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β-blockers increase response to chemotherapy via direct antitumour and anti-angiogenic mechanisms in neuroblastoma

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Background: The use of β-blockers for the management of hypertension has been recently associated with significant clinical benefits in cancer patients. Herein, we investigated whether β-blockers could be used in combination with chemotherapy for the treatment of neuroblastoma.

Methods: Seven β-blockers were tested for their antiproliferative and anti-angiogenic properties alone, and in combination with chemotherapy *in vitro*; the most potent drug combinations were evaluated *in vivo* in the TH-MYCN mouse model of neuroblastoma.

Results: Three β-blockers (i.e., carvedilol, nebivolol and propranolol) exhibited potent anticancer properties *in vitro* and interacted synergistically with vincristine, independently of P-glycoprotein expression. β-blockers potentiated the anti-angiogenic, antimitochondrial, antimitotic and ultimately pro-apoptotic effects of vincristine. *In vivo*, β-blockers alone transiently slowed tumour growth as compared with vehicle only ($P<0.01$). More importantly, when used in combination, β-blockers significantly increased the tumour regression induced by vincristine ($P<0.05$). This effect was associated with an increase in tumour angiogenesis inhibition ($P<0.001$) and ultimately resulted in a four-fold increase in median survival, as compared with vincristine alone ($P<0.01$).

Conclusion: β-blockers can increase treatment efficacy against neuroblastoma, and their combination with chemotherapy may prove beneficial for the treatment of this disease and other drug-refractory cancers.

Neuroblastomas are enigmatic, multifaceted tumours of the peripheral nervous system that represent the most common solid tumour in children under 5, accounting for 8–10% of all paediatric malignancies and 15% of all cancer-related mortalities in children. Stage, age, tumour histology including degree of differentiation, *MYCN* oncogene status and DNA ploidy have been defined as the

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most significant and clinically relevant prognostic markers of outcome (Cohn *et al*, 2009). For high-risk patients, who represent over 36% of all neuroblastoma patients, the 5-year event-free survival is 30% when treated with induction chemotherapy, surgery, radiation therapy and consolidation therapy with bone marrow transplantation (Matthay *et al*, 2009). Recently, immunotherapy was found to increase overall survival when combined with maintenance therapy (Yu *et al*, 2010) and has now become part of the standard of care, despite proving ineffective in patients with recurrent or refractory bulky disease (Shusterman *et al*, 2010). Moreover, these therapeutic options are all extremely aggressive and toxic (Ishola and Chung, 2007; Shusterman *et al*, 2010; Yu *et al*, 2010), suggesting that the limit of therapy intensification may have been reached. Safer and more efficacious therapeutic options are urgently needed, thus warranting the evaluation of innovative therapeutic strategies in clinically relevant models of high-risk neuroblastoma.

Recent retrospective studies have linked the use of β-adrenergic receptor antagonists – also called β-blockers – prior to cancer diagnosis and/or concomitantly with chemotherapy and radiotherapy to reduced rates of progression and/or increased survival in melanoma, breast, ovarian and non-small cell lung cancer patients (Powe *et al*, 2010; Barron *et al*, 2011; De Giorgi *et al*, 2011; Lemeshow *et al*, 2011; Melhem-Bertrandt *et al*, 2011; Diaz *et al*, 2012; Wang *et al*, 2013). Preclinical studies have also showed that β-blockers were able to significantly inhibit stress-induced tumour growth and/or metastasis in models of ovarian cancer (Thaker *et al*, 2006), breast cancer (Sloan *et al*, 2010) and acute lymphoblastic leukaemia (Lamkin *et al*, 2012). Furthermore, the serendipitous discovery of the efficacy of the non-selective β-blocker, propranolol, in the treatment of infantile haemangioma (Leaute-Labreze *et al*, 2008) has completely revolutionized the management of this pathology and shed some light on the anti-angiogenic potential of β-blockers. Collectively, these findings strongly suggest that β-blockers may represent a valuable therapeutic tool in the fight against cancer and may prove beneficial in the treatment of drug-refractory cancers.

Here, we sought to investigate whether β-blockers could increase the efficacy of chemotherapy against neuroblastoma. We evaluated the antiproliferative and anti-angiogenic properties of seven different β-blockers *in vitro* and performed drug combination studies with the major chemotherapy agents currently used in the clinic for the treatment of neuroblastoma. The efficacy of the most potent drug combinations was then evaluated in the TH-MYCN transgenic mouse model of neuroblastoma. β-blockers were found to exert little antitumour efficacy when used alone, but increased treatment efficacy and prolonged median survival when used in combination with vincristine, through antimitochondrial and anti-angiogenic mechanisms.

MATERIALS AND METHODS

Cell culture. The neuroblastoma cell lines BE(2)-C and SHEP were maintained in DMEM medium (Invitrogen, Mulgrave, Victoria, Australia) and SK-N-SH in RPMI-1640 medium (Invitrogen), supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 1% penicillin streptomycin. The NH02A neuroblastoma cell line was isolated from TH-MYCN mouse tumour and grown as previously described (Cheng *et al*, 2007). The culture of MRC-5 lung fibroblasts was carried out in MEM (Invitrogen) containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 2% sodium bicarbonate and 1% nonessential amino acids. BMH29L cells are bone marrow-derived endothelial cells (BMECs) that were immortalised by ectopic expression of human telomerase reverse transcriptase (MacKenzie *et al*, 2002). They were kindly provided by Dr Karen MacKenzie (Children's Cancer Institute Australia) and grown as previously described (Pasquier *et al*, 2013). Cell lines were regularly screened and are free from mycoplasma contamination.

Growth inhibition assay. Growth inhibition assays were performed as previously described (Pasquier *et al*, 2010). After 72-h-drug incubation, metabolic activity was detected by addition of Alamar blue and spectrophotometric analysis. Cell proliferation was determined and expressed as a percentage of untreated control cells. The determination of IC_{50} values was performed using GraphPad Prism 4 software (GraphPad Sofware Inc, La Jolla, CA, USA). Combination indexes (CI) were calculated for all tested drug concentrations according to the Chou and Talalay method (Chou and Talalay, 1984; Chou, 2010).

***In vitro* Matrigel assay.** Matrigel (BD Biosciences, North Ryde, New South Wales, Australia) assay was used to examine the anti-angiogenic effects of β-blockers alone and in combination with chemotherapy, as previously described (Pasquier *et al*, 2011). Cells were treated with different drug solutions 20 min after seeding on Matrigel, and photographs were taken after 8-h-drug incubation using the ×5 objective of an Axiovert 200 M fluorescent microscope coupled to an AxioCamMR3 camera driven by the AxioVision 4.7 software (Carl Zeiss, North Ryde, New South Wales, Australia). The anti-angiogenic effects were then quantitatively evaluated by measuring the total surface area of capillary tubes formed in at least 10 view fields per well using AxioVision 4.7 software.

Cell cycle analysis and apoptosis detection. For cell cycle analysis, adherent and floating cells were harvested, fixed in 80% ethanol and incubated 15 min in the dark with 100 $\mu g\,ml^{-1}$ propidium iodide (PI), immediately before analysis. Amount of DNA present was measured by flow cytometry (FACSCalibur, BD Biosciences) and cytogram analysis was done with FlowJo software version 7.6.5 (Tree Star, Ashland, OR, USA). For apoptosis detection, adherent and floating cells were harvested and incubated for 15 min in the dark with PI and Annexin V-FITC (BD Biosciences), followed immediately by flow cytometry analysis (FACSCalibur, BD Biosciences). Cytogram analysis was performed with Cell Quest Pro software (BD Biosciences), as previously described (Pasquier *et al*, 2004).

Mitochondrial network. Fluorescence labelling of mitochondria was performed by stably transfecting neuroblastoma SK-N-SH cells with the mitochondrial-targeted DsRed (mtDsRed) (Savry *et al*, 2013). The dynamic localisation of mitochondria was analysed in living cells after a 6-h incubation with propranolol alone, vincristine alone or their combination. Cells were observed using the ×40 objective of a Leica DM-IRBE microscope driven by Metamorph software (Princeton Instrument, Trenton, NJ, USA), and 600 cells were analysed for each experimental condition to determine the percentage of cells with a fragmented mitochondrial network.

Gene silencing and quantitative RT–PCR. *ABCB1* gene expression was silenced in BE(2)-C neuroblastoma cells using Lipofectamine 2000 and ON-TARGET Plus SMARTpool siRNA (Millenium Science, Surrey Hills, Victoria, Australia). A non-silencing control siRNA, which has no sequence homology to any known human gene sequence, was used as a negative control (Qiagen, Doncaster, Victoria, Australia). Total RNA was extracted using the Qiagen Mini RNeasy kit (Qiagen) and cDNA synthesis was performed using high-capacity cDNA reverse transcription kit with RNAse inhibitor (Applied Biosystems, Melbourne, Victoria, Australia). Real-time PCR was run on 7900HT Fast Real-time PCR system using Taqman gene expression assays (Applied Biosystems) for *ABCB1* (Hs00184500) and the housekeeper gene *HPRT1*

(4326321E). Gene expression levels were determined using the $\Delta\Delta C_t$ method, normalised to the housekeeper gene and expressed relative to a calibrator (Winer *et al*, 1999).

Radiolabelled drug accumulation. For drug uptake studies, cells, seeded in 12-well plates, were incubated for 4 h at 37 °C with 50 nM [^{3}H]-vincristine in presence or absence of verapamil and β-blockers. Cells were then washed thrice with ice-cold PBS and lysed in 0.1 M NaOH. Intracellular [^{3}H]-vincristine concentration was determined by β-scintillation counting and normalised to protein content, as determined by BCA assay (Haber *et al*, 1989).

Animal study. The generation and maintenance of the human *MYCN* transgenic mouse model of neuroblastoma has been previously described (Weiss *et al*, 1997). Following the development of a palpable abdominal tumour (~5 mm in diameter), TH-MYCN transgenic mice were treated weekly for 3 consecutive weeks with single i.v. injections of vincristine at 0.2 mg kg^{-1} (on days 1, 8 and 15) and/or 5 days a week with single i.p. injections of propranolol (50 mg kg^{-1}), carvedilol (10 mg kg^{-1}) or nebivolol (10 mg kg^{-1}). Saline and DMSO served as vehicle controls. Mice were supplemented with sunflower seeds to prevent severe constipation associated with vincristine treatment. For the tumour growth study and histological analyses, mice ($n=6$ per cohort and per time point) were killed just before the start of treatment (day 1) or after 3 and 6 days of treatment (days 4 and 7, respectively). Tumours were excised, measured, weighed and trisected for subsequent RNA extraction from fresh-frozen tissue or haematoxylin and eosin (H&E), and immunohistochemical staining on formalin-fixed paraffin-embedded tissue. For the survival study, mice ($n \geqslant 8$ per cohort) were killed when tumour size reached 1 cm^3 or when signs of morbidity became apparent. All experimental procedures involving mice met the standards

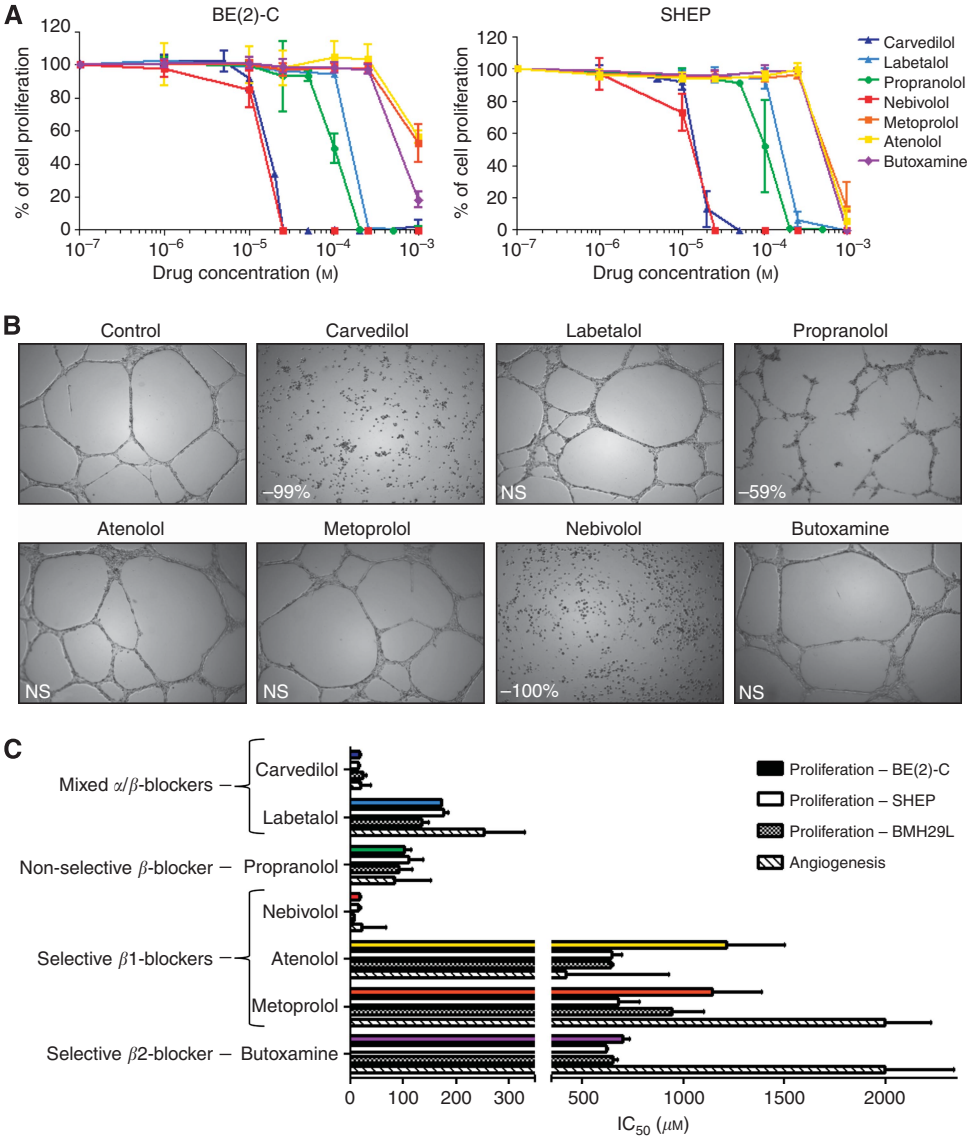

Figure 1. Antiproliferative and anti-angiogenic properties of β-blockers. (**A**) Growth inhibition assay performed on BE(2)-C and SHEP neuroblastoma cell lines using Alamar Blue after 72-h incubation with a range of concentrations of β-blockers. *Points*, % of cell proliferation as compared with untreated control cells, means of at least three individual experiments; *bars*, 95% CI; log scale for *x* axis. (**B**) Representative photographs of BMH29L cells in Matrigel assays. Cells were treated with β-blockers 20 min after seeding on Matrigel and photographs were taken after 8-h-drug incubation. Vascular structures were imaged on a Zeiss Axiovert 200 M using a ×5 objective. *Inset*, % of inhibition as compared with untreated control cells (NS = not statistically significant). (**C**) Histogram representation of the molar concentration of β-blockers required to inhibit 50% (IC_{50}) of cell proliferation after 72-h-drug incubation for all tested cell lines and 50% of vascular structure formation on Matrigel after 8-h-drug incubation. *Columns*, means of at least three individual experiments; *bars*, 95% CI.

required by the UKCCR guidelines (Workman *et al*, 2010), and were approved by the University of New South Wales Animal Care and Ethics Committee according to the Animal Research Act, 1985 (New South Wales, Australia) and the Australian Code of Practice for Care and Use of Animals for Scientific Purposes, 1997.

Statistical analysis. All experiments were performed at least in triplicate and statistical analyses were done using the GraphPad Prism 4 software. Statistical significance was determined using two-sided Student's *t*-test for single comparisons and one-way ANOVA for multiple comparisons, respectively. Median survival comparison was performed using the Mantel–Cox log-rank test. Additional methods are provided as *Supplementary Material*.

RESULTS

***β*-blockers display potent anticancer properties *in vitro*.** Seven β-blockers with different selectivity for adrenergic receptors were used in this study, including two mixed α/β-blockers (carvedilol and labetalol), one non-selective β-blocker (propranolol), three selective β1-blockers (nebivolol, atenolol and metoprolol) and one selective β2-blocker (butoxamine). Growth inhibition assays performed on BE(2)-C and SHEP neuroblastoma cell lines showed very similar results and demonstrated that β-blockers could be divided into three categories based on their potent (i.e., carvedilol and nebivolol), intermediate (i.e., propranolol and labetalol) or weak (i.e., atenolol, metoprolol and butoxamine) antiproliferative properties (Figure 1A). Matrigel assay further revealed that the β-blockers exerting the most potent antiproliferative effects against neuroblastoma cells were also the most anti-angiogenic *in vitro* (Figure 1B). While 100 μM labetalol, atenolol, metoprolol and butoxamine did not significantly alter the formation of vascular structures by BMH29L endothelial cells, the same concentration of carvedilol and nebivolol completely suppressed angiogenesis *in vitro* and propranolol induced a significant inhibition of 59 ± 12% ($P<0.01$). As summarised in Figure 1C, dose-response studies revealed that the three β-blockers with the most potent antiproliferative and anti-angiogenic properties were mixed α/β-blocker carvedilol, selective β1-blocker nebivolol and, to a lesser extent, non-selective β-blocker propranolol. Interestingly, the antiproliferative and anti-angiogenic properties of β-blockers did not correlate with their respective selectivity for adrenergic receptors.

***β*-blockers potentiate the antiproliferative and anti-angiogenic effects of microtubule-targeting agents *in vitro*.** The three β-blockers with the most potent anticancer properties were combined with seven chemotherapy agents commonly used in the clinic for the treatment of neuroblastoma, in order to identify potential synergistic drug interactions. When used at nontoxic concentrations, propranolol, carvedilol and nebivolol did not significantly alter the sensitivity of BE(2)-C neuroblastoma cells to carboplatin, etoposide, mafosfamide (i.e., a derivative of cyclophosphamide active *in vitro*), melphalan or SN-38 (i.e., an active metabolite of irinotecan) (Figure 2A and Supplementary Figure S1). However,

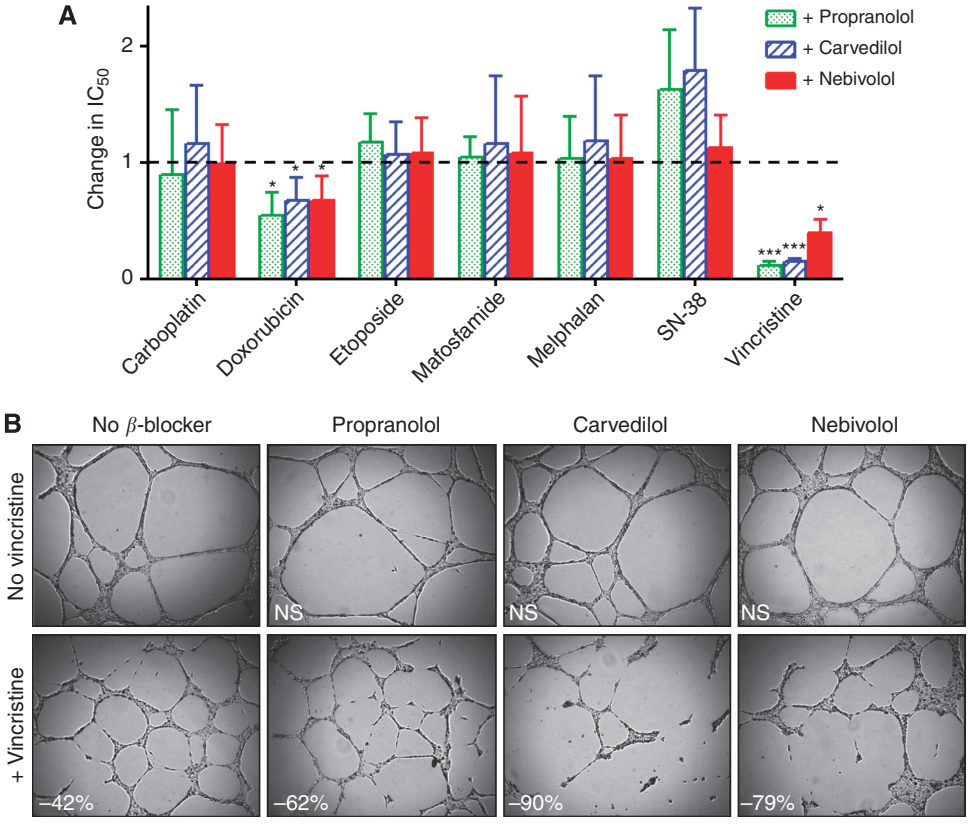

Figure 2. *In vitro* drug combinations. (**A**) Histogram representation of change in IC_{50} values in BE(2)-C neuroblastoma cells when chemotherapy agents are used in combination with nontoxic concentrations of propranolol (10 μM – *green*), carvedilol (1 μM – *blue*) and nebivolol (1 μM – *red*), as compared with chemotherapy alone. *Columns*, means of at least four individual experiments; *bars*, 95% CI. Statistical analysis was performed by comparing the IC_{50} values of chemotherapy alone or in combination with propranolol, carvedilol and nebivolol using Student's *t*-test (*$P<0.05$; ***$P<0.001$). (**B**) Representative photographs of BMH29L cells in Matrigel assays. Cells were treated with 10 nM vincristine alone or in combination with 10 μM propranolol, 1 μM carvedilol or 1 μM nebivolol 20 min after seeding on Matrigel, and photographs were taken after 8-h-drug incubation. Vascular structures were imaged on a Zeiss Axiovert 200 M using a ×5 objective. *Inset*, % of inhibition as compared with untreated control cells (NS = not statistically significant).

they strongly potentiated the antiproliferative effects of vincristine (2.5–9-fold increase in sensitivity; $P<0.01$) and, to a lesser extent, those of doxorubicin (1.5–1.8-fold increase in sensitivity; $P<0.05$). Potentiation was also observed while combining β-blockers with vinblastine and paclitaxel (Supplementary Figure S1), thus showing that β-blockers increased the antiproliferative activity of microtubule-targeting agents against neuroblastoma cells. Similar results were obtained using SHEP cells, and the synergism between propranolol and vincristine was further confirmed in SK-N-SH cells and TH-MYCN mouse-derived neuroblastoma cell line NH02A (Supplementary Figure S2). Importantly, the combination of β-blockers and vincristine did not result in increased cytotoxicity in normal fibroblasts (Supplementary Figure S3).

Matrigel assay further showed that β-blockers also potentiated the anti-angiogenic effects of vincristine (Figure 2B). While 10 nM vincristine alone inhibited the formation of capillary-like structures by BMH29L cells by 42 ± 3% ($P<0.001$) and low concentrations of propranolol, carvedilol and nebivolol alone did not significantly affect it, the combination treatment led to 62 ± 5, 90 ± 6 and 79 ± 5% reduction in angiogenesis, respectively ($P<0.05$ for all three drug combinations as compared with vincristine alone).

β-blockers potentiate the antimitochondrial and antimitotic effects of vincristine. Microtubule-targeting agents are known to inhibit cancer cell proliferation by disrupting mitochondrial functions, inducing mitotic arrest and ultimately triggering apoptosis (Rovini *et al*, 2011). Here, we used SK-N-SH neuroblastoma cells stably transfected with mtDsRed to allow for the visualisation of the mitochondrial network in live cells without affecting mitochondrial metabolism. Propranolol was found to induce mitochondrial network fragmentation only at toxic concentrations (i.e., from 100 μM) and this effect was not inhibited by mDivi-1, a specific inhibitor of dynamin-related protein 1 (Drp1)-mediated mitochondrial fission (Savry *et al*, 2013) (Supplementary Figure S4). Although it did not induce mitochondrial network fragmentation when used alone at low concentration, propranolol strongly potentiated the fragmentation of the mitochondrial network induced by 6-h-vincristine incubation (Figure 3A). While 10 μM propranolol did not significantly induce mitochondrial network fragmentation and 5 nM vincristine resulted in 9.0 ± 1.0% of cells showing a fragmented mitochondrial network, the combination treatment induced mitochondrial fragmentation in 26.3 ± 3.2% of cells ($P<0.01$ as compared with both treatments alone).

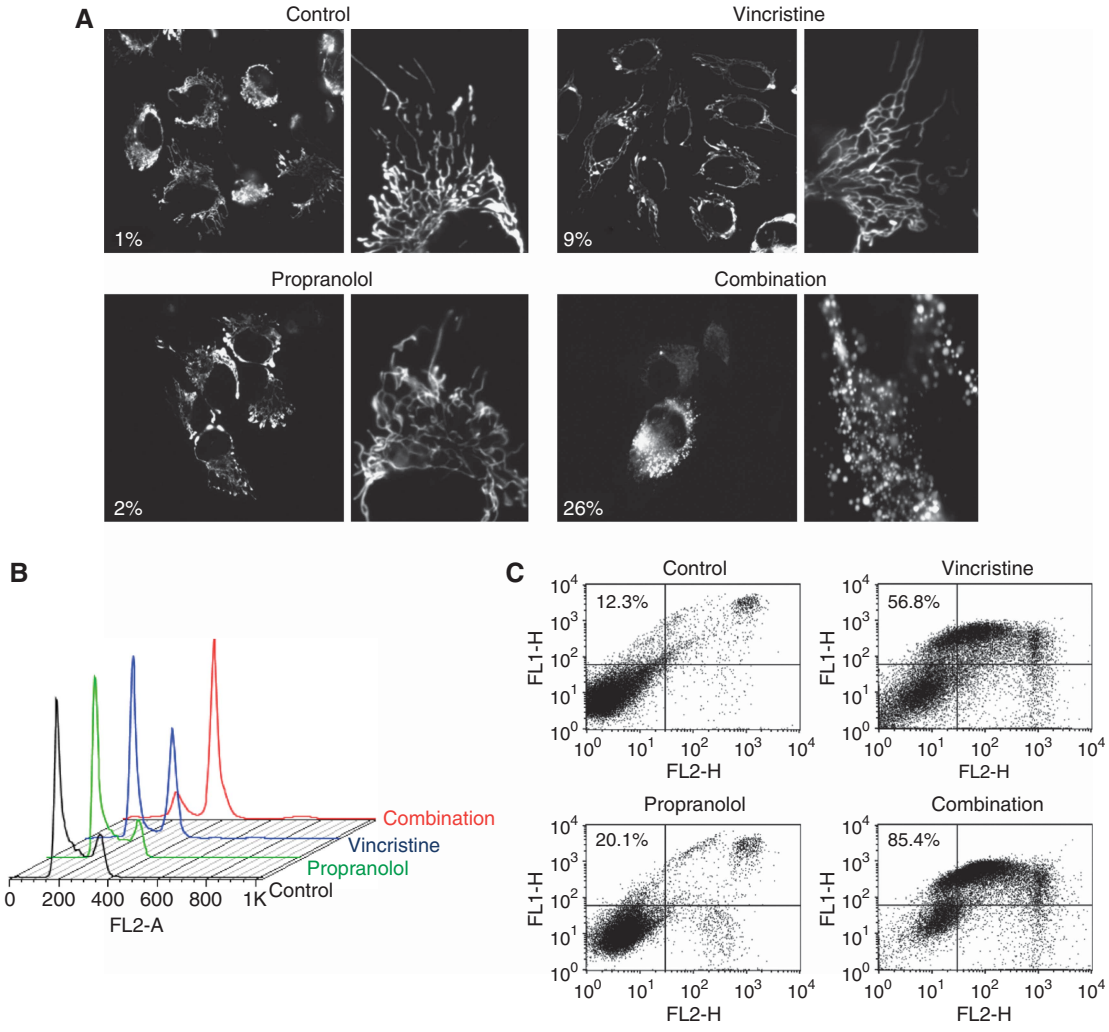

Figure 3. Antimitochondrial and antimitotic effects. (**A**) Representative photographs of SK-N-SH cells stably expressing mtDsRed and incubated for 6 h with propranolol alone (10 μM), vincristine alone (5 nM) or the combination. Photographs were taken using the ×40 objective of a Leica DM-IRBE microscope. Amplified views show the mitochondrial network of an individual cell in more detail. *Inset*, % of cells showing fragmented mitochondrial network. (**B**) Representative cell cycle profiles of SHEP cells incubated for 18 h with propranolol alone (10 μM – *green*), vincristine alone (2 nM – *blue*) or the combination (*red*). (**C**) Representative dot plots of SHEP cells following Annexin V-FITC (*x* axis) and PI (*y* axis) staining after 48-h incubation with propranolol alone (50 μM), vincristine alone (10 nM) or the combination.

Cell cycle analysis further revealed that β-blockers increased the mitotic arrest induced by vincristine in neuroblastoma cells after 18-h-drug incubation (Figure 3B). Although treatment with 10 μM propranolol did not significantly alter the cell cycle distribution of SHEP cells, its combination with 2.5 nM vincristine increased the percentage of cells arrested in mitosis from 38.8 ± 2.4 to 63.3 ± 3.6% ($P<0.01$). Consistently, with the increase in mitochondrial network fragmentation and mitotic arrest, a significant increase in apoptosis induction was detected after 48-h-drug incubation (Figure 3C). Treatment with 50 μM propranolol alone did not significantly induce apoptosis in SHEP cells but it increased the level of apoptosis induced by 10 nM vincristine from 56.8 ± 8.1 to 85.4 ± 5.3% when used in combination ($P<0.01$). Similar results were obtained in BE(2)-C neuroblastoma cells, BMH29L endothelial cells, and using carvedilol and nebivolol (data not shown).

β-blockers increase the antiproliferative effects of vincristine *in vitro*, independently of P-glycoprotein expression. Previous studies have demonstrated that some β-blockers, including carvedilol and propranolol, are potent inhibitors of P-glycoprotein (P-gp) (Bachmakov *et al*, 2006). Here, we showed that low concentrations of propranolol and carvedilol, but not nebivolol, significantly increased the intracellular accumulation of vincristine in neuroblastoma cells (Figure 4A). The increase in vincristine uptake was more pronounced in BE(2)-C cells (+150%) than in SHEP cells (+36–39%), most likely due to higher *ABCB1* gene expression (i.e., the gene encoding P-gp) in these cells (Supplementary Figure S5). Functional analysis of *ABCB1* was therefore undertaken in BE(2)-C cells, and siRNA transfection resulted in 82.1 ± 0.1% decrease in gene expression (Figure 4B). Importantly, knocking down *ABCB1* gene expression did not alter the CI between β-blockers and vincristine in BE(2)-C cells (Figure 4C). Propranolol and carvedilol, thus, synergized with vincristine in neuroblastoma cells, irrespective of P-gp expression.

β-blockers increase the antitumour effect and overall efficacy of vincristine against neuroblastoma *in vivo*. The best characterised genetic abnormality in neuroblastoma is amplification of the *MYCN* oncogene (Weiss *et al*, 1997). The TH-MYCN transgenic mouse model of neuroblastoma is based on targeted expression of human *MYCN* oncogene to mouse neuroectodermal cells via the tyrosine hydroxylase promoter (Weiss *et al*, 1997). Tumour formation in these transgenic mice is dependent on transgene dosage, with 100% of homozygous MYCN mice developing neuroblastoma tumours, with an average latency of 6–7 weeks (Norris *et al*, 2000). Here, we used the TH-MYCN model, which expresses detectable levels of adrenergic receptors β1 and β2 (Supplementary Figure S6) to evaluate the efficacy of β-blockers alone and in combination with vincristine for the treatment of neuroblastoma. When used alone, β-blockers were not able to induce tumour regression, but they significantly slowed tumour progression as compared with vehicle only (Figure 5A; $P<0.01$). Importantly, when used in combination, β-blockers significantly increased the tumour regression induced by vincristine after 3 days of treatment ($P<0.05$). They also slowed tumour regrowth observed 6 days after single vincristine injection ($P<0.05$), except propranolol that did not show a statistically significant difference due to high inter-animal variability.

The histological appearance of vehicle-treated tumours was consistent with that previously reported for *MYCN*-driven neuroblastoma tumours (Moore *et al*, 2008). They were morphologically undifferentiated or poorly differentiated neuroblastomas, with nil or few ganglion-like cells, scant Schwannian stroma and a high mitosis-karyorrhexis index (Supplementary Figure S7). Treatment with β-blockers alone induced little histological change on day 4 except nebivolol, which induced some degree of tumour differentiation, as indicated by a significant increase in the number of ganglion-like cells present in the tumour tissue (Supplementary Figure S7). Propranolol and nebivolol treatment also significantly reduced the extent of haemorrhage within the tumour (Supplementary Figure S7). When combined with vincristine, β-blockers induced further tumour differentiation as evidenced by significant increases in the number of ganglion-like cells and

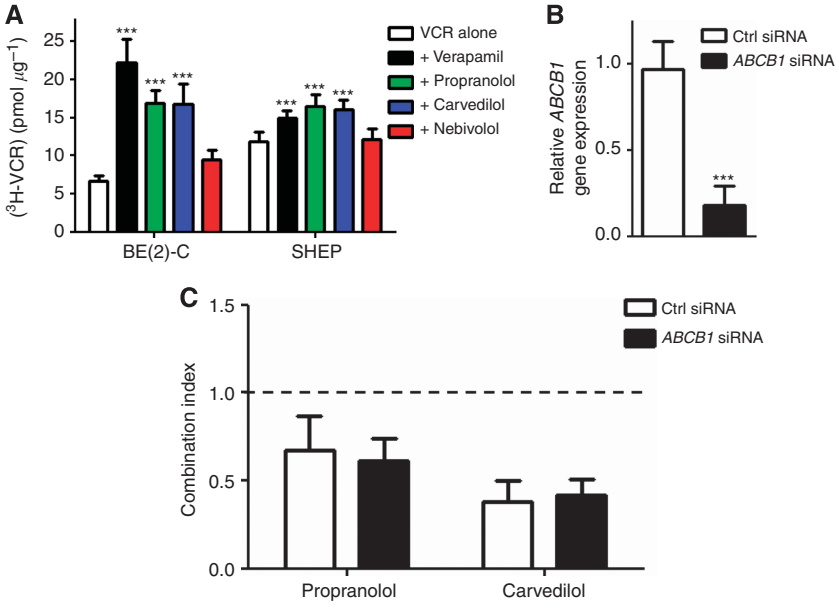

Figure 4. *ABCB1* functional study. (**A**) Intracellular drug accumulation in BE(2)-C and SHEP cells, as determined by scintillation count after 4-h incubation with 50 nM ^{3}H-vincristine alone (*white*) or in presence of 10 μM verapamil (*black*), 10 μM propranolol (*green*), 1 μM carvedilol (*blue*) and 1 μM nebivolol (*red*). *Columns*, means of at least four individual experiments; *bars*, 95% CI. (**B**) *ABCB1* gene expression in BE(2)-C as determined by qRT–PCR, following 72-h-siRNA transfection. *Columns*, means of at least four individual experiments; *bars*, 95% CI. (**C**) Combination index of vincristine and β-blockers in ctrl and *ABCB1* siRNA-treated BE(2)-C cells as determined, using the Chou and Talalay method for a range of drug concentrations (0.1–100 nM, 50 μM and 5 μM for vincristine, propranolol and carvedilol, respectively). *Columns*, means of at least three individual experiments; *bars*, 95% CI. Statistical analyses were performed using Student's *t*-test (***$P<0.001$).

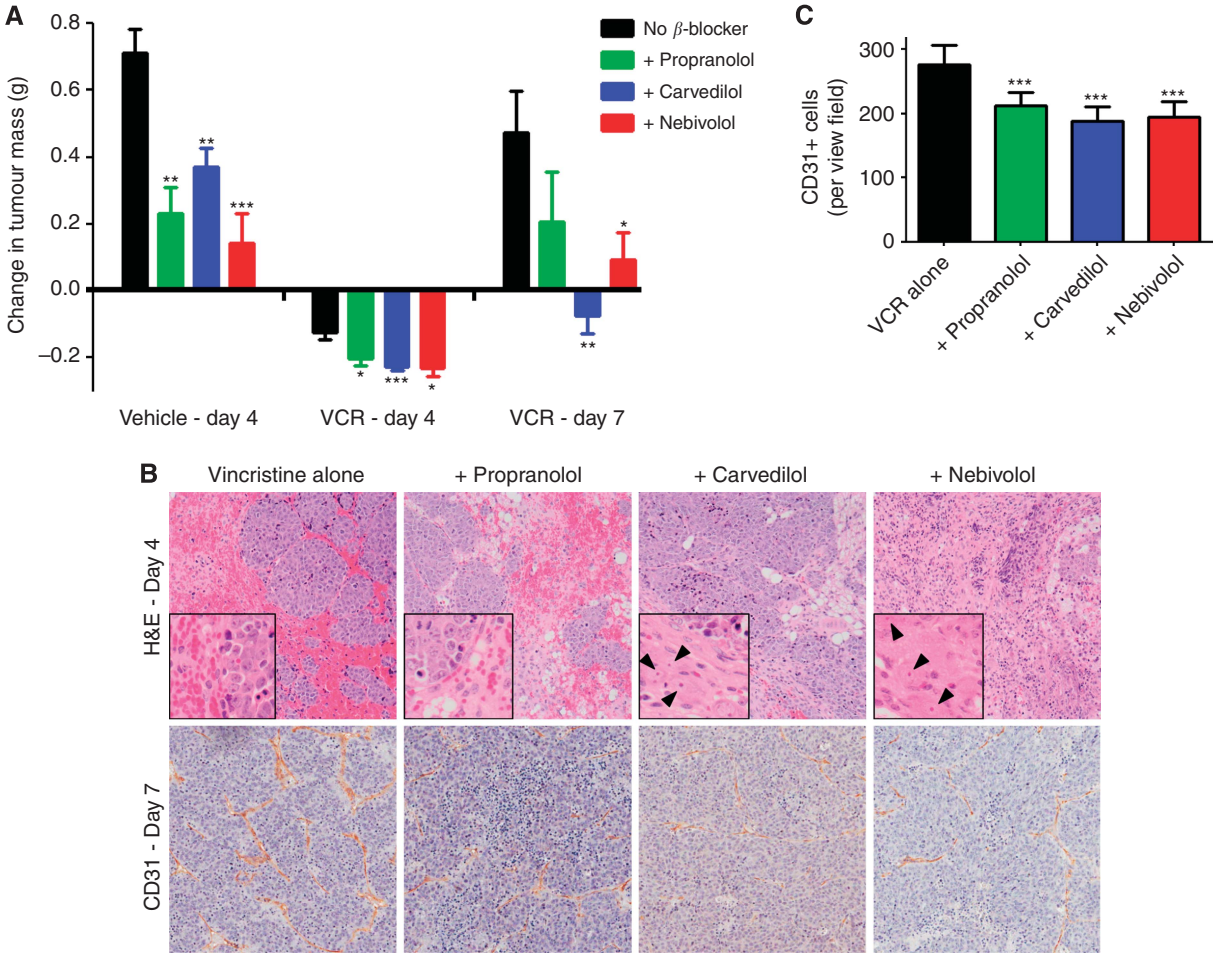

Figure 5. Antitumour effects *in vivo*. (**A**) Change in tumour mass in TH-MYCN mice ($n=6$ per cohort) on days 4 and 7 of drug treatment, as compared with tumour mass at start of treatment (day 1). Treatment consisted of vehicle or vincristine only (single i.v. injection on day 1 at 0.2 mg kg^{-1}; *black*) or in combination with propranolol (daily i.p. injection at 50 mg kg^{-1}; *green*), carvedilol (daily i.p. injection at 10 mg kg^{-1}; *blue*) or nebivolol (daily i.p. injection at 10 mg kg^{-1}; *red*). *Columns*, means of at least six animals; *bars*, s.e.m. (**B**) Representative photographs of tumour sections following H&E staining on day 4 (*top*), and CD31 staining on day 7 (*bottom*) of treatment with either vincristine alone (*left*) or the combination of vincristine and β-blockers. Insets show magnified views of H&E staining; arrowheads point to ganglion-like cells. (**C**) Histogram representation of the number of CD31 positive cells per view field on day 7. *Columns*, means of at least eight view field per tumours ($n=5$ per cohort); *bars*, 95% CI. Statistical analysis was performed using Student's *t*-test by comparing vincristine alone with the combination ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$).

Schwannian stroma (Figure 5B and Supplementary Figure S7). Furthermore, the increased antitumour effect induced by the combination of vincristine and β-blockers was associated with an increase in tumour angiogenesis inhibition (Figure 5B). Indeed, all the three β-blockers were found to significantly decrease the number of CD31 positive cells in neuroblastoma tumours by 23–32%, as compared with vincristine alone (Figure 5C; $P<0.001$).

The survival study showed that treatment with β-blockers alone or in combination with vincristine was well-tolerated and did not result in increased toxicity, as evidenced by a lack of significant change in animal weight gain (Supplementary Figure S8). Not surprisingly, the weak antitumour effects of β-blockers alone translated into little or no increase in median survival (Figure 6 and Table 1). However, the combination of propranolol and carvedilol with vincristine resulted in a substantial increase in median survival. While the median survival of mice treated with vincristine alone was 7 days ($P<0.0001$ as compared with vehicle-treated mice), it increased to 30 and 29 days when vincristine was combined with propranolol and carvedilol, respectively ($P<0.01$ as compared with mice treated with vincristine alone). Interestingly, only the combination of vincristine and carvedilol resulted in sustained complete remission in one animal, which remained tumour-free until study completion (day 60). Collectively, our results show that β-blockers increase the antitumour and anti-angiogenic effects of vincristine against neuroblastoma *in vivo*, ultimately resulting in increased treatment efficacy and prolonged median survival.

DISCUSSION

In the past 3 years, an increasing number of retrospective studies have suggested that the use of β-blockers may be associated with clinical benefits in cancer patients (Powe *et al*, 2010; Barron *et al*, 2011; De Giorgi *et al*, 2011; Lemeshow *et al*, 2011; Melhem-Bertrandt *et al*, 2011; Diaz *et al*, 2012; Wang *et al*, 2013). The present study revealed that β-blockers exert strong antiproliferative effects against neuroblastoma cells along with potent anti-angiogenic effects *in vitro*, irrespective of their selectivity for adrenergic receptors. The β-blockers with the most potent antineuroblastoma properties *in vitro* were mixed α/β-blocker

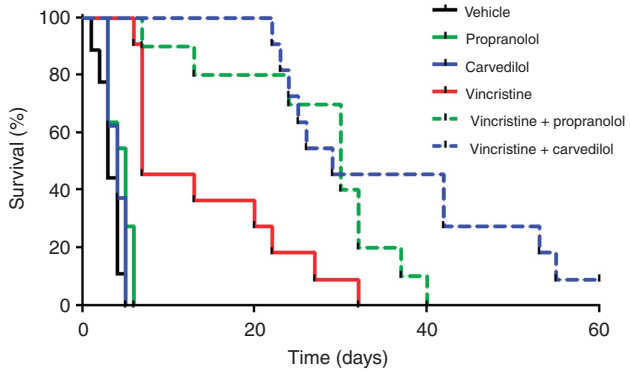

Figure 6. Survival benefits *in vivo*. Kaplan–Meier survival curves of TH-MYCN mice ($n \geqslant 8$ per cohort) following treatment with vehicle only (*black*), vincristine alone (weekly i.v. injection at 0.2 mg kg^{-1} for 3 consecutive weeks on days 1, 8 and 15; *red*), propranolol alone (i.p. injection at 50 mg kg^{-1}, 5 days a week; *solid line, green*), carvedilol alone (i.p injection at 10 mg/kg, 5 days a week; *solid line, blue*), or the combination of vincristine and propranolol (*broken line, green*) or vincristine and carvedilol (*broken line, blue*) following the same schedule. Mice were killed when tumour reached 1 cm^3 or when showing signs of morbidity.

Table 1. Median survival of TH-MYCN mice

Treatment (mice per cohort)	Median survival (days)	Log-rank test (*vs* vehicle)	Log-rank test (*vs* vincristine)
Vehicle ($n=9$)	3	N/A	$P<0.0001$
Propranolol ($n=11$) i.p. 50 mg kg^{-1}, 5 days per week	5	$P=0.031$	$P<0.0001$
Carvedilol ($n=8$) i.p. 10 mg kg^{-1}, 5 days per week	4	$P=0.136$	$P<0.0001$
Vincristine ($n=11$) i.v. 0.2 mg kg^{-1}, weekly	7	$P<0.0001$	N/A
Vincristine + propranolol ($n=10$)	30	$P<0.0001$	$P=0.0061$
Vincristine + carvedilol ($n=10$)	29	$P<0.0001$	$P=0.0009$

carvedilol, selective β1-blocker nebivolol and, to a lesser extent, non-selective β-blocker propranolol. Besides their direct anticancer properties *in vitro*, all three β-blockers synergistically interacted with microtubule-targeting agents, and particularly with vincristine. Mechanistically, this synergism appears to be mediated by enhanced mitochondrial network fragmentation and increased cell cycle arrest, ultimately resulting in increased apoptotic cell death. Importantly, the synergism observed between β-blockers and vincristine *in vitro* translated into increased antitumour and anti-angiogenic effects *in vivo*, and resulted in prolonged median survival in neuroblastoma-bearing mice.

A number of studies have recently highlighted the potential anti-angiogenic and anticancer properties of β-blockers. First, the serendipitous observation of the efficacy of propranolol in treating severe haemangioma of infancy has revolutionized the clinical management of these vascular tumours (Leaute-Labreze *et al*, 2008). In addition, preclinical studies have shown that propranolol could significantly inhibit stress-induced tumour growth and/or metastasis in animal models of breast cancer, ovarian cancer and acute lymphoblastic leukaemia (Thaker *et al*, 2006; Sloan *et al*, 2010; Lamkin *et al*, 2012). Here, we found that β-blockers alone were able to transiently slow down the growth of *MYCN*-driven neuroblastoma tumours but this only translated into a marginal increase in median survival. In contrast, when combined with vincristine, β-blockers were able to significantly increase the antitumour and anti-angiogenic effects of the treatment, ultimately resulting in a substantial increase in median survival.

Out of the chemotherapy agents tested in this study, β-blockers were found to selectively potentiate the antiproliferative effects of two of the chemotherapeutics most commonly used in the clinic for the treatment of neuroblastoma: vincristine (up to nine-fold) and doxorubicin (up to 1.8-fold). Interestingly, vincristine and doxorubicin are often used in combination in the clinic for the treatment of various cancers, such as lymphomas and acute lymphoblastic leukaemia, in addition to neuroblastoma. Furthermore, preclinical studies recently reported synergisms between β-blockers and cancer therapy in a broad range of tumour cell lines, such as radiotherapy in gastric cancer cells (Liao *et al*, 2010), gemcitabine in pancreatic cancer cells (Shan *et al*, 2011), tyrosine kinase inhibitor imatinib in glioma cells (Erguven *et al*, 2010) and paclitaxel and 5-FU in an orthotopic model of triple-negative breast cancer (Pasquier *et al*, 2011).

Surprisingly, there was no clear correlation between the selectivity of β-blockers for the different β-adrenergic receptors and their antiproliferative effects against neuroblastoma cells, suggesting that this effect may be independent from adrenergic receptor blockade and involve off-target effects. One of the major off-target effects of some β-blockers, including carvedilol and propranolol, is the inhibition of P-gp (Wigler and Patterson, 1994; Kwon *et al*, 1996; Hamilton *et al*, 2001; Bachmakov *et al*, 2006), which can increase cell sensitivity to P-gp substrates, such as vinblastine, paclitaxel and doxorubicin (Jonsson *et al*, 1999; Kakumoto *et al*, 2003). Here, we confirmed that carvedilol and propranolol, but not nebivolol, significantly increased the accumulation of vincristine inside neuroblastoma cells. However, functional analysis using siRNA demonstrated that the synergism between vincristine and β-blockers was independent from P-gp expression. This results show that β-blockers can potentiate the antiproliferative effects of vincristine in the absence of P-gp expression, and this synergism involves additional mechanisms that remain to be determined.

A range of mechanisms have been proposed to mediate the influence of β-adrenergic signalling on tumour progression, metastasis and response to treatment, including angiogenesis, inflammation, resistance to apoptosis/anoikis, stimulation of tumour cell invasion and epithelial–mesenchymal transition (Cole and Sood, 2011). In addition, several mechanisms have also been proposed to explain the anti-angiogenic and antitumour effects of β-blockers. For instance, the inhibitory effects of propranolol on stress-induced tumour growth and metastasis have been attributed to anti-angiogenic mechanisms, inhibition of stress-induced macrophage infiltration and inhibition of β2-adrenergic receptor signalling (Thaker *et al*, 2006; Sloan *et al*, 2010). Propranolol was also shown to induce the regression of haemangioma cells through HIF-1α-mediated inhibition of VEGF signalling (Chim *et al*, 2012). In addition, inhibition of NF-κB signalling, downregulation of Bcl-2 and induction of Bax were reported in the synergistic interaction between β-blockers and gemcitabine in pancreatic cancer cells (Shan *et al*, 2011). Functional assays used in the present study revealed that β-blockers could potentiate the key mechanisms of action of vincristine: mitochondrial network fragmentation, mitotic arrest and apoptosis induction. Interestingly, in contrast with a large majority of apoptosis-inducing stimuli, including microtubule-targeting agents (Savry *et al*, 2013), the pharmacological inhibition of dynamin-related protein Drp1 did not attenuate mitochondrial fragmentation induced by cytotoxic concentrations of propranolol. This suggests that propranolol may activate the mitochondrial fission machinery in a Drp1-independent manner, and/or that the mitochondrial fragmentation induced by propranolol rather results

from a disruption of the mitochondrial fusion machinery. Since engaging the mitochondrial fission machinery usually triggers tumour cell apoptosis (Oakes and Korsmeyer, 2004; Reis *et al*, 2012), this mechanistic difference may contribute to the selective synergism observed between β-blockers and vincristine against neuroblastoma cells.

CONCLUSIONS

By using approved drugs with known toxicity and pharmacokinetic profiles, drug repositioning can save time and remove substantial risks and costs from the pathway to the clinic. Here, we provide the first evidence to encourage the repositioning of β-blockers in paediatric oncology. Dose-escalation studies may be required to define the most effective and safe doses of β-blockers to use in combination with chemotherapy in neuroblastoma patients. The feasibility of this innovative therapeutic strategy is supported by the fact that β-blockers have been used in children for decades for the management of hypertension and cardiac arrhythmia. Moreover, β-blockers have been used in the perioperative management of patients with catecholamine-secreting neuroblastoma (Hernandez *et al*, 2009; Pappas *et al*, 2010) and the specific combination of vincristine and propranolol has been recently used with success in a 6-week-old boy with Kaposiform haemangioendothelioma and Kasabach–Merritt syndrome (Hermans *et al*, 2011). Collectively, the results of the present study together with recent epidemiologic data and clinical case reports provide a strong rationale for the clinical evaluation of a combination of β-blockers and chemotherapy for the treatment of neuroblastoma and other drug-refractory cancers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

EP, TT, NA and MK conceived and designed the experiments and coordinated the work presented. EP and CP performed flow cytometry analyses, proliferation and matrigel assays with β-blockers alone and in combination with chemotherapy agents. MC performed the mitochondrial network analysis. EP performed the functional study of β-adrenergic receptors and P-gp in neuroblastoma cells. EP, JS and JM were responsible for the animal studies. JS was also responsible for histological studies and qRT–PCR from both cell lines and tumour tissues. AJG performed the histological analysis of tumour sections in blind. EP collected and analysed the data and wrote the manuscript. MC, AJG, JM, MDN, TT, NA and MK commented on and edited the manuscript.

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