Dissecting the role of cell signaling versus CD8+ T cell modulation in propranolol antitumor activity

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Abstract

Preclinical and early clinical mechanistic studies of antitumor activity from the beta-adrenergic receptor (β-AR) blocker propranolol have revealed both cell signaling and immune function pathway effects. Intertumoral studies were performed using propranolol, a β1-AR selective agent (atenolol), and a β2-AR selective agent (ICI 118,551) in a preclinical in vivo model, as a step to dissect the contribution of cell signaling and CD8+ immunological effects on anticancer activity. We found that repression of β2-AR but not β1-AR signaling selectively suppressed cell viability and inhibited xenograft growth in vivo. Moreover, western blot analysis indicated that the phosphorylation levels of AKT/MEK/ERK were significantly decreased following the inhibition of β2-AR. Furthermore, propranolol was found to activate the tumor microenvironment by inducing an increased intratumoral frequency of CD8+ T cells, whereas neither selective β1 nor β2-AR blockers had a significant effect on the tumor immune microenvironment. Thus, the results of this mechanistic dissection support a predominant role of tumor cell signaling, rather than the accumulation of CD8+ T cells, as the basis for propranolol antitumor activity.

Key messages

• Molecular signaling of AKT/MAPK pathway contributes to propranolol caused cancer control.
• CD8+ T cells in tumor microenvironment were activated upon propranolol exposure.
• The basis for propranolol antitumor activity was predominantly dependent on cell signaling, rather than the activation of CD8+ T cells.

Keywords Propranolol · Immunotherapy · Cell signaling · AKT/MAPK pathway · CD8+ T cells
**Introduction**

Propranolol is a non-selective β-adrenergic receptor (β-AR) blocker and is widely used in the treatment of hypertension, atrial tachycardia, migraine, and infantile hemangiomas [1–4]. Several studies have demonstrated propranolol has the potential antitumor effects in patients with gastric cancer, prostate cancer, colorectal cancer, breast cancer, and cutaneous malignant melanoma [5–8]. Recently reported clinical studies have found that administering propranolol may reduce tumor progression and recurrence [9–14].

The mechanisms by which propranolol exhibits antitumor activity are beginning to unfold. The presence of β1- and β2-ARs has been reported for a number of human solid tumors, including breast, colorectal, ovarian, and melanoma [8, 12–16]. β-ARs stimulate multiple signaling cascades, including the cAMP/PKA, MAPK/ERK1/2, p38/MAPK, PI3K/AKT, VEGF, and Src/STAT pathways and arachidonic acid (AA) cascade, which thus induces cancer cell growth and invasion [1]. Propranolol has activity against β1- and β2-ARs [17]. Our previous work confirmed that propranolol induced G0/G1/S phase arrest and apoptosis in colorectal, gastric, and melanoma cells and xenograft models through AKT/MAPK pathway [8, 9, 18]. Our perioperative clinical studies found that compared with the control patients, the expression of phosphorylated AKT/ERK/MEK protein in tumor tissues had been significantly reduced in patients administering oral propranolol [8, 18]. Other studies demonstrated that propranolol inhibits norepinephrine stimulation of colon carcinoma, ovarian cancer, breast cancer, and pancreatic cancer to exert anti-proliferative, anti-migratory, and cytotoxic effects on cells [19–22].

Stimulation of adrenergic receptors on immune cells helps regulate immune cell development, survival, proliferative capacity, circulation, trafficking for immune surveillance, and recruitment [23]. Memory CD8+ T cells primarily express β2-AR, which can inhibit CD8+ T cell effector function. More recently, it has also become clear that stress-induced adrenergic signaling can play a key role in promoting tumor growth [17, 24]. We and other groups have found that propranolol can promote CD8+ T cell activation [8, 25, 26]. Other studies have also confirmed that activated memory CD8+ T cells express β2-ARs, and its functions are impaired by β2-AR signaling [27]. As propranolol has prominent effects on both β1- and β2-ARs, the interaction between ARs and either AKT/ERK/MEK signaling and/or CD8+ immune effects are relatively undefined.

**Materials and methods**

**Cell lines and reagents**

All cell lines were obtained from American Type Culture Collection (ATCC). The A375 cell lines were cultured in DMEM (Gibco, Life Technologies, China) supplemented with 10% FBS (Gibco, Life Technologies Australia) at 37 °C and 5% CO2 in a tissue culture incubator. Murine B16F10 melanoma cell line was maintained in RPMI 1640 medium (Gibco, Life Technologies, China) supplemented with 10% FBS (Gibco, Life Technologies Australia) at 37 °C and 5% CO2 in a tissue culture incubator.

**Cell viability assay**

CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay, Promega, G3582, USA) was used to determine the effects of β-adrenoceptor blockers on cancer cell proliferation [8]. A375 and B16F10 cell lines were plated at a density of 2.0 × 103 cell per well, in 96-well plates, and then treated with propranolol (Sigma-Aldrich, P0884, China), atenolol (MCE, HY-17498, China), or ICI 118,551 hydrochloride (MCE, HY-13951, China) over a concentration range from 0.1 to 200 μM for 24, 48, and 72 h. At the end of treatments, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H–tetrazolium (MTS) reagent was added to each well. All plates were incubated for 40 min at 37 °C in the dark. Then, the fluorescence was measured using a compact microplate spectrophotometer (BioTek® Eon, Synergy™) at 490 nm.

**In vivo model and treatment**

Syngeneic female C57BL/6 J mice (8 weeks old) from Hunan Silaïe Experimental Animals Inc (China) were kept on a 12-h light/12-h dark cycle with the room temperature maintained at 22 °C. Experimental research on mice was performed with the approval of the ethics committee on animal research of the Xiangya Hospital (China).

The B16F10 mouse melanoma cells (6 × 105 cells) in 100 μL RPMI 1640 medium without FBS were injected s.c. into the flank of each mouse. A week later, animals were randomly divided into six groups of six mice each: one PBS group and five treatment groups. Propranolol was dissolved in PBS solution and administered i.p. 20 mg kg−1 day−1, and ICI 118,551 was given 20 and 50 mg kg−1 day−1. Atenolol was given 80 and 100 mg kg−1 day−1, and ICI 118,551 was given 20 and 50 mg kg−1 day−1. Tumor length (L) and width (W) were measured every 3 days, and the tumor volume was calculated as L × W2 × 0.5. At the end of the experiment, tumor tissues were collected, weight, fixed in formalin solution, or frozen at −80 °C.

**Western blotting analysis**

Effects of β-ARs blockers on signaling in cancer cells were examined by western blotting. For the in vitro studies, A375
and B16F10 cell lines were treated with or without β-AR blockers for 24 h. After the treatment, cells were lysed, protein was extracted, and the total protein concentration was measured using BCA assays (Beyotime Biotechnology, China). Protein samples were resolved on NuPAGE 12% Bis–Tris gels with MOPS buffer or 3–8% Tris acetate gels with Tris acetate buffer (Life Technologies) and then transferred to a 0.45-mm nitrocellulose membrane (Bio-Rad). After saturation in Tris-buffered saline supplemented with 5% BSA, the membranes were incubated with antibodies (diluted at 1:2000) overnight at 4 °C. Antibodies specific for the following proteins were purchased from Cell Signaling Technology: AKT (rabbit, 9272), MEK1/2 (L38C12) (mouse, 4694), ERK1/2 (rabbit, 9102), phospho-ser473-AKT (rabbit, 9271), phosphor-ser221-MEK (rabbit, 2338), phospho-ERK1/2-Thr202/Tyr204 (rabbit, 4370). The antibody specific for GAPDH (mouse, clone 6C5, MAB374) was purchased from Millipore. Quantification of the bands was done with ImageJ.

**Flow cytometry**

Single-cell suspensions were created by excising and cutting mouse tissues into 2–3-mm pieces. Tumor and spleen were mechanically disrupted and directly passed through a 70-μm nylon cell strainer (Corning). Cells were then washed with flow running buffer (0.1% BSA in PBS) and incubated with Zombie NIR™ (Biolegend, 1:100) at room temperature for 20 min, which were used to gate out dead cells. Cells were then stained with the following extracellular antibodies: anti-mouse CD3 FITC, anti-mouse CD8a APC, anti-mouse CD4 PerCP/Cy5.5, anti-mouse CD 279 (PD-1) Brilliant Violet 421™, or anti-mouse CD45 Brilliant Violet 510™ all purchased from Biolegend. For intracellular staining, cells were surface-stained as above, then fixed and permeabilized using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) as per the manufacturer’s protocol. Cells were then stained with anti-mouse FoxP3 PE or anti-mouse IFNγ Brilliant Violet 510™ from Biolegend. All data were collected on a flow cytometer (BD Biosciences, Canto II) and analyzed with FlowJo v10 software (Tree Star, Inc.).

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed for CD8 and IFN-γ to evaluate immune status. Deparaffinized tissue sections were treated with Antigen Retrieval Solution (made from citrate buffer, pH 6.0, concentrated 103, T0050 Diapath). Tissue sections were then incubated with Peroxidase Blocking Solution (S2023, Dako) for 15 min and Protein Block (X0909, Dako) for 20 min. All sections were incubated with CD8 (ab4055, Abcam) antibody and IFN-γ (MAB285-sp, Novus) antibody respectively at 4 °C overnight. A conventional streptavidin–biotin method was used to detect binding antibodies according to the manufacturer’s protocol (S-A/HRP kit, ZSGB-BIO). The reaction was visualized using 3,3′-diaminobenzidine + Chromogen, and nuclei were counterstained using hematoxylin. Finally, the slides were covered with coverslips using gelatin.

H-SCORE = \[\sum (\text{PI} \times I) = (\text{percentage of cells of weak intensity} \times 1) + (\text{percentage of cells of moderate intensity} \times 2) + (\text{percentage of cells of strong intensity} \times 3)\]. In this formula, PI indicates the percentage of positive cells in the section and I represents the intensity of staining [28–30].

**Statistical analysis**

Student’s t test was used to compare data between two groups and tumor growth statistics were calculated using two-way ANOVA with Tukey’s analysis using GraphPad Prism software. All one-way data are depicted as mean ± SEM.

**Results**

**Antitumor activity of propranolol, ICI 118,551, and atenolol in vitro and in vivo**

The effect of propranolol, the β2-AR selective agent ICI 118,551, and the β1-AR selective agent atenolol on tumor growth was assessed in vitro and in vivo. Propranolol and ICI 118,551 significantly reduced in vitro cell viability of A375 and B16F10 in a concentration-dependent and time-dependent manner, whereas the effect of atenolol was not notable in B16F10 cell line. But the high concentrations of atenolol can also reduce cell viability of A375 cells (Fig. 1).

To further evaluate whether the therapeutic activity in vitro could be transferred to xenografts, the B16F10 melanoma model was engrafted in C57BL/6 mice. The mean tumor sizes of propranolol and ICI 118,551 groups were smaller than those of the PBS group on day 14 in B16F10 xenografts (1339 ± 232.9 mm³ vs 2915 ± 386.2 mm³, p = 0.0058; 606.2 ± 60.89 mm³ vs 2915 ± 386.2 mm³, p < 0.0001), but there is not a significant difference between the atenolol and PBS groups (2238 ± 659.4 mm³ vs 2915 ± 386.2 mm³, p = 0.37; Fig. 2A and B). Notably, no mice in any of the three drug groups lost body weight (more than 10%) during this treatment (Fig. 2C) and there was an absence of any gross toxicity from this treatment.

**The expression of AKT/MAPK proteins was inhibited by propranolol and ICI 118,551 in vitro**

The expression and phosphorylation of AKT, ERK and MEK were evaluated by western blotting in two cancer cell...
The expression of AKT, ERK, and MEK was decreased 8.5% \((p > 0.05)\), 36.0% \((p < 0.05)\), and 35.0% \((p < 0.01)\) by propranolol and 1.0% \((p > 0.05)\), 57.0% \((p < 0.01)\), and 60.5% \((p < 0.001)\) by ICI 118,551 in A375 cells. The phosphorylation levels of AKT, ERK, and MEK were also significantly decreased 61.0% \((p < 0.01)\), 68.0% \((p < 0.001)\), and 47.2% \((p < 0.001)\) by propranolol and 69.0% \((p < 0.001)\), 65.9% \((p < 0.001)\), and 52.1% \((p < 0.001)\) by ICI 118,551 in A375 cells. Interestingly, atenolol also inhibited the expression of p-ERK and p-MEK in A375 cells by 34.7% \((p < 0.01)\) and 21.1% \((p < 0.01)\); however, it increased the expression of AKT by 21.0% \((p < 0.05)\) (Fig. 3C). The expression of MEK, p-MEK, and p-AKT was also significantly inhibited by propranolol and ICI 118,551 in B16F10 cells \((p < 0.05)\). These data implied inhibition of \(\beta\)-AR has impact on the AKT/ERK/MEK pathway.

**Inhibited tumor growth following \(\beta\)-AR blockade results from enhanced CD8\(^+\) T cell-mediated antitumor immune response**

The frequency of CD4\(^+\) and CD8\(^+\) T cells in the tumor microenvironment (TME) of the xenograft tumors was assessed via flow cytometry. The frequency and relative number of CD8\(^+\) T cells in the propranolol group were significantly
higher than those in the control group (58.53 ± 4.595 vs 41.37 ± 0.6566, \( p = 0.0209 \)). However, there was no significant difference in either of the selective \( \beta \)-AR blocker groups (Fig. 4A). The frequency of CD4\(^+\) regulatory T cells in TME was also assessed via flow cytometry. In this context, the frequency of CD4\(^+\) T cells with a Treg phenotype was significantly reduced in propranolol-treated mice and ICI 118,551-treated mice but not atenolol group (propranolol 48.26 ± 7.856, \( p = 0.019 \), ICI 118,551 59.12 ± 4.724, \( p = 0.023 \), atenolol 76.75 ± 0.7343, \( p = 0.673 \) vs control 77.58 ± 2.499; Fig. 4B). To further explore the CD8\(^+\) T cell infiltration in tumor, IHC was performed on tumor sections from all experimental groups. A significant increase of CD8 index was observed in the propranolol-treated xenografts, but no effect was found in the selective \( \beta \)-AR blocker groups (Fig. 4C). Although propranolol and ICI 118,551 increased the expression of IFN\( \gamma \) in TME, it is not statistically different when compared with the control group (Fig. 4C).

Fig. 2 Effects of \( \beta \)-blockers on tumor growth in vivo. The B16F10 mouse melanoma cells (6 \( \times \) 10\(^5\) cells) in 100 \( \mu \)L RPMI 1640 medium without FBS were injected s.c. into the flank of each mouse 1 week prior to drug treatment. A, B Quantitative analysis of tumor volume from days 0 to 14. C Quantitative analysis of tumor weight of each group. D Quantitative analysis of mouse weight of each group, indicating the absence of gross toxicity for the treatment. The growth curves of tumor measured by average volume of 6 mice in each group. The data are presented as the mean ± SEM of three independent experiments. *\( p < 0.05 \); **\( p < 0.01 \); ***\( p < 0.001 \); ns, not significant. \( N = 6 \) per group

Fig. 3 The effect of \( \beta \)-blockers on AKT/MAPK pathway in vitro. Representative images and quantitative analysis of western blot data for ERK, MEK, AKT, p-ERK, p-MEK, and p-AKT in A375 cells A–C and B16F10 cells D–F which were exposed to propranolol (100 \( \mu \)M), atenolol (100 \( \mu \)M), and ICI 118,551 (100 \( \mu \)M) for 24 h. The data are presented as the mean ± SEM of three independent experiments. *\( p < 0.05 \); **\( p < 0.01 \); ***\( p < 0.001 \); ns, not significant
Fig. 4 The effect of β-blockers on antitumor immune response. A Representative images and quantitative analysis of flow cytometry analysis for CD8+ T cells and CD4+ T cells in the spleen of B16F10 mouse melanoma cells (6 × 10⁵ cells) implanted C57BL/6 J mouse model. B Representative images and quantitative analysis of flow cytometry analysis for CD4+ regulatory T cells in the spleen. C Representative images and quantitative analysis of immunohistochemistry analysis of CD8+ T cells and IFN-γ of xenograft tumors harvested from mice. The data are presented as the mean ± SEM of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant. N = 3 to 5 per group.

Discussion

Propranolol has demonstrated anticancer activity in a number of preclinical and clinical models, including colorectal, ovarian, and breast carcinoma; hemangioma; and melanoma. In addition to decrease in tumor size, biochemical and molecular measures of apoptosis were observed. This has heightened interest in the mechanistic basis for propranolol’s antitumor effects. As propranolol is known to inhibit both β₁- and β₂-adrenergic receptors and these receptors are expressed in many human cancers, it is important to examine the relative importance in intermediate biomarkers and observed measures of tumor size.

In our previous studies, propranolol was found to have antitumor effects by regulating AKT/MAPK pathway [9]. Results of this study demonstrated that propranolol was more cytotoxic to cancer cell lines than the β₁-blocker atenolol, but less cytotoxic than the β₂-blocker ICI 118,551, with the findings replicated in mice bearing tumor xenografts. Similar in vitro results were reported by Chin et al. which suggested that colorectal cancer cell lines were more sensitive to propranolol and ICI 118,551, but not atenolol [31]. Furthermore, ICI 118,551 was thought to be more effective in tumor growth inhibition than atenolol as demonstrated in mouse xenograft of colon carcinoma [32]. Zhang et al. also found that ICI 118,551 caused inhibition of pancreatic carcinoma cell proliferation and induction of apoptosis, and these effects were stronger than the β₁-AR blocker metoprolol [33]. This data implied the β₂-AR, rather than β₁-AR signaling, is required for melanoma cell survival and tumor progress.

The mechanisms of antitumor effects of propranolol have been examined in other investigations. Propranolol and other β-blockers reduced MAPK in pancreatic cancer and hemangioma [34, 35]. Another study demonstrated that propranolol used alone inhibited migration and invasiveness of epithelial progenitor cells through AKT and MAPK/ERK pathway [36]. In a breast cancer study, propranolol and ICI 118,551 were suggested to inhibit the viability of MDA-MB-231 cells by targeting ERK1/2 phosphorylation and COX-2 expression [37]. Our study found ICI 118,551 and propranolol reduced...
the expression and phosphorylation of AKT, ERK, and MEK, whereas atenolol showed no effects on these proteins. This further supports the modulation of the AKT/MAPK signaling pathway by inhibiting the β2-AR, but not β1-AR. A recent study reported that the overexpression of ADRB2 may play an important role in the development and prognosis of gastric cancer, indicating ADRB2 may be a potential prognostic biomarker of gastric cancer [24].

It is known that β2-AR is the primary subtype expressed on immune cells, including T cells, dendritic cells, B cells, and macrophages [38, 39]. Studies have shown that increasing activating β-ARs on immune cells can significantly suppress immune cell function [40–42]. Qiao et al. demonstrated that β2-AR stimulation suppressed metabolic reprogramming by both glycolysis and oxidative phosphorylation, which revealed a new mechanism by which adrenergic stress can suppress the effector activity of immune cells [26]. Our previous study confirmed that propranolol activated autologous CD8+ T cells in patients with colorectal cancer [8]. Another study revealed that in breast and melanoma mouse models, stress promoted tumor growth and propranolol can reverse this promotion via increasing frequency of effector CD8+ T cells [25]. In addition, propranolol decreases MDSC accumulation in the spleen and recruitment in tumor tissue by blocking STAT3 signaling [23] or deactivating CXCL5-CXCR5-Erk signaling [43] which indirectly strengthen the function of cytotoxic T cells in TME. Another study showed that activation of the adrenergic receptors can promote the expression of IL-6 in breast cancer cells, and they found that tumor-derived IL-6 promoted the differentiation of MDSCs in vitro, which was a positive correlation [44]. Interestingly, in our study, we found that propranolol does improve increasing the frequency of CD8+ T cells, but neither the β1 selective blocker atenolol nor the β2 selective blockerICI 118,551 had a significant effect observed. This discordance between the potency of antitumor effect and the increasing of CD8+ T cells suggests that the immune response is not necessarily required for antitumor effect.

Conclusions

In a summary, this study explored the dual mechanism of propranolol anticancer activity. We observed that propranolol may mainly inhibit the AKT/MAPK pathway through β2-receptors; meanwhile, the increasing frequency of CD8+ T cells could be independent of β-ARs blockade. The use of β-blockers, even the selective β2-blockers, alone or in combination with first-line antitumor drugs may be a promising therapeutic strategy to treat patients with solid tumors.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest Prof McLeod is on the board of directors for Vyant Bio. He is one of the founders of Interpares Biomedicine and Clarifi and a consultant to Pharmazam and eviCORE Health Solutions. All other authors declared no competing interests for this work.

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