Nebivolol, an antihypertensive agent, has new application in inhibiting melanoma

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Abbreviations

DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate-buffered saline
FCM	flow cytometry
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
ΔΨm	mitochondrial membrane potential
Rh123	rhodamine123
PI	propidium iodide
WB	western blot
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PVDF	polyvinylidene difluoride
MMP	matrix metalloproteinase
H&E	hematoxylin-eosin

Materials and methods

Reagents and materials

Nebivolol was purchased from Chengdu Baiote Technology Co. Nebivolol was prepared as 20 mM stock solution in dimethyl sulfoxide (DMSO) and placed at -20 ^{o}C. Working solution was prepared by mixing the stock solution and relevant assay medium freshly. 0.1% DMSO (*v/v*) served as a vehicle control. DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT), and 2′,7′-dichlorofluorescein diacetate (DCFH-DA), as well as 2-(6-Amino-3-imino-3H-xant -hen-9-yl) benzoic acid methyl ester (Rh123) were obtained from Sigma Chemical Co. Hoechst33258, DAPI staining solution, JC-1 and Cell-LightTM EDU DNA Cell Proliferation Kit were obtained from Beyotime. The Annexin V-FITC/PI Apoptosis Detection Kit was purchased from KeyGen Biotech. For western blot (WB) experiments, the primary antibodies against Bax (Item No. #2772), Bcl-2 (Item No. #3498), casepase3 (Item No. #14220), cleaved-casepase3 (Item No. #9664), cyclin-dependent kinase (CDK) 2 (Item No. #18048),

CDK4 (Item No. #23972), CD31 (Item No. #3528), Matrix metalloproteinase (MMP) 2 (Item No. #87809), MMP9 (Item No. #13667) and Tissue inhibitor of metalloproteinase (TIMP) 2 (Item No. #5738) were purchased from Cell Signaling Technology. β-actin (Item No. #ab8226) was obtained from Abcam, meanwhile, the secondary antibodies were also purchased from Abcam, including Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) (Item No. #ab150113), Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (Item No. #ab150077), HPR Anti-Rabbit IgG antibody (Item No. #ab288151), and HPR Anti-Mouse IgG antibody (Item No. #ab150117). Caspase3-siRNA (Si-caspase3) were provided by Chengdu Jingmai Biotechnology Co., Ltd. The sequences of Si-caspase3 were 5'-GCGUGAUGUUUCUAAAGAATT-3' for A2058 cells, 5'-GGAUAHUGUUUCUAAGGAATT-3' for B16 cells. cAMP (Cycle adenosine monophosphate) ELISA Kit was obtained from UpingBio technology Co., Ltd.

Cell culture

Melanoma cell lines A2058 and B16 (B16-F10) were purchased from the American Type Culture Collection (ATCC). A2058 cells and B16 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) plus penicillin (100 U/mL) and streptomycin (0.1 mg/mL). All cells were incubated at 37 ^{o}C in humidified incubator containing 5% CO_2.

Cell morphology

A2058 cells and B16 cells were plated in 6-well plates at 5×10^5 cells/well and cultured for 24 h. After treated with nebivolol, cells were washed by phosphate-buffered saline (PBS). Cell morphologies were evaluated using an inversed fluorescent microscope (Zeiss, Axio Observer 3, Germany).

EDU incorporation assay

EDU dye was used to label proliferating cells. In a nutshell, melanoma cells seeded in 96-well plates at 2×10^3 cells/well for 24 h were treated with different concentrations of nebivolol for 24 h, and detected by the Cell-LightTM EDU Cell Proliferation Kit

according to the manufacturer's explanations. Images were acquired by Two-photon confocal laser scanning microscopy (Zeiss, LSM800, Germany).

Wound-healing assay

When A2058 cells and B16 cells in 6-well plate reached 80% confluence, cell monolayers were scratched swiftly by a sterile pipette tip. Then a series of concentrations of nebivolol was slowly added to substitute the premier medium. After incubation at 37 ºC for 24 h, A2058 cells and B16 cells were photographed using an inversed fluorescent microscope.

Safety evaluation

In order to investigate safety of nebivolol on mice during treatment, the blood samples were harvested from eyeball and significant tissue samples (heart, liver, spleen, lung and kidney) were collected when mice were sacrificed. Blood samples were performed with blood routine analysis to detect the influence of the conventional indexes such as main cells in the blood. At the same time, blood samples were centrifuged (8000 rpm, 15 min) to obtain serum for further blood chemical analysis. The tissues of heart, liver, spleen, lung, and kidney were sectioned and stained with hematoxylin and eosin (H&E) for imaging by a digital pathology slide scanner (KF-PRO-005-EX, Ningbo Konfoong Biotech International Co., LTD., China).

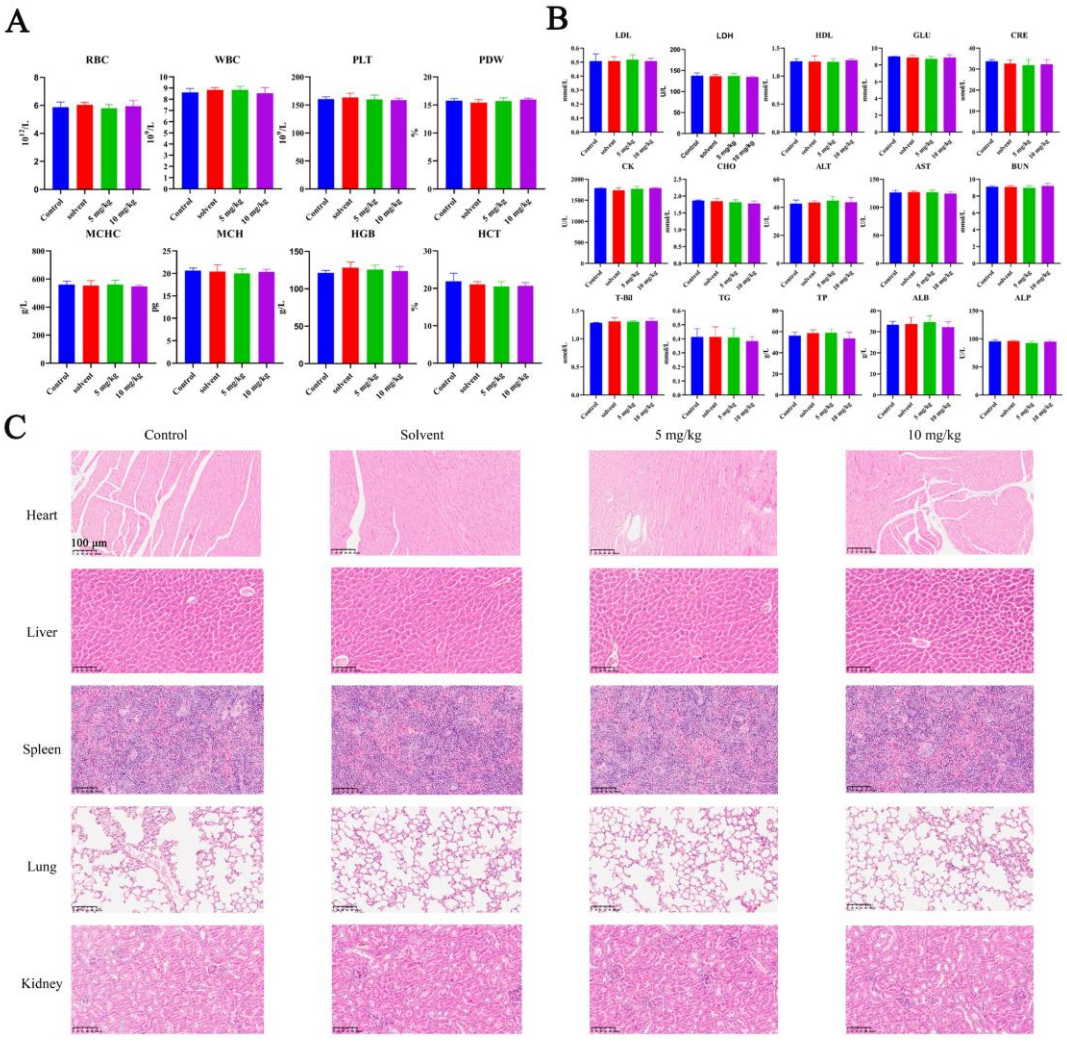
Results

Safety profile of nebivolol

To further evaluate the safety of nebivolol, we collected blood samples and tissue samples from A2058 tumor-bearing nude mice at the end of treatment. Following, some blood samples were used for routine blood test. Synchronously, other blood samples were centrifuged to obtain serum for the detection of blood biochemical. As described in Figure S1A and B, nebivolol could not cause blood system's abnormality and exhibited outstanding biocompatibility. Besides, no pathological alteration after nebivolol treatment were observed in the heart, liver, spleen, lung and kidney compared

with the control group (Figure S1C). Consequently, the results mentioned above implied that nebivolol might be a safe agent for melanoma therapy in xenograft models.

Figure S1. The safety evaluation of nebivolol *in vivo*. (A) Hematological analysis of blood samples. Indicators of detection were as following: RBC (red blood cell), PLT (platelet), PDW (platelet distribution width), MCHC (mean corpuscular hemoglobin concentration), HGB (hemoglobin), HCT (hematocrit), WBC (white blood cell), MCH (Mean Corpuscular Hemoglobin). (B) Serum biochemistry analysis. Indicators of detection were as following: ALB (albumin), AST (aspartate aminotransferase), ALT (alanine transarninase), ALP (alkaline phosphatase), BUN (blood urea nitrogen), CRE (creatinine), CK (Creatine Kinase), CHO (cholesterol), GLU (glucose), HDL (high density lipoprotein), LDH (lactate dehydrogenase), TG (triglyceride), T-BiL (total bilirubin), LDL (low density lipoprotein), TP (total protein). (C) Hematoxylin and eosin (H&E) staining of visceral organs including heart, liver, spleen, lungs and kidneys. Scale bar = 100 μm.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5 mg/kg
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CK
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AST
BUN
T-Bil
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Solvent
5 mg/kg
10 mg/kg
Heart
Liver
Spleen
Lung
Kidney
100 μm