

Carvedilol blocks neural regulation of breast cancer progression *in vivo*

and is associated with reduced breast cancer mortality in patients

Ryan D. Gillis ¹, Edoardo Botteri ^{2,3}, Aeson Chang ¹, Alexandra I. Ziegler ¹, Ni-Chun Chung¹,

Cindy K. Pon ¹, David M. Shackelford ⁴, Bettina K. Andreassen ², Michelle L. Halls ¹,

Jillian G. Baker ⁵, Erica K. Sloan ^{1,6}

1. Drug Discovery Biology Theme, Monash Institute of Pharmaceutical Science, Monash University, Parkville VIC 3052, Australia

2. Department of Research, Cancer Registry of Norway, Oslo, Norway

3. Section for Colorectal Cancer Screening, Cancer Registry of Norway, Oslo, Norway

4. Centre for Drug Candidate Optimisation, Monash Institute of Pharmaceutical Science, Monash University, Parkville VIC 3052, Australia

5. Cell Signalling Research Group, School of Life Sciences, University of Nottingham, Nottingham, United Kingdom

6. Peter MacCallum Cancer Centre, Division of Cancer Surgery, Melbourne VIC 3000, Australia.

Running Title: Carvedilol reduces breast cancer metastasis

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Corresponding author: Erica K. Sloan, 399 Royal Pde, Parkville Victoria 3052, Australia.

erica.sloan@monash.edu, +61 399039707

Abstract

Purpose: The sympathetic nervous system drives breast cancer progression through β -adrenergic receptor signalling. This discovery has led to the consideration of cardiac β -blocker drugs as novel strategies for anti-cancer therapies. Carvedilol is a β -blocker used in the management of cardiovascular disorders, anxiety, migraine and chemotherapy-induced cardiotoxicity. However, little is known about how carvedilol affects cancer-related outcomes.

Methods: To address this, we investigated the effects of carvedilol on breast cancer cell lines, mouse models of breast cancer and in a large cohort of breast cancer patients (n=4,014).

Results: Treatment with carvedilol blocked the effects of sympathetic nervous system activation, reducing primary tumour growth and metastasis in a mouse model of breast cancer and preventing invasion by breast cancer cell lines. A retrospective analysis found that women using carvedilol at breast cancer diagnosis (n=136) had reduced breast cancer-specific mortality compared to women who did not (n=3,878) (5-year cumulative incidence of breast cancer deaths: 3.1% vs. 5.7%; p=0.024 and 0.076 from univariate and multivariable analyses, respectively) after a median follow-up of 5.5 years.

Conclusions: These findings provide a rationale to further explore the use of the β -blocker carvedilol as a novel strategy to slow cancer progression.

Highlights:

- Carvedilol blocks the effects of endogenous catecholamines on growth of primary mammary tumours
- Carvedilol antagonises cancer cell invasion *in vitro* and metastatic dissemination in mice
- Use of carvedilol at cancer diagnosis is associated with reduced breast-cancer specific mortality

Introduction

The sympathetic nervous system is a branch of the autonomic nervous system that has been shown to increase metastasis and enhance cancer progression (1-3). Sympathetic nerves are present in solid tumours (4-6). When activated, sympathetic nerves release catecholaminergic neurotransmitters including adrenaline and noradrenaline that bind to β -adrenergic receptors within the tumour microenvironment (7,8). Activation of β -adrenergic receptors within tumour cells drives a signalling cascade that modulates expression of genes involved in epithelial-mesenchymal transition and invasion (9-12), immunosuppression (13-15), and vasculature remodelling (1,16,17).

Drugs that block neural signalling through β -adrenergic receptors are emerging as novel anti-cancer therapeutics (18,19). To date, both mechanistic and clinical studies have focused on the effects of propranolol, a non-selective β -adrenergic receptor antagonist (or β -blocker) that was originally developed for the treatment of angina and hypertension and is now used in the management of arrhythmias, hypertension, migraine, and anxiety (20). Recent clinical trials in patients with cancer have shown that propranolol reduced the risk of recurrence (21,22), and reduced biomarkers of cancer invasion and progression (23-25). A series of preclinical studies has demonstrated the protective mechanisms of propranolol, which include reducing invasion and angiogenesis (9,16) and improving anti-cancer immunity (26). However, it is currently unknown if other β -blockers such as carvedilol protect against cancer progression.

Carvedilol is a third generation non-selective β -blocker that is used for treatment of chronic heart failure (27). Carvedilol is currently used in cancer patients to treat chemotherapy-induced cardiotoxicity (28,29). However, little is known about its effects on cancer-related outcomes. To address that, we investigated the effect of carvedilol treatment in preclinical models of breast cancer. To explore the clinical relevance of findings, we investigated carvedilol use and cancer-related outcomes in a large cohort of breast cancer patients.

Materials and Methods

Cell culture

The highly metastatic triple negative human breast cancer cell line MDA-MB-231^{HM} was a generous gift from Prof Zhou Ou, Fudan University Shanghai Center, China and is described after this as MDA-MB-231. 4T1.2 mouse breast cancer cell line was a kind gift of Prof Robin Anderson, Olivia Newton John Cancer Centre, Melbourne, Australia. Both cell lines were stably transduced to express codon-optimized firefly luciferase and mCherry under control of the ubiquitin-C promoter as previously described (30). MDA-MB-231 cells were stably transfected with pCRE-luc reporter construct (Stratagene) and a stable clonal line was selected. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)-GlutaMAX (Invitrogen, USA) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO, USA) and maintained at 37°C with 5% CO₂. Cells were tested for mycoplasma prior to use, as described (31). All experiments were performed within 10 passages of cell thawing. Cell line identity was confirmed by short tandem repeat profiling (Cellbank, Australia).

cAMP assay

cAMP accumulation was measured using the CisBio Gs dynamic cAMP kit, according to the manufacturer's instructions. Isoprenaline, carvedilol and vehicle control (0.1% v/v DMSO) were diluted in stimulation buffer containing 0.5 mM IBMX. MDA-MB-231 cells (5,000 cells/well in stimulation buffer with IBMX) were added to a 384-well white opaque plate, prior to addition of vehicle control (for isoprenaline and carvedilol concentration-response curves) or 1 μM carvedilol (Tocris, United Kingdom)(for isoprenaline concentration-response curves in the presence of carvedilol) for 10 min at 37°C. The cells were then stimulated with increasing concentrations of isoprenaline (1 pM to 100 nM), carvedilol (10 pM to 1 μM) or vehicle controls, for 10 min at 37°C. Cells were lysed and cAMP detected by the addition of cAMP-d2 and anti-cAMP-cryptate antibody diluted in lysis buffer. After 60 min, time-resolved FRET was measured using the EnVision plate reader (Perkin Elmer, USA) according to the manufacturer's instructions. Data were analysed against

a cAMP standard curve to determine the pmol cAMP produced per well, and expressed as the mean \pm SE of 4 biological repeats, each performed in triplicate.

ERK1/2 phosphorylation

Changes in ERK1/2 phosphorylation (pERK) were detected using the AlphaScreen SureFire pERK1/2 Kit (PerkinElmer). Cells were treated with carvedilol or phorbol-12,13-dibutyrate (PDBu) diluted in serum-free medium for 30 min at 37°C. Medium was aspirated, cells were lysed in 100 μ l lysis buffer, and cell lysates (4 μ l) were transferred to 384-well ProxiPlates (PerkinElmer) for detection. Data are expressed relative to the effect of serum-free medium.

cAMP Response Element reporter assay

MDA-MB-231 cells stably transfected with pCRE-Luc were seeded in a 96 well plate overnight. 24 hours later the cells were serum starved overnight. Following serum starving cells were treated with isoprenaline and/or carvedilol (all 1 μ M, in 0.1% DMSO in serum-free medium) for 6 hours. When used as an antagonist, cells were treated with carvedilol for 30 minutes prior to the addition of isoprenaline. After incubation, the media was removed and luminescence was quantified using the Bright Glo luciferase reagent (Promega, USA) and an EnVision plate reader.

Gene expression

MDA-MB-231 cells were incubated with isoprenaline and/or carvedilol (all 1 μ M, in 0.1% v/v DMSO vehicle) for 6 hours. When used as an antagonist, cells were treated with carvedilol prior to the addition of isoprenaline. Total RNA was extracted using the Nucleospin RNA Plus kit (Machery-Nagel, Germany) using the manufacturer's instructions. The concentration and quality of the extracted RNA was determined using a NanoDrop spectrophotometer and gel electrophoresis. qRT-PCR was used to quantify gene expression in 100 ng of total RNA using the one-step Taqman qRT-PCR kit (Bio-Rad, USA) and gene specific Taqman probes (*MMP2*-Hs01548727_m1, *GAPDH*-Hs02758991_g1) using a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). Cycle

parameters were 10 min at 50°C for cDNA amplification, 3 min at 95°C for strand separation, followed by 45 cycles of 15 sec at 95°C and 30 sec at 60°C for annealing and extension, respectively. The threshold was set at 100 RFU within the range of linear amplification. Each sample was run in triplicate and the genes of interest were compared to the housekeeper gene *GAPDH* via the $2^{-\Delta\Delta C_t}$ method.

Invasion assay

MDA-MB-231 or 4T1.2 cells were seeded into a 24-well plate. The following day the media was aspirated and replaced with 2% (v/v) FBS supplemented DMEM. After 6h, each well was scratched using a 200 μ L pipette tip, and washed with DPBS. DMEM containing 2mg/ml Matrigel and isoprenaline and/or carvedilol (all 1 μ M, vs. vehicle) was added as described. 1 μ M isoprenaline has previously been shown to induce maximal invasive capabilities. Cell movement was tracked by Operetta imaging system (PerkinElmer, USA) at 37°C and 5% CO₂. After 32h the wound area closure was quantified using the formula $((\text{wound area at } T_0 - \text{wound area at } T_{32}) / \text{wound area at } T_0) \times 100\%$.

Animal studies

All animal experiments were approved by the Monash Animal Ethics Committee and adhered to the National Health and Medical Research Council and ARRIVE guidelines. Six-week old female BALB/c nu/nu mice (Animal Resources Centre, Western Australia) were housed in specific pathogen free cages and kept on a 12/12-hour light dark cycle. Temperature and humidity were controlled, and mice had access to food and water *ad libitum*. To model metastatic breast cancer, MDA-MB-231 cells (3×10^5 in 20 μ l PBS) were injected into the fourth left mammary fatpad of anesthetized mice. Tumour growth was monitored twice weekly using calliper measurements and tumour volume was calculated by $\text{length} \times \text{width}^2 / 2$. To physiologically activate the sympathetic nervous system, we used repeated restraint as previously described (1,16). Metastasis development was monitored twice weekly using imaging of bioluminescent metastatic tumour cells with IVIS Lumina II (Perkin Elmer, USA) by an investigator blinded to treatment condition. Carvedilol (2 mg/kg/day versus vehicle, 20%

w/v hydroxypropyl- β -cyclodextrin in 1% glacial acetic acid, Sigma-Aldrich, USA) was delivered to mice using 28-day osmotic pumps (Model 1004, Alzet, USA), implanted 7 days prior to tumour cell inoculation to model long-term carvedilol use in people. The capacity of carvedilol to affect physiological readouts of sympathetic nervous system activity was confirmed by measuring heart rate using a SC1000 analysis system (Hatteras Instruments, USA), and by video recording mice during restraint and counting nose-pokes to quantify anxiety-like escape behaviour. Results were confirmed in 3 independent studies. Representative findings are shown.

Clinical Evaluation of Carvedilol in Breast Cancer Patients

Investigation of the relationship between carvedilol usage and cancer outcomes was done using linkage between the Cancer Registry of Norway, the Norwegian Prescription Database and the Norwegian Cause of Death Registry (32,33). Data were available on all prescribed drugs between January 2004 and April 2017 and all cancers diagnosed between January 2007 and April 2017 in Norway. The population was limited to women with an initial diagnosis of non-metastatic breast carcinoma (stage I-III) and no history of previous cancer (Figure 4). As no information was available on menopausal status, a possible confounder, analysis was limited to women aged 55 years or older to exclude most pre- and peri-menopausal women. Patients prescribed carvedilol at diagnosis were identified using the Anatomical Therapeutic Chemical code C07AG02, and compared to users at diagnosis of other antihypertensives, namely diuretics, calcium channel blockers and agents acting on the renin-angiotensin system (C03/08/09). A woman was considered a drug user if the length of the last prescription before diagnosis covered the date of diagnosis. Women prescribed non-carvedilol β -blockers including anti-glaucoma preparations (C07 excluding C07AG02, and S01ED) were excluded from the study population as we hypothesized that the use of other β -blocker drugs may obscure an effect of carvedilol (23). Analyses considered possible confounders including region of residence in Norway and other prescriptions for drugs used in diabetes (A10) and cardiac therapy (C01), lipid modifying agents (C10), psycholeptics including anxiolytics (N05), psychoanaleptics including antidepressants and psychostimulants (N06), and drugs for obstructive airways diseases

(R03). The study was approved by the South East Norway Ethical Committee (2016/352) and the Norwegian Data Protection Authority.

Statistical analysis

Quantitative data are expressed as mean \pm standard error (SE). Statistical significance in preclinical studies was determined using one-way Analysis of Variance (ANOVA) with Tukey's *post-hoc* test, or 2-way ANOVA followed by Sidak's *post-hoc* test, or unpaired T-test with Welch's correction, as appropriate. Statistical analyses were performed using GraphPad Prism 8.2 (Graphpad Software, USA).

Differences in the population characteristics between carvedilol users and non-users were evaluated by the Chi-square test or the T-test, as appropriate. Cumulative incidence of deaths from breast cancer and deaths from causes other than breast cancer was calculated in a competing risk framework and compared using the Gray test. In case of statistical significance at the univariate analysis, a multivariate Cox proportional hazard model was used, adjusted by age and tumour stage, classified as localized or regionally advanced, based on the absence or presence of metastasis in the regional lymph nodes. We also included in the model those variables that showed some significance ($p < 0.10$) with carvedilol use and/or the outcome. Hazard ratio (HR) with 95% confidence interval (CI) was reported. These analyses were carried out with SAS software, version 9.4 (SAS Institute, Cary, NC).

Results

Carvedilol prevents *in vitro* tumour cell invasion

β AR signalling activates adenylate cyclase, resulting in cAMP accumulation and modulation of gene transcription (Figure 1A)(34). Carvedilol is used clinically as a β -blocker. However, it has also been shown to stimulate a low level of cAMP in some cell systems (35) and stimulate ERK through β -arrestin signalling and transactivation of the epidermal growth factor receptor in others (36-38) As its effect on breast cancer cells is unknown, we first we treated MDA-MB-231 breast cancer cells with carvedilol and examined the effect on cAMP levels and ERK phosphorylation. The β AR-agonist isoprenaline was used to activate β AR signalling. Carvedilol had no effect on basal cAMP but blocked isoprenaline-induced cAMP accumulation (Figure 1B), indicating carvedilol antagonises isoprenaline-induced signalling in these cancer cells. Carvedilol had no effect on ERK phosphorylation (Figure 1C) in these cells. To define the effect of carvedilol on downstream signalling, we used a reporter assay to quantify cAMP Response Element (CRE)-mediated gene transcription. Carvedilol had no effect on basal CRE-mediated gene transcription, but effectively blocked isoprenaline-stimulated CRE activation (Figure 1D). To determine the effect on expression of cancer-related genes, we used qRT-PCR to quantify the effect of carvedilol on expression of matrix metalloproteinase-2 (*MMP2*), a CRE-regulated protease that facilitates matrix degradation during tumour cell invasion (39). Carvedilol treatment prevented isoprenaline-stimulated *MMP2* expression (Figure 1E).

To determine if these molecular changes translate to functional effects, we investigated the effect of carvedilol on invasion of tumour cells through Matrigel. As expected from previous studies (9,10), isoprenaline stimulation accelerated cell movement through the extracellular matrix, resulting in >2-fold increased invasion after 32h ($9.0\% \pm 0.9$ vs. $21.6\% \pm 3.2$, $p < 0.01$) (Figure 1F). Treating cells with carvedilol effectively blocked this effect on invasion (Figure 1F). Carvedilol similarly mitigated

isoprenaline-stimulated invasion by 4T1.2 cancer cells (Figure 1G). Collectively, these findings show that carvedilol inhibited isoprenaline-stimulated intracellular signalling and invasion in cancer cells.

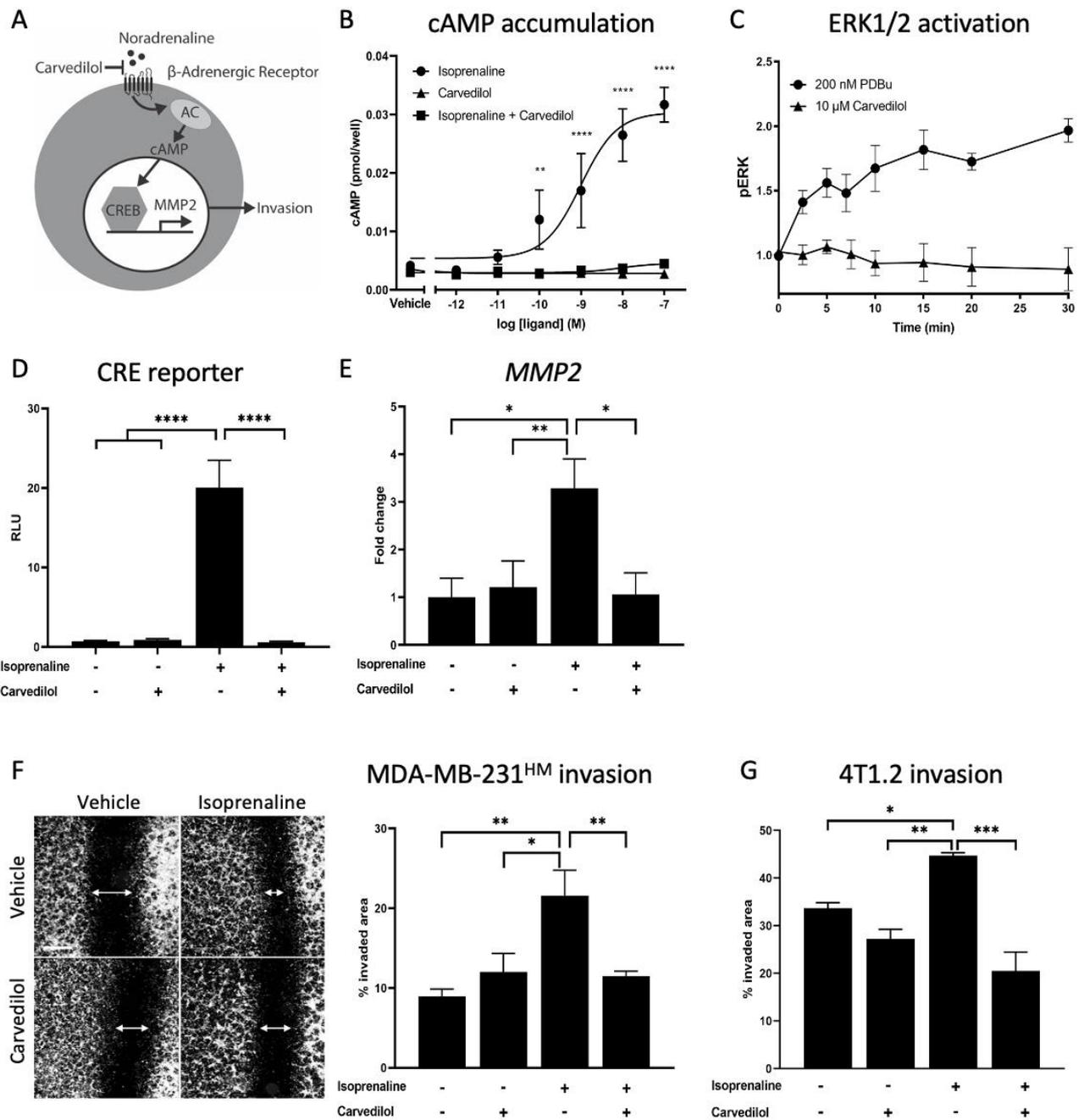


Figure 1: Carvedilol antagonises isoprenaline-induced cAMP production, CRE-reporter gene and *MMP2* gene expression and tumour cell invasion. (A) Schematic of signalling from β AR in tumour cells. AC: Adenylate cyclase. cAMP: cyclic adenosine monophosphate. CREB: cAMP Response Element Binding Protein. MMP2: matrix metalloprotease 2. (B) cAMP accumulation induced by activation of endogenous β AR was measured in MDA-MB-231 tumour cells in response to increasing concentrations of β AR agonist isoprenaline, β AR antagonist carvedilol or isoprenaline in the presence of carvedilol (1 μ M) (n=4). (C) Phosphorylation of ERK was measured in MDA-MB-231 cells in response to carvedilol (10 μ M), or phorbol 12,13-dibutyrate (PDBu). Data are presented relative to serum-free medium. (n=4). (D) MDA-MB-231 cells were stably transduced with CRE reporter construct and cAMP-dependent transcription was measured by bioluminescence (relative luminescence units, RLU) in response to 1 μ M isoprenaline and 1 μ M carvedilol (n=4). (E) Quantitative RT-PCR was used to measure *MMP2* gene expression in MDA-MB-231 cells following treatment with 1 μ M isoprenaline and/or 1 μ M carvedilol vs. vehicle. (n=3). (F) Invasion through Matrigel by MDA-MB-231 tumour cells treated with 1 μ M isoprenaline and/or 1 μ M carvedilol vs. vehicle. Assessed at 32h. (n=2). Scale bar: 500 μ m. (G) Invasion by 4T1.2 tumour cells treated with 1 μ M isoprenaline and/or 1 μ M carvedilol vs. vehicle. ** = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$ by two-way ANOVA with Sidak's *post-hoc* test (Fig. 1B, C) or one-way ANOVA with Tukey's *post-hoc* test (Fig. 1D-G).

Carvedilol slows tumour growth and metastasis *in vivo*.

Carvedilol is used clinically for its cardiovascular protective effects that include reduction in mortality in people with heart failure (27) and portal hypertension (40,41). To confirm that carvedilol exposure was sufficient to modulate cardiovascular function, we measured heart rate in mice treated with 2 mg/kg carvedilol. As expected, isoprenaline increased heart rate and carvedilol antagonised the effect of isoprenaline on heart rate, confirming an effect on cardiovascular function (Figure 2A).

We also evaluated the capacity of carvedilol to antagonise physiological activation of the sympathetic nervous system induced by repeated restraint. Mice treated with carvedilol showed reduced escape-motivated behaviour (nose-pokes) during restraint compared to vehicle-treated mice, confirming an effect of carvedilol on anxiety-like behaviour (Figure 2B).

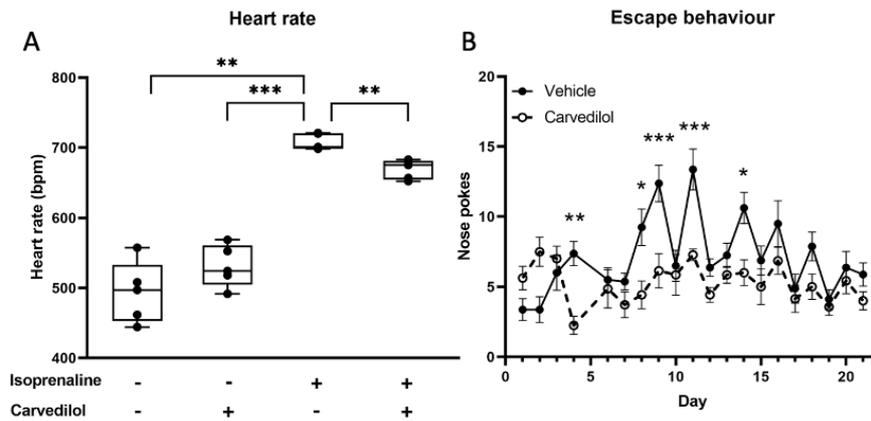


Figure 2: Carvedilol antagonises β -adrenergic activation of heart rate and anxiety. (A) Heart rate was quantified in mice following carvedilol treatment at baseline and in the presence of isoprenaline. Each datapoint represents one mouse. The midline shows the median and the whiskers show the minimum and maximum values (n=5 for each condition). (B) Nose pokes were assessed as a measure of anxiety-like behaviour during restraint stress (n=7-8 per group). Days after commencing restraint. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$ by one-way ANOVA with Tukey's *post-hoc* test (Fig. 2A) or two-way ANOVA with Sidak's *post-hoc* test (Fig. 2B).

To investigate the effect of carvedilol on cancer progression, mice were inoculated with MDA-MB-231 tumour cells in the fourth left mammary fatpad and bioluminescence imaging was used to track the effect of carvedilol on tumour growth and metastatic dissemination. To activate the sympathetic nervous system we used repeated restraint, which has been previously shown to drive cancer progression by elevating β AR signalling (1,16,17). Under these conditions, carvedilol treatment reduced primary tumour growth, and resulted in >30-fold reduction in distant metastasis (Figure 3B,

D). To determine the effect of carvedilol treatment in the absence of elevated β AR signalling, we also investigated the effect in mice maintained in their homecage. Under baseline conditions, carvedilol had no effect on either primary tumour growth or metastasis (Figure 3C, D). These results show that carvedilol inhibits stress-induced elevation of cancer progression *in vivo*.

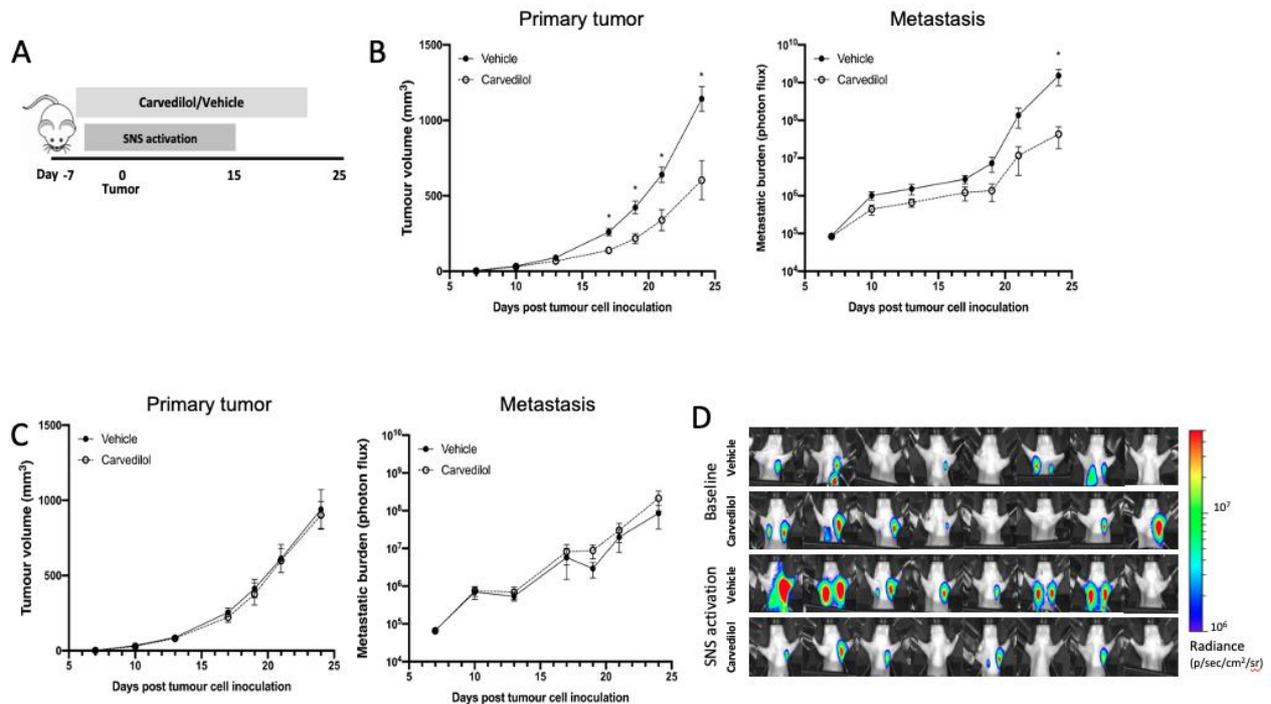


Figure 3: Carvedilol reduces primary tumour growth and metastasis in the MDA-MB-231 mouse model of breast cancer. (A) Timeline of *in vivo* experiment. SNS was activated using daily restraint commencing 5 days prior to tumour cell injection. Carvedilol administration started the day prior to restraint. (B) The effect of carvedilol (2mg/kg/day) vs. vehicle on cancer progression was assessed by caliper measurement of primary tumour growth and bioluminescence imaging (BLI) detection of metastasis in mice exposed to daily restraint to activate the sympathetic nervous system. (C) The effect of carvedilol (2mg/kg/day) vs. vehicle on cancer progression under baseline conditions was assessed by caliper measurement of primary tumour growth and bioluminescence imaging detection of metastasis. (D) Representative images of metastatic burden after 24 days of tumour growth. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ by 2-way ANOVA with Sidak's *post-hoc* test

Carvedilol use is associated with improved breast cancer outcomes in patients

To investigate the clinical validity of these findings, we examined carvedilol use and cancer-related outcomes in a large cohort of women with breast cancer (Figure 4). Women with non-carcinoma or with metastatic cancer at diagnosis were excluded, as the goal was to evaluate cancer progression. The majority of carvedilol users (83.1%) also used one or more non- β -blocker cardiovascular medications. Therefore, to match for cardiovascular risk, we excluded women not using cardiovascular drugs, including diuretics, calcium channel blockers, or renin-angiotensin system modulators. This resulted in a cohort of 4,014 women with stage I, II or III breast cancer (Figure 4).

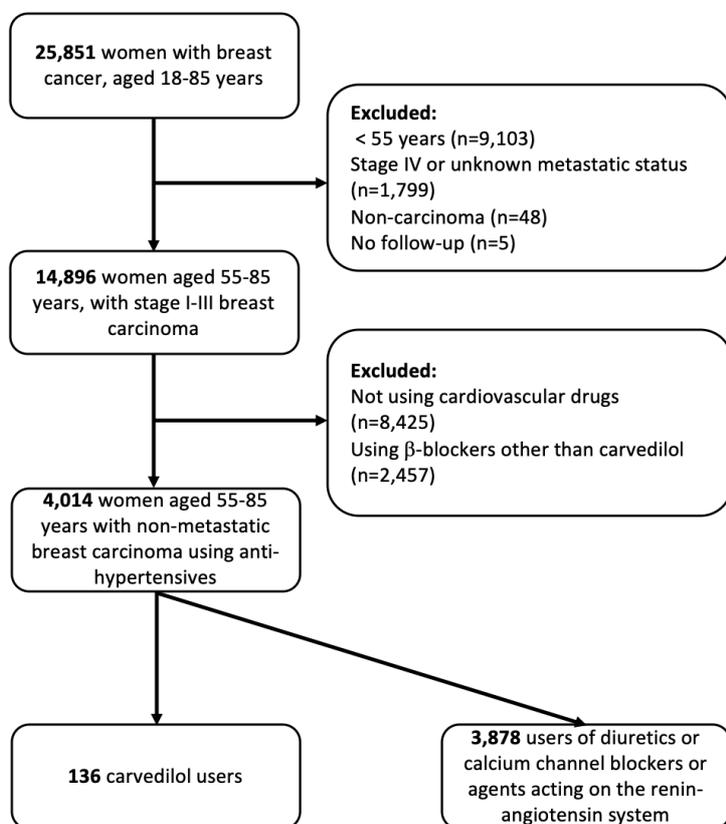


Figure 4. Clinical study inclusion criteria

Age, education, use of psycholeptics or psychoanaleptics, use of drugs for obstructive airways diseases, and tumour characteristics were similarly distributed in carvedilol users and non-users at diagnosis ($p \geq 0.10$; Table 1). Carvedilol users and non-users shows some differences in region of

residence and use of antidiabetics, cardiac therapy and lipid modifying agents ($p < 0.10$). During a median follow-up of 5.5 years (range 0.1-12.0), 4 breast cancer deaths were observed in 136 carvedilol users and 285 in 3,878 non-carvedilol users, corresponding to a 5-year cumulative incidence of breast cancer deaths of 3.1% vs. 5.7%, respectively (Gray test $p = 0.024$; adjusted HR 0.41; 95% CI 0.15-1.09; $p = 0.076$) (Table 1; Figure 5). Mortality from other causes and overall mortality was similar between the two groups.

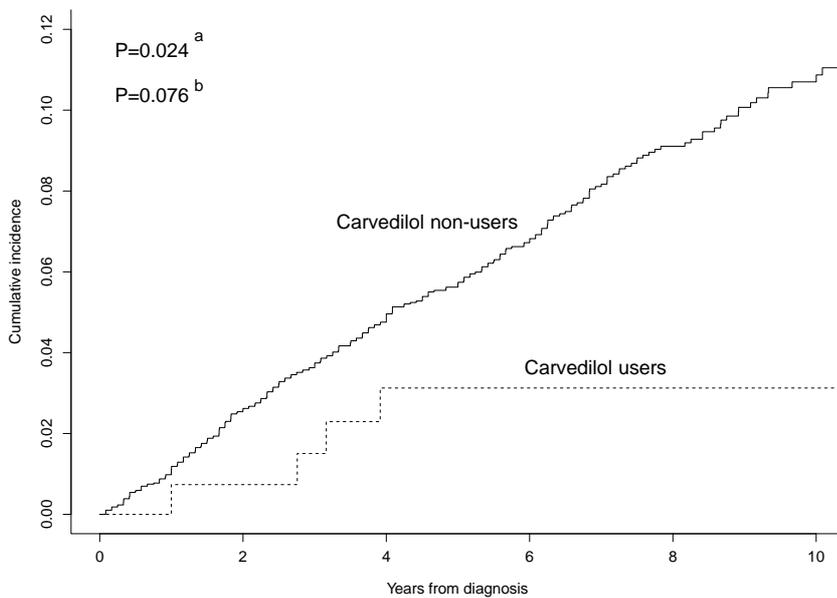


Figure 5. Cumulative incidence of breast cancer deaths by carvedilol use

P-value was calculated using: ^a Gray test, ^b Cox model adjusted for age, education, region of residence, tumour stage and use of the following drugs: drugs used in diabetes, cardiac therapy and lipid modifying agents

Table 1. Characteristics at diagnosis and outcome of the population according to carvedilol use

	Carvedilol users	Carvedilol non-users	P-value

			n=136	n=3,878	
Age	Years	Mean (IQR)	68.5 (63-74)	68.4 (62-75)	0.866 ^c
Region of residence	South-East	N (%)	74 (54.4)	2,295 (59.2)	0.002 ^d
	West	N (%)	39 (28.7)	658 (17.0)	
	Mid	N (%)	17 (12.5)	566 (14.6)	
	North	N (%)	6 (4.4)	359 (9.3)	
Education ^a	Primary	N (%)	40 (29.9)	1,105 (28.8)	0.144 ^d
	Secondary	N (%)	76 (56.7)	1,957 (51.0)	
	University or more	N (%)	18 (13.4)	777 (20.2)	
Use of drugs	Drugs used in diabetes	N (%)	22 (16.2)	408 (10.5)	0.036 ^d
	Cardiac therapy	N (%)	8 (5.9)	118 (3.0)	0.062 ^d
	Lipid modifying agents	N (%)	64 (47.1)	1,435 (37.0)	0.017 ^d
	Psycholeptics or psychoanaleptics	N (%)	52 (38.2)	1,447 (37.3)	0.827 ^d
	Drugs for obstructive airways diseases	N (%)	16 (11.8)	621 (16.0)	0.183 ^d
Tumour histology	Ductal carcinoma	N (%)	110 (80.9)	3,098 (79.9)	0.605 ^d
	Lobular carcinoma	N (%)	18 (13.2)	465 (12.0)	
	Other	N (%)	8 (5.9)	315 (8.1)	
Tumour stage	Positive regional lymph nodes	N (%)	37 (27.2)	1,277 (32.9)	0.162 ^d
Tumour grade ^b	Poorly differentiated (G3)	N (%)	35 (26.9)	935 (25.4)	0.701 ^d
Deaths	Breast cancer deaths	N (5-year cum inc %)	4 (3.1)	285 (5.7)	0.024 ^e 0.076 ^f
	Deaths from other causes	N (5-year cum inc %)	18 (10.8)	404 (7.3)	0.716 ^e
	All deaths	N (5-year cum inc %)	22 (13.9)	689 (13.0)	0.219 ^g

IQR: Interquartile range; ^a Missing for carvedilol users (n=2) and non-users (n=39); ^b Missing for carvedilol users (n=6) and non-users (n=201). P-value was calculated using: ^c T-test, ^d Chi-square test, ^e Gray test, ^f Cox model adjusted for age, education, region of residence, tumour stage and use of the following drugs: drugs used in diabetes, cardiac therapy and lipid modifying agents; ^g Log-rank test

Discussion

Carvedilol is a well-tolerated β -blocker that blocks the actions of endogenous catecholamines and has been used for many years as a long-term treatment to reduce mortality in cardiovascular disease. Here, we found that treatment with carvedilol blocked the effects of endogenous catecholamines on growth of primary mammary tumours and metastatic dissemination in mice. Within the tumour microenvironment, catecholaminergic signalling through β -adrenergic receptors has been shown to drive cancer cell invasion (9,10,12). We found here that carvedilol blocked expression of matrix metalloprotease-2 and reduced invasion by breast cancer cell lines that was induced by the β -adrenergic receptor agonist isoprenaline. In a retrospective examination of patients with breast cancer, we found that carvedilol use was associated with reduced breast cancer-specific mortality, compared to non-use. The association between carvedilol use and reduced breast cancer-specific mortality was statistically significant at univariate analysis, and borderline statistically significant after adjusting for cancer stage and other factors. These findings provide provisional clinical support for the hypothesis that carvedilol may be leveraged as a novel strategy to slow breast cancer progression.

The *in vivo* findings suggest that carvedilol may be most effective in cancer in the context of stress, where elevated SNS activity drives β -adrenergic signalling. Denervation studies found that circulating adrenaline is not required for the effects of stress on cancer progression (42). Therefore, β -adrenergic receptors signalling is likely induced by elevated catecholamines including noradrenaline that are released from SNS nerve terminals, plausibly in the tumour itself (4,5). In addition to acting on tumour cells to drive invasion (Fig 1) (9,10,12), β -adrenergic signalling also contributes to metastasis by increasing inflammation (1), suppressing anti-cancer immunity (13), and controlling remodelling of the vasculature that provides pathways for tumour cell dissemination (1,16). In future work it will be important to determine the effects of carvedilol on these processes.

While β -adrenergic agonist effects have been reported for carvedilol (35-37), we saw no evidence for this in MDA-MB-231 breast cancer cells (Figure 1). This has important implications as the intracellular signalling that results from activation of β -adrenergic receptors is potentially detrimental

in the cancer setting, as described above. While β -adrenergic agonism by carvedilol has been reported for both cAMP and pERK in other cell lines (35,43,44), we found no evidence for agonist effects of carvedilol at cAMP or pERK in this human breast cancer cell line that expresses native β AR. Furthermore, carvedilol had no agonist effect on downstream CRE-luciferase or matrix metalloproteinase gene expression. In future work it will be important to extend analysis of the antagonist and agonist effects of carvedilol to other cancer cell lines using sensitive readouts of downstream effectors including cAMP. Nonetheless, the clinical data presented here suggest no adverse effect of carvedilol use in patients, consistent with carvedilol acting as a β -blocker in the tumour microenvironment.

In addition to its β -blocker properties, carvedilol has α -adrenergic blocking and antioxidant properties. In future studies it will be important to define the relative contributions of the β -blocker, α -blocker, and antioxidant properties of carvedilol on cancer progression. Here, we demonstrated that carvedilol reduced β -adrenergic-regulated invasion (Figure 1). We also show that carvedilol treatment blocked the adverse effects of physiological activation of the sympathetic nervous system on tumour growth *in vivo*. β -adrenergic receptors are the predominant mediator of sympathetic neural activity in the tumour microenvironment (1,6,13,16,17). However, the findings do not rule out an effect of the α -blocking and antioxidant properties of carvedilol on cancer progression. α -blockers are used in the treatment of benign prostatic hyperplasia and have been shown to modulate cancer cell invasion (45,46). The antioxidant properties of carvedilol are thought to contribute to its cardioprotective effects, although the effects of antioxidants in cancer is unclear (47-49).

The findings provide guidance for future clinical evaluation of the anti-cancer effects of carvedilol. The sympathetic nervous system is elevated by stressors, and both a diagnosis of cancer and cancer treatments including surgery are significant stress-inducing events (19,23). Thus, carvedilol may have the most beneficial effect in patients with heightened anxiety, both to slow cancer progression and for anxiety management. However, it would be important to evaluate carvedilol in all patients as a recent randomized trial of the β -blocker propranolol found decreased expression of mesenchymal

genes in all patients treated with propranolol regardless of whether propranolol treatment induced clinical evidence of β -blockade (i.e., heart rate and blood pressure reduction) (23). Furthermore, preclinical findings that carvedilol reduced primary tumour growth in mouse models of skin cancer and oral cavity squamous cell carcinoma (50,51) raise the possibility that carvedilol may slow progression of diverse solid tumour types. Clinical studies are currently evaluating prophylactic use of carvedilol in cancer patients to prevent cancer therapy-induced cardiotoxicity with mixed results (52,53). The findings presented here suggest that future evaluation of carvedilol for primary prevention for cardiotoxicity may be an ideal opportunity to also evaluate biomarkers of its effect on cancer progression.

An important limitation of our clinical study in addition to its observational nature, is the absence of detailed information on cancer stage, cancer molecular subtype and treatment. This may have contributed to incomplete adjustment in the multivariable model thereby affecting the comparison between carvedilol users and non-users. Moreover, the small number of carvedilol users and cancer-related events in the treatment group likely contributed to an imprecise association estimate (i.e., large confidence interval) and precluded performing sensible subgroup analyses.

Overall, the findings support further clinical evaluation of carvedilol in cancer patients for its effects on cancer progression in addition to its effects on the heart.

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