Abstract. Treatment options for advanced metastatic or progressive thyroid cancers are limited. Although targeted therapy specifically inhibiting intracellular kinase signaling pathways has markedly changed the therapeutic landscape, side-effects and resistance of single agent targeted therapy often leads to termination of the treatment. The objective of the present study was to identify the antitumor property of the non-selective β-adrenergic receptor antagonist propranolol for thyroid cancers. Human thyroid cancer cell lines 8505C, K1, BCPAP and BHP27 were used in the present study. Broad β-blocker propranolol and β2-specific antagonist ICI118551, but not β1-specific antagonist atenolol, inhibited the growth of 8505C and K1 cells. Propranolol treatment inhibited growth and induced apoptosis of 8505C cells in vitro and in vivo, which are closely associated with decreased expressions of cyclin D1 and anti-apoptotic Bcl-2. Expression of hexokinase 2 (HK2) and glucose transporter 1 (GLUT1) also decreased following propranolol intervention. 18F-FDG PET/CT imaging of the 8505C xenografts validated shrinkage of the tumors in the propranolol-treated group when compared to the phosphate-buffered saline treated group. Finally, we found that propranolol can amplify the cytotoxicity of vemurafenib and sensitize thyroid cancer cells to cytotoxic effect of vemurafenib. Our present results suggest that propranolol has potential activity against thyroid cancers and investigation of the combination with targeted molecular therapy for progressive thyroid cancers could be beneficial.

Introduction

Despite intensive initial treatments that include surgery, radioiodine therapy, thyroid-stimulating hormone (TSH) suppression with levothyroxine, poorly differentiated thyroid cancer including anaplastic thyroid cancer are aggressive and refractory to conventional therapies (1). As the knowledge of molecular pathogenesis of thyroid cancer increases, targeted therapies are being innovated for these patients (2). Unfortunately, side-effects and cellular resistance of single agent multikinase or BRAFV600E inhibitor often leads to termination of the targeted therapy. To overcome resistance and to reduce side-effects, combined administration containing multikinase or BRAFV600E inhibitor should be investigated. In contrast to development of new compounds, screening FDA-approved drugs may identify anticancer drugs and facilitate initiation of early clinical trials. Propranolol hydrochloride, although originally used for the treatment of hypertension, cardiovascular disorders and hemangiomas, has been shown to have anticancer property for many cancers including pancreatic, breast and gastric cancer, leukemia, neuroblastoma, and head and neck squamous cell carcinoma (3-9). In addition, retrospective studies have demonstrated that cancer patients taking β-blockers may have improved outcomes and decreased incidence of secondary malignances (9-11). Based on the basic research and clinical evidence, we hypothesized that the β-adrenergic propranolol may have potential efficacy in inhibiting the proliferation and/or inducing the apoptosis of anaplastic thyroid cancer.

In the present study we observed that β-adrenergic receptors (ADRBs), particularly β2-AR (ADRB2), are expressed in both well-differentiated and poorly-differentiated thyroid cancer cell lines. Propranolol reduced the viability of 8505C cells through inhibition of proliferation and induction of apoptosis. Propranolol induced apoptosis was associated with suppressed expression of anti-apoptotic Bcl-2 while inhibited proliferation was the result of decreased expression of cyclin D1. In addition, the levels of glucose metabolism proteins, hexokinase 2 (HK2) and glucose transporter 1 (GLUT1), also decreased in the propranolol-treated group. Furthermore, propranolol inhibited the growth of ATC xenografts in vivo at a dose of 10 mg/kg/day. Consistently 18F-FDG PET/CT imaging revealed tumor shrinkage in the propranolol-treated group. Immunohistochemistry of the tumor specimens validated...
the downregulation of Bcl-2 and cyclin D1 observed in vitro. Notably, we found that propranolol pretreatment sensitized K1 cells to the cytotoxicity of vemurafenib.

Taken together, our present findings suggest that propranolol is an effective agent in inhibiting the growth of thyroid cancers, and that the combination therapy consisting propranolol and BRAFV600E inhibitor may provide clinical benefits with minimal side-effects to BRAFV600E mutant advanced thyroid cancer patients.

Materials and methods

Cell culture. Human PTC cell line K1, BCPAP and ATC cell line 8505C were purchased from the Institute of Biochemistry and Cell Biology (SIBS, CAS, Shanghai, China). BHP27 cell line was a kind gift from Professor Li-Bo Chen in our hospital. Both the cell lines used in the present study have been confirmed for identity (Fumed Biotech Bio-Medicine; SIBS, CAS). All cell lines used in these experiments were maintained in RPMI-1640 medium (cat. #11875-093) supplemented with 10% fetal bovine serum (FBS) (cat. #16000-044) (both from Gibco, Carlsbad, CA, USA) in a 5% CO₂-95% air atmosphere at 37°C.

Cell Counting Kit-8 (CCK-8), colony formation and apoptosis assay. In order to determine the half maximal inhibitory concentration (IC₅₀) of propranolol, atenolol and ICI118551, both from Sigma (St. Louis, MO, USA); ICI118551 from MedChem Express (Monmouth Junction, NJ, USA), and the effect of isoprenaline hydrochloride (Sigma) on growth, a CCK-8 assay (Yeasen, Shanghai, China) was performed. The cells were seeded into 96-well plates at a density of 1x10⁴ cells/well for 24 h, and then incubated for 24 h with increasing concentrations of the various compounds under study (10 and 40 µM for isoprenaline; 50, 100, 150, 200, 250, 300, 350 and 400 µM for propranolol, atenolol and ICI118551). Control cells were allowed to grow in the absence of any inhibitors for the same period of time. The samples were assayed in sextuplet and at least in three independent experiments, and the mean value for each experiment was calculated. Results are presented as mean (± SEM) and are expressed as percentage of the control group. For clonogenic survival studies, K1 cells were pretreated with ABT-737 (1 µM) or propranolol (200 µM) for 24 h before treatment with vemurafenib (PLX-4032) (5 µM) for 72 h, and then the media were changed and colonies were stained with crystal violet 10 days after treatment and imaged. For nuclear fragmentation assay, 8505C cells were treated with ABT-737 (1 µM) or propranolol (200 µM) for 24 h before treatment with vemurafenib (PLX-4032) (5 µM) and incubated in 300 µl PBS (containing 20 µl RNase A) at 37°C for 30 min. In addition, 400 µl propidium iodide (PI) solution was added and incubated at 4°C for 30-60 min in the dark. Samples were analyzed on a flow cytometer (Beckman Coulter, Brea, CA, USA).

Flow cytometric analysis. Before flow cytometric apoptosis analysis, 8505C cells were treated for 24 h with increasing concentrations of propranolol (0, 100, 200, 300, 400 and 500 µM). At each concentration half a million cells were collected, washed 2 times with cold phosphate-buffered saline (PBS). Fixed cells were stained with Annexin V-FITC/PI (Yeasen). For cell cycle analysis, cells (10⁶) were treated with propranolol (100 and 200 µmol/l) for 0, 24 and 48 h. After the treatment, the cells were trypsinized and centrifuged at 800 rpm for 5 min. Subsequently, cells were collected and washed 2 times with PBS. Cell pellets were resuspended in 300 µl ice-cold PBS and fixed overnight by adding 700 µl ethanol. After washing with PBS, cell pellets were resuspended and incubated in 300 µl PBS (containing 20 µl RNase A) at 37°C for 30 min. In addition, 400 µl propidium iodide (PI) solution was added and incubated at 4°C for 30-60 min in the dark. Samples were analyzed on a flow cytometer.

Western blot analysis. 8505C cells were plated at a density of 20x10⁴ cells/well in 6-well plates. At confluence, propranolol and ICI118551 at doses of 0, 25, 50 and 100 µM were incubated with 8505C cells for 24 h. Cells were harvested in RIPA lysis buffer containing proteinase and phosphatase inhibitors. Protein was quantified using a protein assay kit (Bicinchoninic Acid; Yeasen). Equal amounts of cell lysates were separated by 12% SDS-PAGE, and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with Tris-buffered saline (TBS) containing 5% skimmed milk powder for 1 h, and then probed with specific antibodies [anti-ADRB1 from Aviva Systems Biology Corporation (San Diego, CA, USA); anti-ADRB2, anti-Akt, anti-Bcl-2, anti-MCL1, anti-Bcl-xL, anti-Bax, anti-CCND1, anti-HK2 and anti-GAPDH from ProteinTech (Chicago, IL, USA); anti-mTOR, anti-phospho-mTOR and anti-phospho-Akt from Signalway Antibody (SAB; Signalway Antibody, College Park, MD, USA); anti-GLUT1 from Novus Biologicals (Littleton, CO, USA); anti-β-actin from Sigma] overnight at 4°C and followed by horseradish peroxidase (HRP)-labeled goat anti-mouse IgG or HRP-labeled goat anti-rabbit IgG (both from Abcam, Cambridge, MA, USA) for 1 h. The membranes were developed using the enhanced chemiluminescence assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Images were analyzed using Image-Pro Plus 6.0.

Xenograft studies and PET/CT imaging. All protocols involving mice were evaluated and approved by our Institutional Animal Care and Use Committee and performed under veterinary supervision. Nude mice (5-week-old) were injected with 2x10⁶ 8505C cells in RPMI-1640 medium subcutaneously in the left flank. When tumors reached 50 mm³ mice were injected subcutaneously with propranolol (Sigma) dissolved in PBS alone (n=15) or PBS alone (n=10) at a dose of 10 mg/kg/day for up to 15 days. Tumor growth was monitored every two days and tumor volume was calculated (volume = length x width²/2). Following in vivo pharmacologic intervention, 130-200 µCi 18F-FDG in 200 µl of saline were injected into the tail vein of each mouse. Anesthesia was performed with isoflurane anesthesia system. The PET/CT data acquisition procedure was performed on Siemens Inveon PET-CT when the mice were fully anesthetized. Body temperature was maintained using a heating pad equipped with the micro PET/CT system. All PET/CT images were processed and analyzed using Intrasense software. After PET/CT imaging, mice bearing ATC were sacrificed and resected tumors were weighted, followed by fixation of the specimens.

Histopathology and immunohistochemistry. The resected tumor specimens of ATC xenografts were fixed in 10% neutral...
buffered formalin and embedded in paraffin. Sections were cut on a microtome and mounted on glass slides. Sections were dewaxed and hydrated in graded alcoholic solutions and then distilled water. Routine hematoxylin and eosin (H&E) staining was carried out. Immunohistochemical staining for Bcl-2, CCnD1 and Ki-67 were performed using the SABC kit according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using the Statistical Package for the Social Sciences, version 20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant result.

Results

β-adrenergic receptors are expressed in 8505C and K1 cell lines. As is shown in Fig. 1A, western blotting of 8505C and K1 lysates demonstrated that ADRB 1/2 could be detected in both 8505C and K1 cell lines. ADRB2 is relatively higher expressed in 8505C cells than that expressed in K1 cells. It seems that the expressions of ADRB1 between the two cell lines are comparable. Positive expression of ADRB1/2 suggests that propranolol may influence the viability of thyroid cancer cells through targeting ADRB1/2.

Propranolol inhibits the growth of 8505C and K1 cells in vitro. To determine the effect of propranolol on thyroid cancer cells, CCK-8 assay was used to determine the half-maximal inhibitory concentration (IC₅₀). Growth curves of 8505C and K1 cells treated with increasing doses of propranolol for 24 h are shown in Fig. 1B. For 8505C and K1, the IC₅₀ was 200 and 280 µM, respectively, which were higher than that reported in neuroblastoma (5) and similar to those reported in other cancer cells (3).

Growth inhibition of propranolol is specific to β2-AR. Next, we explored whether β1-AR or β2-AR is involved in the inhibitory effect of propranolol. 8505C cells were treated with non-selective β-blocker propranolol, β1-specific antagonist atenolol and β2-specific antagonist ICI118551 hydrochloride. With the concentrations increased, propranolol and ICI118551 showed equivalent inhibitory function on the growth of 8505C while atenolol had no effect, suggesting that propranolol induces cell death via blocking β2-AR rather than β1-AR (Fig. 1C). In contrast to the growth suppression in response to β-blockers, we observed that isoproterenol induced a dose-dependent cell growth in both 8505C and K1 cells and this effect can be reversed by pretreatment using propranolol (50 µM) (Fig. 1D).

Propranolol induces apoptosis of 8505C cells in vitro. In order to study the mechanism of propranolol-induced inhibition on the viability of 8505C cells, we detected cell apoptosis in the normal control and propranolol-treated 8505C cells as depicted in Fig. 2. Fig. 2A-F shows that following the propranolol treatment (0-500 µM) for 24 h, FCM revealed a dose-dependent increase in Annexin V-FITC/PI-positive apoptotic cells (apoptosis rate from 0 to 500 µM: 1.8, 7.8, 13, 17.5, 27.8 and 27.6%). In addition, propranolol-induced apoptosis was further characterized by nuclear fragmentation, a hallmark feature of apoptosis (Fig. 2G-I). These data suggested that propranolol was able to induce apoptosis of 8505C cells in a dose-dependent manner in vitro.

Propranolol treatment decreases the expressions of Bcl-2, p-Akt and p-mTOR. Bcl-2 family members, including Bcl-2,
Mcl-1, Bcl-xL, Bim, Bax and Bak, have been reported to be involved in the mitochondrial manner of apoptosis, of which anti-apoptotic Bcl-2 is overexpressed in certain solid tumors and inhibition of Bcl-2 may enhance apoptosis and primary responses to targeted therapy (12-14). Following treatment with increasing concentrations of propranolol and ICI118551 for 24 h, Bcl-2 decreased after treatment with propranolol and ICI118551, and these two drugs yielded similar inhibitory effect in 8505C cells. Notably, expression levels of other Bcl-2 family members did not change following propranolol treatment (Fig. 3D). Given the central role of PI3K/Akt/mTOR pathway in tumorigenesis, the fact that AKT directly phosphorylates pro-apoptotic BAD and restores anti-apoptotic Bcl-xL and Bcl-2 (15-17), we next investigated the expression profiles of Akt and mTOR. Western blotting of 8505C lysates validated that propranolol and ICI118551 significantly suppressed the expression levels of p-Akt and p-mTOR in 8505C cells at a relatively higher concentration (Fig. 3C).

Propranolol induces 8505C cell cycle arrest through down-regulating cyclin D1. Furthermore, we performed cell cycle analysis in the propranolol-treated 8505C cells as shown in Fig. 3A and B. Exposure of 8505C cells to propranolol resulted in the enrichment of G0/G1 phase accompanied by a decrease in the S phase in a dose- and time-dependent manner. The cell cycle regulation protein cyclin D1, which is responsible for cell cycle progression (18), was also investigated in the present study. The expression level of cyclin D1 was dose-dependent and significantly decreased in both propranolol and ICI118551 treated groups when compared to the normal controls (Fig. 4C). Similarly Zhang et al also reported decreased expression of Bcl-2 and cyclin D1 induced by propranolol in pancreatic cancer cells (8).

Propranolol intervention downregulates the expression of HK2 and GLUT1. During dedifferentiation process from differentiated thyroid cancer to anaplastic thyroid cancer, an inverse relationship between radioiodine and fluorodeoxyglucose uptake was observed (19). Among the major proteins regulating the transportation and metabolism of glucose, GLUT-1 and HK-2 are closely related to the rate of 18F-FDG uptake in cancers. Therefore, we investigated the relationship between the blockage of ADRB and the expression of GLUT-1 and HK-2 in 8505C cells in vitro. The results of the in vitro propranolol and ICI118551 intervention revealed that the expression of both GLUT-1 and HK-2 significantly decreased after treatment using β-blockers (Fig. 5A).

In vivo propranolol intervention and 18F-FDG PET/CT imaging. Finally we investigated the effect of propranolol on the growth of 8505C xenografts in vivo. Under the circumstances that ATC is high metabolic and the tumors in the
treatment group shrunk (Fig. 5B), first we assessed the nude mouse tumors using 18F-FDG PET/CT scan. Coronal computed tomography validated that tumor of the control group was bigger than that of the propranolol treated group (Fig. 5C and D). SUV\textsubscript{max} of the control group is statistically higher than that of the propranolol intervention group (SUV\textsubscript{max} 8.9 vs. SUV\textsubscript{max} 2.1). It was noteworthy that the mean body weights of the two groups were not statistically significant (data not shown), suggesting limited toxicity of propranolol in the short-term. Although no low density areas appeared on
the CT scans of the control mice, H&E staining of the tumor specimens of the control group showed necrosis in the middle area of the slice (Fig. 6A), partially reflecting the aggressive property of ATC in the control group mainly caused by rapid tumor progression. Consistent with western blot results, immunohistochemistry analysis of the resected tumor tissue showed...
decreased cyclin D1 and Bcl-2 in the propranolol treated group when compared with normal controls (Fig. 6B). Delayed tumor proliferation in the experimental group was further supported by immunohistochemical staining of tumor cells with the proliferation marker Ki-67 (Fig. 6B).

Propranolol sensitizes thyroid cancer cells to vemurafenib. The present study confirmed the previous observation that BRAF<sup>V600E</sup> inhibitor vemurafenib induces cell cycle arrest of BRAF mutant cell lines K1 and BCPAP, but had little impact on 8505C and BRAF-WT BHP27 cells (Fig. 7A and B). In addition, we found that G0/G1 cell cycle arrest was correlated with reduced phosphorylation of ERK1/2 and reduced expression of cyclin D1 in K1 cells time-dependently (Fig. 7C). However, as was seen from the colony formation assay, vemurafenib (PLX-4032) alone only inhibited the prolif-
erative of K1 cells, but was insufficient to induce apoptosis. Neither ABT-737 (a Bcl-2 inhibitor) nor the combination of ABT-737 and vemurafenib resulted in profound synergism or extensive tumor cell death (Fig. 7D; data not shown), although ABT-737 has been proven to be potent when used along with MEK inhibitor or vemurafenib in melanoma (13,20). Notably propranolol alone induced apoptosis of K1 cells at a relatively higher concentration (Fig. 8A). Most importantly, propranolol pretreatment (200 µM) sensitized K1 cells to the cytotoxicity of vemurafenib characterized by the loss of clonogenic survival and enhanced apoptosis (Fig. 8B and C). These preliminary results demonstrated that propranolol may enhance the cytotoxicity and minimize the side-effects of the targeted molecular therapy.

Discussion

While stress-induced activation of β-adrenergic signaling stimulates tumor cell proliferation, migration, invasion and suppresses apoptosis (21,22), treatment with β-antagonist propranolol reversed these stress-induced effects (23,24). Population based studies have also demonstrated that breast cancer patients with propranolol intake history were less likely associated with a T4 or N2/N3/M1 tumor stage at initial diagnosis and breast cancer-specific mortality was significantly lower for propranolol users, and that propranolol usage was also associated with improved relapse-free survival for triple-negative breast cancer patients (10,25).

In the present study, we found that β-adrenergic receptors are expressed in both 8505C and K1 cell lines and that blockage of β2-AR, but not β1-AR, inhibited the growth of 8505C cells in vitro and in vivo. We have also explored the many potential underlying mechanisms, and discovered that inhibition of β2-AR using either propranolol or ICI118551 was inversely correlated with the expressions of p-Akt, p-mTOR, Bcl-2, cyclin D1, HK2 and GLUT1. Furthermore, we clarified the impact of propranolol on ATC xenografts and validated the shrinkage of tumors using 18F-FDG PET/CT imaging. Immunohistochemistry of the tumor specimens affirmed the downregulation of Bcl-2 and cyclin D1 revealed by western blotting. Finally we highlighted the potential possibility of the combination therapy consisting of propranolol and BRAF specific inhibitor.

Previous studies have demonstrated that propranolol had an negative effect on the 18F-FDG uptake of brown adipose tissue and expression of HK2 was mediated by propranolol in breast cancer model (26-28), similarly we found that propranolol suppressed both the expressions of HK2 and GLUT1 in ATC cell line in vitro despite the intriguing PET imaging of the ATC xenografts. The altered expression of HK2 was probably regulated at the post-transcriptional level (28). HK2 has been reported to be associated with lung and breast cancer development and its deletion was therapeutic in mice bearing lung tumors (29,30), therefore we suppose that downregulation of HK2 may account for the propranolols anti-ATC properties to some extent. In addition, vascular endothelial growth factor (VEGF) plays an important role in thyroid carcinogenesis and its expression level correlates with advanced disease (31). Propranolol has been reported to inhibit VEGF and capillary vessel formation in vivo (32,33), suggesting synergistic effects of propranolol through various mechanisms. In addition, this kind of effect may also rationalize the relative high concentration to decrease the levels of target proteins in vitro (100 µM) to that needed to suppress tumor growth in vivo (10 mg/kg/day).

Although the excellent prognosis of most thyroid cancer cases, there are few treatment options for radioiodine-resistant, metastatic differentiated thyroid cancer and anaplastic thyroid cancer (34). Targeted therapy has shown promise in clinical trials but cellular resistance occurs, and sometimes termination of the targeted therapy is unavoidable due to adverse effects (AE). Combination therapy containing BRAFV600E inhibitor or multikinase inhibitor is a promising option to prevent resistance and to reduce AEs. Decreasing anti-apoptotic BCL-2 family members and lowering the cellular threshold for apoptosis is highlighted by recent studies (35,36). Serasinghe et al found that although inhibition of BRAFV600E by PLX-4032 sensitized melanoma cells to the mitochondrial manner of apoptosis but only a fraction of cells eventually underwent apoptosis. Addition of ABT-737 (a Bcl-2 and Bcl-xL inhibitor) to PLX-4032 promoted apoptosis and reduced development of resistance to targeted therapy (13). Cragg et al demonstrated that addition of ABT-737 to MEK inhibitor converted cytostatic effect of MEK inhibition to a cytotoxic effect and induced long-term tumor regression in mice bearing melanoma, successfully overcoming apoptotic resistance caused by overexpression of Bcl-2 (20). However, some preclinical studies have investigated effects of β-AR signaling in the regulation of tumor cell apoptosis and anoikis. Sastry et al determined that epinephrine via the β2-AR reduces the sensitivity of prostate and breast cancer cells to apoptosis (22). Sood et al showed that the β-AR agonists epinephrine and norepinephrine not only enhance the invasive potential but also protected ovarian tumor cells from apoptosis and that this effect was inhibited by the β1/β2-non-selective antagonist propranolol (37). Two further studies have shown that inhibition of β2-AR signaling by propranolol or combined usage of a β2-adrenergic receptor specific antagonist and gemicitabine induces apoptosis in pancreatic cancer cells via downregulation Bcl-2 (7,38). In response to propranolol blockade we also detected decreased levels of Bcl-2 and the phosphorylated Akt in 8505C cells, along with previous studies we tend to believe that propranolol mediate and induce apoptosis through lowering the expression of the anti-apoptotic protein Bcl-2. Considering its well-tolerated property, its function in inhibiting growth, inducing apoptosis and lowering Bcl-2 level in thyroid cancer, we supposed that propranolol may play a role in combination with targeted agent in inhibiting refractory or progressive thyroid cancer.

In conclusion, our results indicated that propranolol, in addition to its primary action on cardiovascular diseases such as hypertension and arrhythmias, has potential anti-thyroid cancer properties. Studies investigating the combined administration of propranolol and targeted molecular agent in suppressing thyroid cancer should be conducted in the future.

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References


