechanism is illustrated in Figure 5. The exact point at which β2-AR signalling pathway feeds into the α1-AR signalling pathway remains to be established. Fourth, several studies have reported that PRO has antimelanoma effects (Barbieri et al., 2012; Bucsek et al., 2017; Calvani et al., 2019; de Giorgi et al., 2018; Glasner et al., 2010; Hasegawa & Saiki, 2002; Maccari et al., 2017; Wrobel & Le Gal, 2015). In the present study we used PRO at the dose of about 60 $mg \cdot kg^{-1} \cdot day^{-1}$ and found it to have no anticancer effects in the B16F10 melanoma model. This result is in agreement with our previous study showing that high-dose PRO has no antimelanoma effects (Maccari et al., 2017). A possible explanation of this paradoxical effect of PRO on B16F10 tumour growth is that its antitumour activity depends on the levels of circulating catecholamines. If the β-adrenergic tone is high, the effects of PRO will be evident. On the contrary, if the β-adrenergic tone is low, the effects will be negligible. For example, it is well known that the effects of PRO on cardiac output and heart rate are more dramatic during exercise, that is, when the adrenergic tone increases. Under our experimental conditions, it is possible that the β-adrenergic tone is relatively low after chronic continuous administration of PRO and consequently PRO effects on B16F10 tumour growth are not evident. However, these again appear after coadministration of the catecholamine ADR. This hypothesis would be in accordance with the observation that PRO can cause a reduction in central sympathetic nervous activity (Lewis & Haeusler, 1975).

In conclusion, the results of the present study suggest that stimulation of α-AR subtypes negatively affects the growth of B16F10 melanoma, but β2-AR stimulation extinguishes this effect.

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AUTHOR CONTRIBUTIONS

G.M., P.M. and L.G. designed the experiments and wrote the paper. S.M., P.M., L.G., B.A., L.C. and M.B. carried out the experiments and analysed the data. T.S., D.M. and S.F. contributed to the pharmacological studies in animals.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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