[6]-Gingerol: A Novel AT₁ Antagonist for the Treatment of Cardiovascular Disease

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Abstract

Considering the prevalence of cardiovascular disease in public health and the limited validated therapeutic options, this study aimed to find novel compounds targeting the angiotensin II type 1 receptor, accepted as a therapeutic target in cardiovascular disease. A small library consisting of 89 compounds from 39 Chinese herbs was profiled using a cell-based calcium mobilization assay which was developed and characterized for high-throughput screening. [6]-Gingerol derived from Zingiber officinale Roscoe (ginger) was identified as a novel angiotensin II type 1 receptor antagonist, with an IC₅₀ value of 8.173 µM. The hit was further tested by a specificity assay indicating that it had no antagonistic effects on other evaluated GPCRs, such as endothelin receptors. The major ingredient of ginger, [6]-gingerol, could inhibit angiotensin II type 1 receptor activation, which partially clarified the mechanism of ginger regulating blood pressure and strengthening heart in the cardiovascular system.

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Introduction

Cardiovascular disease (CVD) is associated with high morbidity, mortality, and financial burden to healthcare services [1,2]. The annual cost of CVD is estimated to be €169 billion a year in the enlarged European Union and $394 billion (€296) in the USA [3,4]. In China, CVD is expected to increase with further economic development and urbanization, aging of the population, changes in diet and physical activity [5–7]. AT₁ (angiotensin II type 1) receptor as a therapeutic target in CVD mediates most of the known actions of angiotensin II. Angiotensin II is the primary product of the renin–angiotensin–aldosterone system (RAAS) enzyme cascade and binds to the AT₁ receptor, thus mediating vasoconstriction of vascular smooth muscle, aldosterone secretion from adrenal glomerulosa cells, cellular proliferation, and hypertrophy [8]. AT₁ antagonists are drugs useful for hypertension [9], diabetic nephropathy [10], and congestive heart failure [11]. As CVD has become the major killer of people’s health and life all over the world and there are limited validated therapeutic options to treat these diseases [12], the need to develop new drugs which can target the AT₁ receptor is urgent.

Chinese herbs have been used for thousands of years in the East and have had a recent resurgence in popularity among consumers in the West. Patients are increasingly using herbal products for purportedly preventive and therapeutic purposes [13]. It is known that many Chinese herbs promoting blood circulation and removing blood stasis are widely used in the treatment of coronary heart disease, hypertension, angina, and other CVDs, such as Salvia miltiorrhiza [14–16] and Rhi zoma Chuanxiong [16]. While Chinese herbs are considered a big chemical library and plant extracts are valuable collections offering a huge diversity of compounds, potential drugs of single molecules are worthy of development and utilization.

To obtain new active AT₁ antagonists, a cell-based calcium mobilization high-throughput screening (HTS) assay was established and performed. This study screened 89 compounds from 39 Chinese herbs; one hit was selected in the primary screening with a hit rate of 1.1%. We report here for the first time to our knowledge that [6]-gingerol (Fig. 1) acts as an AT₁ receptor antagonist. And

Key words
- [6]-gingerol
- AT₁ antagonist
- calcium assay
- high-throughput screening
- cardiovascular disease
- Chinese herb

Bibliography
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the screening approach presented here offers a reliable method for the discovery of novel AT1 antagonists.

Materials and Methods

Compounds preparation

Based on the traditional use of Chinese herb against CVD, 89 compounds from 39 Chinese herbs were purchased from the National Institutes for Food and Drug Control (China) with purities greater than 98% (Table 1S, Supporting Information). In the following assays, compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mM as the stock concentration. They were diluted to the indicated concentrations in Hanks’ buffered salt solution (HBSS) before HTS screening, and the final DMSO concentration in each well was 0.3%.

Cell culture and generation of the HEK293/Gα15/AT1 cell line

HEK293/Gα15 cells provided by the Beijing Institute of Genomics, Chinese Academy of Science, were cultured in Dulbecco’s modified Eagles medium supplemented with 10% fetal bovine serum, 500 U/mL penicillin, and 500 µg/mL streptomycin in a humidified atmosphere of 5% CO2 incubator at 37°C. To generate cell lines stably expressing the human AT1 receptor gene, the coding region was subcloned into pcDNA3.1 (Invitrogen). The resulting construct was transfected into the HEK293/Gα15 cells using FuGene HD (Roche) following the manufacturer’s instructions. Selection of stably transfected cells was carried out by treatment with culture medium containing 800 µg/mL G418 (Sigma-Aldrich) in 6-cm dishes (Costar). After 2 weeks of selection pressure, single-cell active clones were isolated and measured using fluorescence detection of calcium mobilization. The stably transformed clone that presented the highest expression level was chosen for functional studies and named HEK293/Gα15/AT1 cell line. The cell line was maintained in culture medium supplemented with 400 µg/mL G418.

Calcium mobilization HTS assay

HEK293/Gα15/AT1 cells (passage 4–6) were plated at a density of 40000 cells/well in 96-well clear-bottom black plates (Costar) coated with matrigel (BD) and incubated at 37°C overnight. The following day, growth media were removed from the cell plates before testing and replaced with 100 µL assay buffer containing a final concentration of 4 µM calcium-sensitive dye Fluo-4-AM (Molecular Probes) and 2.5 mM probenecid (purity ≥ 98%, Sigma-Aldrich) in HBSS. Cells were incubated in a humidified atmosphere of 5% CO2 at 37°C for 30 min. The intracellular Ca2+ flux was assayed using a fluorescent imaging plate reader (FlexStation II; Molecular Devices) to simultaneously monitor Fluo-4-AM fluorescence in all wells (λ excitation = 485 nm, λ emission = 525 nm). Cells were challenged with agonist angiotensin II (purity ≥ 93%, Sigma-Aldrich), and the fluorescence intensity was captured every 1.52 secs for 80 secs. For antagonist studies, the cells were preincubated with the test compounds for 10 min prior to calcium-flux measurement; telmisartan (purity ≥ 98%, Sigma-Aldrich) was used as a reference compound.

To assess the robustness of the HTS assay, the Z’ value was calculated. Positive (1 µM angiotensin II) and negative (0.3% DMSO) controls were included in each 96-well plate for this assay. In addition, the HEK/Gα15/AT1 cell line was tested for its response to the treatment of angiotensin II at every 5 passages for cell line stability test. The sample was identified as a primary hit when its inhibition rate was over 50%. For validation of the screening results, tested samples identified as hits in HTS were reevaluated in triplicate.

Compound cytotoxicity assay

Cytotoxicity of compound was tested using the luciferase-coupled ATP assay (CellTiter-Glo; Promega) coupled with the cell-based HTS assay. HEK293/Gα15/AT1 cells were seeded at 10000/well in 96-well clear-bottom black plates and incubated in a humidified atmosphere of 5% CO2 at 37°C overnight. The following day, 20 µL cell culture medium was aspirated off; different concentrations of the compound under the same conditions used in the calcium assay were added into 96-well plates and incubated for 1 h in a humidified atmosphere of 5% CO2 at 37°C. Luminescence was recorded by Envision 2100 multilabel reader (PerkinElmer) following incubation with CellTiter-Glo reagent.

Compound specificity assay

HEK293 cell lines stably expressing human ET/A ETB endothelin receptors, A1/A2B adenosine receptors, PAR1 protease-activated receptor, a1D-adrenoceptor, and platelet-activating factor receptor (PAFR) genes provided by the Beijing Institute of Genomics, Chinese Academy of Science, were separately plated at a density of 40000 cells/well in 96-well clear-bottom black plates coated with matrigel and incubated at 37°C overnight. The compound was tested at a final concentration of 10 µM using a calcium assay when cells were challenged with their selective agonists for antagonist identification. The HEK293 cell line was set up as the naïve group, and cells were challenged with 10 µM ATP (purity ≥ 99%, Sigma-Aldrich) as an agonist.

Data analysis

All calcium-flux data was expressed as the maximum RFU value per well minus the minimum value (MAX–MIN) after ligand addition. Dose-response curves were fitted and EC50/IC50 values were determined using GraphPad Prism 5 software. EC50/IC50 data were presented as the mean of at least triplicate independent determinations ± SEM.

Supporting information

Compounds investigated and their respective medicinal plants and Chinese herbs are available as Supporting Information.

Results and Discussion

HEK293/Gα15/AT1 cell line was validated with a known agonist and antagonist for correct pharmacology. Consistent with previously published reports [17,18], the EC50 value of the AT1 reference agonist angiotensin II was calculated to be 783 pM (Fig. 2A), and the signal of angiotensin II can be inhibited by the AT1 reference antagonist telmisartan in a dose-dependent manner with an IC50 value of 18.99 nM (Fig. 2B). The assay performance was quantitatively assessed using a Z’ factor described by Liu Q et al. [6]-Gingerol: A Novel Chemical structure of [6]-gingerol.
previously by Zhang et al. [19]. The $Z'$ factor value of 0.82 confirmed that this cell-based assay was excellent with large separation bands between the negative and positive controls (Fig. 3A). In addition, the signal of this assay was stable, and the calculated EC$_{50}$ value was consistent for each passage of the HEK/G$_{a_15}$/AT$_1$ cell line (range from 918 pM to 2.4 nM). These data demonstrated the robustness of this cell-based HTS assay and its suitability for use in screening applications.

To demonstrate the utility of this cell model, a library consisting of 89 Chinese herb-derived compounds was screened for searching antagonists targeting AT$_1$ in a 96-well format. The screen was performed based on their inhibition of 50 nM angiotensin II-induced calcium influx at the concentration of 10 µM; telmisartan and 0.3% DMSO were used as the positive and negative control,
respectively. [6]-Gingerol was selected for further research with inhibition rate greater than 50% (Fig. 4). The hit was confirmed by retesting and IC50 determination. [6]-Gingerol was identified as a novel AT1 antagonist with an IC50 value of 8.173 µM (Fig. 5).

To exclude the possibility of false positive results, a luciferase-coupled ATP quantitation assay was used to determine the cytotoxicity of [6]-gingerol. Compared to the control group, [6]-gingerol did not show cytotoxicity on HEK293/Gα15/AT1 cells at concentrations up to 30 µM (Fig. 6), which clarified that [6]-gingerol inhibits AT1 receptor activation induced by angiotensin II.

The AT1 receptor belongs to the G-protein coupled receptors super family (GPCRs). Some compounds can act on the diversity of GPCRs. Opipramol dihydrochloride, for example, acts as an α1/α2 opioid receptor agonist and also as a low to moderate affinity antagonist for the D2, 5 HT2, and H1 receptors [20]. To study the specificity of the action target of [6]-gingerol, response profiles of 8 GPCRs related to CVD antagonized with [6]-gingerol were detected. Besides the AT1 receptor, effects profiles of [6]-gingerol at ETα/ETβ receptors, A1/A2B receptors, PAR1 receptor, α1D-adrenoceptor, and PAFR were evaluated. Results indicated that [6]-gingerol cannot act on the other GPCRs that we tested (Fig. 7).

Interestingly, [6]-gingerol was assessed as an agonist of the capsaicin-activated VR1 (TRPV1) receptor [21]. TRPV1 belongs to the recently discovered transient receptor potential (TRP) superfamily of ion channels. TRPs often functionally associate with GPCRs and phospholipase C (PLC) [22]. TRPV1 plays a key role in cardiovascular health and disease by acting as a sensor and regulator of cardiovascular homeostasis and a protector against cardiovascular injury [23]. Consistent with our results, these findings further reveal that [6]-gingerol has an effect on CVD.

[6]-Gingerol is isolated from a commonly used Chinese herb, Zingiber officinale Roscoe (ginger). Ginger is pungent in flavor and warm in nature; it is used to improve the flow of body fluids and stimulate blood circulation throughout the body by diluting the blood and has a powerful stimulatory effect on the heart muscle [24]. Ginger also could be used for prevention and treatment of cardiovascular disorders by day-to-day dietary intake [25]. Gingerols are the main pungent and active ingredients of ginger in the treatment of cardiovascular disorders with [6]-gingerol having the highest concentration among them [26], but very little is known about ginger’s biological targets and the molecular mechanisms underlying its activity. The present study provides molecular evidence of the interaction between [6]-gingerol and the AT1 receptor.
gerol and CVD. AT₁ receptor mediates most of the known actions of angiotensin II that contribute to hypertension, volume dysregulation, and cardiovascular damage [27, 28]. Angiotensin signaling downstream of AT₁ can occur through several different pathways. The AT₁ receptor can mobilize Ca²⁺ from the sarcoplasmic reticulum and promote the interaction of actin and myosin filaments to allow for contraction and migration of cells. The other major pathways activated by G-protein interaction with the AT₁ receptor leads to the activation of PKC and the ERK pathway, implicated in sustenance of contraction and cellular growth. Moreover, angiotensin II-induced arachidonic acid metabolism via phospholipase A₂ also maintains a balance between vasoconstriction and vasodilation in various vascular beds [29]. In the present study, [6]-gingerol was discovered as a novel AT₁ antagonist that may contribute to the medicinal properties of ginger acting on the cardiovascular system.

In conclusion, the current study provided a cell-based assay for application in HTS and identified a novel and selective AT₁ receptor antagonist, [6]-gingerol, which explained the effect of Zingiber officinale Roscoe on the cardiovascular system from the molecular drug target level. [6]-Gingerol can be considered as a lead for the development of a novel AT₁ target drug to cure hypertensive disease or some other CVDs. Even though the IC₅₀ of [6]-gingerol in inhibition of the AT₁ receptor is relatively high, we believe that, through further examination of the structural basis of the [6]-gingerol-target interaction, small molecular analogs of [6]-gingerol with more potent AT₁ blockade activity will be developed in this direction. Moreover, further investigation is required to clarify the signaling pathway of [6]-gingerol in order to evaluate the available options for future drug development.

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Conflict of Interest

None

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