

# Naringin as a natural bitter tastant promotes proliferation of cultured human bronchial epithelial cells via activation of TAS2R signaling

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## Research

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# Abstract

Bitter tastants can activate bitter taste receptors (TAS2Rs) and thus initiate the relaxation of airway smooth muscle cells, which have great potential in the development of novel asthma therapy. However, recent study shows that canonical bitter substance denatonium induces apoptosis of bronchial epithelial cells (BECs), indicating the toxic effect of bitter tastants on airways. Considering the diversity of bitter tastants in nature and TAS2Rs expressed in airway cells, it is thus necessary to carefully evaluate the bitter tastant for its effect on the proliferation of BECs, if aimed to treat airway disease. Here we first screened a group of bitter flavonoids, including apigenin, hesperetin, kaempferol, naringenin, naringin and quercetin which are commonly used in food and traditional medicine, and then quantitatively evaluated the effects of this group of bitter flavonoids on the proliferation of BECs (i.e. 16HBE14o- cells) cultured *in vitro*. The results show that five of the six tested bitter tastants inhibited, but only naringin promoted the proliferation of 16HBE14o- BECs *in vitro* at the dose of a few hundred micromoles. Furthermore, the naringin-promoted cell proliferation was associated with enhanced cell cycle progression, mRNA expression of cyclin E, and evoked calcium signaling/ERK signaling. Inhibition of the TAS2R signaling pathways with specific blockers attenuated the naringin-enhanced cell proliferation, cyclin E expression and calcium signaling/ERK activation. Taken together, these findings indicate that although many bitter flavonoids may inhibit the proliferation of BECs, naringin emerges as one of the kind that promotes the proliferation of BECs via cell cycle progression and TAS2R-activated intracellular signaling. Only such bitter tastant proven to be unharmed to the epithelial structure and function could be further developed as safe and effective TAS2Rs-based bronchodilator in asthma therapy.

## Introduction

Bitter taste receptors (TAS2Rs) are usually considered to be expressed in gustatory tongue to mediate bitter taste sensing to prevent toxic damage from foods [1]. Interestingly, in the last decade, it has been verified that TAS2Rs are also widely expressed in non-gustatory cells including airway smooth muscle cells (ASMCs) and bronchial epithelial cells (BECs), and consequently mediate beneficial functions such as relaxation of ASMCs and the ciliary beating of BECs when the cells are exposed to bitter substances [2, 3]. Subsequently, there has been a great surge of interest in investigation of bitter tastants for their potential as novel drug candidates for bronchodilation in asthma therapy because of their alternative pharmacological mechanisms as opposed to the conventional  $\beta$ 2-adrenergic agonists, and their wide availability in nature [4].

However, a recent study has reported that the canonical bitter substance such as denatonium can induce apoptosis of BECs [5]. Since BECs form the first-line physical barrier in the bronchial airways to protect them from harmful insults of inhaled pathogens and pollutants such as airborne particulates, the healthy integrity of BECs plays a vital role in maintaining the airway health [6–8], and otherwise the accumulation of BECs damage may lead to asthma-associated pathologies including airway inflammation, airway hyper-responsiveness and airway narrowing. For example, it has been well known that asthma occurrence and severity is directly attributable to the shedding of BECs [9, 10]. It is also known that local injury of

BECs can cause global airway smooth muscle contraction and airway constriction, which may be detrimental to asthma symptoms [11]. Therefore, it is very important to examine the cytotoxic effect on BECs of bitter tastants when they are considered to be used as bronchodilators in asthma therapy.

It is fortunate, however, that there are at least 25 human TAS2Rs bitter taste receptors known in existence and each of them can recognize a variety of bitter tastants [12]. On the other hand, there are thousands of bitter tastants that have been well characterized and documented in data bases [13]. Of particular interest are the numerous natural bitter substances used in food and traditional medicine that provides a gold mine to search for potent bronchodilators [14]. Combining such diverse resources of TAS2Rs and bitter tastants makes it highly possible to find bitter tastants that are not only potent in inducing relaxation of ASMCS but also nontoxic to the normal function of BECs. With a literature review following this principle, a group of bitter flavonoids including apigenin, hesperetin, kaempferol, naringenin, naringin and quercetin emerges as the first group of bitter tastants that may meet the requirement of being beneficiary to the functioning of both ASMCS and BECs in asthma therapy, as suggested by their reported alleviating effects on experimental models of asthma [15].

However, none of these bitter tastants has been well evaluated in regards of its cytotoxicity to BECs. In view of the lack of such critical knowledge for drug development of TAS2Rs-based bronchodilators, we aimed to screen these six bitter flavonoids in this study for their effects on the specific functions of BECs related to cell viability and cell cycling. We demonstrate that at least in this group of flavonoids the majority of the bitter tastants inhibited the proliferation of BECs, but only naringin turned out to promote the proliferation and cell cycle via canonical signaling pathway correlated with TAS2Rs.

## Materials And Methods

### Materials

Apigenin (#A106676), hesperetin (#H107700), kaempferol (#K107145), naringin (#N107344), naringenin (#N164488), quercetin (#Q111274) and PD98059 (#P126620, ERK inhibitor) were purchased from Shanghai Aladdin Bio-Chem Technology Co., LTD (Shanghai, China). 6-Methylflavanone (6Met, #M1403, TAS2R inhibitor) was obtained from TCI Development Co., Ltd (Shanghai, China). U73122 (#662035, PLC $\beta$  inhibitor) was obtained from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and trypsin were purchased from Gibco (#27250018, Grand Island, NY). Cell culture flasks and plates were purchased from Corning Incorporated (Corning, NY).

### Cell Cultures And Drug Treatment

The human BEC 16HBE14o- cells, one kind of basal cell featured with functional tight junction and well-developed barrier properties [16], were obtained as a gift from Prof. Gruenert of University of California (San Francisco) [17]. They were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented

with 10% FBS, L-glutamine and antibiotics at 37 °C in a 5% CO<sub>2</sub> humidified incubator. Cells were passaged at 90% confluence using 0.05% trypsin in 1:3 or 1:4 proportions. The healthy cells with cubic-like morphology were used for the following experiments.

Stock solutions (1 M) of apigenin, hesperetin, kaempferol, naringin, naringenin, and quercetin were prepared in DMSO. The stock solution (1 M) of denatonium was dissolved in phosphonate buffer solution (PBS). All those stock solutions were kept frozen at -20 °C. The cells cultured in basal DMEM medium without FBS were exposed to different concentrations of bitter tastants, with or without inhibitors (6Met, U73122, and PD) for TAS2R signaling. For each experiment, an appropriate vehicle control was run in parallel, with the final concentration of DMSO at 0.1% (v/v) to avoid the cytotoxicity of DMSO.

## Cell Morphological Assessment

To directly present the effect of six bitter flavonoids on cell morphology, the cells were treated with different concentrations of bitter flavonoids for 48 h and then imaged by phase contrast microscopy using an inverted optical microscope with a 20x objective (Primovert, Carl Zeiss, Jena, Germany). The view-field for imaging was chosen randomly to prevent subjective bias.

## Cell Proliferation Assay

The cell proliferation was evaluated by using trypan blue exclusion cell counting method. To this end, 16HBE14o- cells were seeded at the density of  $2 \times 10^4$  cells/cm<sup>2</sup> into 6-well plates for 24 h. Then the medium was replaced with fresh serum free DMEM containing bitter tastant at different concentrations. After the cells were incubated for 48 h with/without inhibitors for TAS2Rs signaling or ERK activity, cell number was counted by using cytometer after staining with 0.4% trypan blue for living cells. The cell proliferation is presented as a ratio of cell number in experimental groups to that of the vehicle group (% of control).

## Cell Viability Assay

To verify the cell proliferation analysis, the cell viability of 16HBE14o- cells were tested by using ATP luminescence assay, which can efficiently measure metabolic activity in the cells [18]. Briefly, subconfluent, exponentially growing 16HBE14o- cells at the density of  $1 \times 10^4$  cells/cm<sup>2</sup> were seeded in 96-well plates and cultured for 24 h. Then the medium was replaced with fresh serum free DMEM containing bitter tastant at different concentrations. After the cells were incubated in this medium for 48 h, the medium was replaced again with fresh serum free DMEM so that the ATP secreted from the cells into the culture medium during culture was excluded from the cell viability measurement. Then the cell viability was determined using ATP luminescent cell viability assay (#C0065M, Beyotime Biotechnology, China), which directly presents the metabolic activity of cells based on the quantitation of

the amount of ATP in cells. The cell viability is expressed as the percentage of luminescence (RLU) in experimental groups to that of the vehicle group (% of control).

## Cell Cycle Analysis

Cells were seeded in 6-well plate at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> and then exposed to 0.1, 0.5 and 1 mM naringin with/without inhibitors (6Met, U73122, and PD) for 48 h. Cells were collected and fixed with 70% ethanol. Following incubation on ice for 30 min, samples were washed with ice-cold phosphate-buffered saline (PBS) and then centrifuged at 1200 rpm for 5 min. Pellets were resuspended in PBS with RNase A (#ST578, Beyotime) (100 µg/mL) and propidium iodide (#ST511, Beyotime) (40 µg/mL). Samples were kept at 37 °C for 30 min in the dark. The cell cycle profile was analyzed using a flow cytometer (BD Accuri C6, BD Biosciences, San Jose, CA) and analyzed with ModFit software (Verity Software House, Topsham, ME).

## Assessment Of Intracellular [Ca<sup>2+</sup>]<sub>i</sub> In Cultured BECs

Intracellular calcium signals were visualized as described previously, using the membrane permeable [Ca<sup>2+</sup>]<sub>i</sub>-sensitive fluorescent dye Fluo4 acetoxymethyl ester (Fluo4/AM) (Sigma, St. Louis, MO). Briefly, cultured BECs were inoculated (~ 10<sup>4</sup> cells per dish) into confocal Petri dishes with a glass bottom. Next, the cells were incubated with 5 µM Fluo4/AM for 45 min at 37°C in a 5% CO<sub>2</sub> incubator. The cells were then washed with Tyrode's solution and incubated for 20 min to allow complete deesterification of the cytosolic dye. The fluorescence intensity of the sample (Fluo4/AM labeled BECs) was measured by laser scanning confocal microscopy (LSM710, Carl Zeiss) using an excitation wavelength of 488 nm and an emission wavelength of 505 nm, which represented the influx of the [Ca<sup>2+</sup>]<sub>i</sub>. Subsequent image processing and analysis were performed off-line using Image J software. Baseline Fluo-4 fluorescence (F<sub>0</sub>) was determined by averaging the first 10 frames of each experiment. [Ca<sup>2+</sup>]<sub>i</sub> was represented as F/F<sub>0</sub>, with F calculated by integrating Fluo-4 over entire cells for global [Ca<sup>2+</sup>]<sub>i</sub>.

## Real-time qPCR Assessment Of Cyclin Protein Expression

Cyclin proteins, including Cyclin A, B, D and E are molecules that regulate cell proliferation and cell cycle. In order to determine whether naringin influences these molecules in bronchial epithelial cells, we used real-time qPCR to measure the mRNA expression level of Cyclin A to E in 16BEC14o- cells with/out exposure to naringin at 0.1 or 1 mM for 24 h. Total RNA from cultured BECs was extracted using the TRI Reagent RNA Isolation Reagent (#T9424, Sigma). 500 ng total RNA was used to generate 1st strand cDNA using the Revert Aid First Strand cDNA Synthesis Kit (#K1622, Thermo, MA, USA). Primers for Cyclin A, Cyclin B, Cyclin D, and Cyclin E as target genes and GADPH as reference gene were purchased from General Biosystems (Anhui, China). PowerUp SYBR Green Master Mix (#A25742, Applied

Biosystems, CA, USA) was used. The reaction was run in the Real-time PCR system (StepOnePlus, Applied Biosystems) using 1  $\mu$ l of the cDNA in a 10  $\mu$ l reaction according to the manufacturer's instructions in triplicate. Calibrations and normalizations were done using the  $2^{-\Delta\Delta CT}$  method, where  $\Delta\Delta CT = C_T$  (target gene) -  $C_T$  (reference gene). Fold changes in mRNA expression were calculated from the resulting CT values from three independent experiments.

## FRET Analysis

The FRET biosensor ERK, EKAREV, was prepared as reported previously [19]. Cells were seeded in 6-well plates and grown to reach 40% of confluency. Cells were then transfected with 2.5  $\mu$ g of DNA per well using Lipofectamine 3000 transfection kit (#L3000-008, Thermo). After 12 h, the medium was replaced with 10% FBS DMEM without antibiotics overnight. After transfection for 24 h, cells were seeded on 10  $\mu$ g/ml fibronectin-coated glass-bottom dishes in 10% FBS DMEM for 12 h. Before imaging on microscope, cells were starved in DMEM without FBS overnight. Live cell imaging was performed as previously reported [19, 20]. By using a live cell image system (Cell Observer 1A, Carl Zeiss), cells were excited at a 436 nm wavelength, and the fluorescence of the CFP (emission at 480) channel and YFP channel (emission at 535) were recorded, respectively. FRET/CFP, was used to represent the level of FRET ON state.

Images were acquired every 2 min. After 10 min, drugs were added to the cell medium by gentle pipetting. The ECFP and FRET images were processed and quantified by Fluocell, a MATLAB-based Open Source image analysis software package(<http://github.com/lu6007/fluocell> [21, 22]).

## Statistical analysis

Statistical analysis was performed by using GraphPad Prism (Graph Pad Software, La Jolla, CA). Unless stated otherwise, data were reported as means  $\pm$  s.e.m, and n represents the number of samples. One-way analysis of variance (ANOVA) followed by Post Hoc test was carried out for multiple comparisons. The significance level was set at  $p < 0.05$  ( $*p < 0.05$ ;  $**p < 0.01$ ).

## Results

### Naringin promoted the proliferation of 16HBE14o- cells

We first analyzed the effect of denatonium on the proliferation of 16HBE14o- cells by cell counting (Figures S1A) and ATP luminescent assay (Figures S1B). The result shows that denatonium inhibited the proliferation and decreased cell activity of the epithelial cells, which confirmed the data reported by Wen et al. [5]. To explore bitter tastant that is not only useful for relaxation of ASMCs but also unharmed for proliferation of BECs, we first focused on six bitter flavonoids that are commonly used in both food and TCM and also known to alleviate asthma symptoms (Table S1). We then analyzed these bitter flavonoids

for their effect on cell proliferation at the concentration of a few hundred micromoles that is considered as maximal safe dosage for effective bronchodilation via activating TAS2Rs [14]. As shown in Fig. 1A, we found that like denatonium the majority of these bitter flavonoids including apigenin, hesperetin, kaempferol, naringenin, and quercetin dramatically decreased proliferation of 16HBE14o- cells cultured in DMEM without FBS. Only naringin appeared to enhance the proliferation of 16HBE14o- cells cultured in DMEM without FBS. To clarify the abovementioned effect, we quantified the cell activity of 16HBE14o- cells treated with these bitter flavonoids for 48 h by using cAMP luminance assay. As shown in Fig. 1B, we also found that only naringin enhanced the cell activity of 16HBE14o- cells while apigenin, hesperetin, kaempferol, naringenin, and quercetin all inhibited it. Morphological observation under light microscope further confirmed this phenomenon by showing cellular injury in 16HBE14o- cells treated with apigenin, hesperetin, kaempferol, naringenin, and quercetin, but not in those treated with naringin (Figure S2).

## Naringin Promoted The Cell Cycle Progression Of 16HBE14o- Cells

Since naringin emerged from this group of flavonoids as the only bitter tastant to promote proliferation of 16HBE14o- cells which were cultured with basal DMEM medium without FBS, we then investigated whether naringin promotes cell cycle progression of the cells. Representative cell cycle profiles of 16HBE14o- cells treated with naringin in the presence or absence of TAS2R inhibitor as shown in Figure S3 clearly indicate that naringin caused dose dependent decrease in the percentage of G0/G1 phase and increase in the percentage of S phase and G2/M phase. Figure 2A shows the quantified percentage of cell cycle. Compared with the vehicle control group, a 10% reduction of G1 phase was got in group incubated with the dose of 1 mM naringin. However, cells in S phase increased from 9–14% and cells in G2 phase increased from 20–25%.

The real-time qPCR results show that except for cyclin A, naringin seemed to influence cyclin B, D, E in a dose dependent manner (Fig. 2B). At 0.1 mM, exposure to naringin increased the mRNA expression of cyclin B and E, but decreased that of cyclin D in the 16HBE14o- cells, as compared to the untreated cells. On the other hand, exposure to 1 mM naringin only increased the mRNA expression of cyclin E but not cyclin B while maintaining the decreased mRNA level of cyclin D, as compared to the untreated cells. Considering the Cyclin proteins promote cell cycle transition through checkpoint, these results indicate that naringin may promote the cell cycle progression in cultured 16BEC14o- cells through influence on the mRNA expression of *cyclin B* and *cyclin E*, and the latter may be more significant.

## Naringin Evoked Calcium Signaling And Downstream ERK Signaling

Ca<sup>2+</sup> signaling is known to be evoked by the activation of TAS2Rs with bitter tastant in ASMCs, which plays an essential role in regulation of ASMC function. To ascertain whether Ca<sup>2+</sup> signaling is also

involved in naringin-induced BEC response, the  $[Ca^{2+}]_i$  of 16HBE14o- cells was evaluated with Fluo-4/AM before and after exposure to naringin at 0.01, 0.1, 1 mM, respectively. As shown in Fig. 3A, calcium signaling, as indicated by the fold change of fluorescence intensity of Fluo-4, was activated instantaneously upon exposure to naringin in all cases, but the peak magnitude increased from ~ 1.5 to ~ 3.0 fold as the concentration of naringin increased from 0.01 to 1 mM.

ERK is another downstream signaling of TAS2Rs, which also plays essential role in regulating animal cell proliferation[23, 24]. Figure 3B shows that naringin could induce remarkable activity of ERK phosphorylation in 16HBE14o- cells, as assayed with FRET analysis. When 16HBE14o- cells expressing ERK FRET biosensor EKARE V were stimulated with 1 mM naringin and imaged by time lapse FRET microscopy, the cells exhibited an apparent increase of FRET fluorescence intensity within the cell as shown in the representative FRET images (upper left vs. upper right panel). The bottom panel of Fig. 3B displays the time-course of changing ERK activity in response to 1 mM naringin, quantified as FRET/CFP ratio of fluorescence intensity in each cell normalized to the averaged value of FRET/CFP prior to naringin exposure. It appears that the FRET/CFP increased about 1.3 fold at 1 h after exposure to 1 mM naringin. These data indicate that naringin evoked  $Ca^{2+}$  signaling/ERK activation in cultured 16HBE14o- cells.

### **Naringin promoted proliferation of 16HBE14o- cells via activation of TAS2Rs**

To further determine whether naringin-enhanced proliferation in cultured 16HBE14o- cells did involve TAS2Rs signaling, we cultured the 16HBE14o- cells either in the absence or presence of TAS2Rs inhibitors (6Met, U73122, PD, respectively) and subsequently evaluated the cell proliferation, calcium signaling, ERK activity, the mRNA expression of cyclin E in response to 1 mM. The results show that the 6Met (TAS2R inhibitor) and U73122 (PLC $\beta$  inhibitor) blocked naringin-enhanced calcium signaling (Fig. 4A) and ERK activation (Fig. 4B). Similarly, 6Met, U73122, and PD (ERK inhibitor) blocked naringin-enhanced mRNA expression of Cyclin E (Fig. 4C) and proliferation (Fig. 4D) of 16HBE14o- cells. Thus, the naringin-induced proliferation and related  $Ca^{2+}$  signaling/ERK activation were indeed dependent on the activation of TAS2Rs.

## **Discussion**

The primary finding of this study is that although the majority of the tested bitter flavonoids including apigenin, hesperetin, kaempferol, naringenin, and quercetin inhibited proliferation of the BECs cultured *in vitro*, naringin emerged as the only one to promote proliferation of the BECs. This effect of naringin on proliferation of BECs was mediated at least partially via enhancing the cyclin E expression and intracellular calcium level that are all dependent on TAS2Rs signaling. We further verified that the naringin-enhanced proliferation of BECs may be owing to the acceleration of cell cycle transition.

Our data on the inhibitory effect of the majority of the tested bitter flavonoids on the proliferation of BECs are consistent with that of the canonical bitter tastant denatonium, as previously reported by Wen *et al.* [5]. Additionally, these bitter flavonoids exhibited inhibitory effect on the proliferation of BECs at a dose of

a few hundred micromoles, which falls within the dosage level for the bitter tastant to be used to relax ASMCs [25]. Thus, many bitter flavonoids may be pharmacologically harmful to the integrity of BECs if used at the concentration effective for ASMC relaxation. Therefore, for any bitter tastant to be considered in the development of TAS2Rs-based bronchodilators, it must be carefully evaluated for its potential not only to relax ASMCs but also to proliferate BECs.

Fortunately, however, it is well-known that BECs express diverse TAS2Rs and there are numerous bitter flavonoids in the natural world [13]. It is highly possible that these different TAS2Rs and agonists may mediate very different pharmacological reactions in BECs. Indeed, we found in this study that naringin, a natural bitter tastant mainly from grapefruits, could enhance the proliferation of BECs, at least in the 16HBE14o- cells cultured *in vitro*. Considering that naringin has already been shown *in vitro and in vivo* to relax ASMCs in culture and dilate bronchial airways in ovalbumin-induced asthmatic mice, respectively [26], these beneficial effects of naringin on the proliferation of BECs as presented in this report indicate that naringin may be the one promising bitter substance that can be practically used as drug agent of bronchodilator in asthma therapy.

The airways are lined by three types of cells in the epithelium including ciliated cells, secretory cells, and basal cells [27, 28]. Damages to these cells due to environmental hazards can disrupt the barrier integrity of the bronchial airways and lead to various respiratory diseases. In particular, the basal cells appear to be pluripotent stem cells which are important in maintaining the structural and functional integrity of bronchial epithelial layers [28]. However, there is currently a lack of therapies for promoting the recovery of damaged BECs, especially those aiming at regulating basal cell proliferation to help repairing the damaged airway epithelium and restore its normal phenotype. In this study, naringin promoted the proliferation of 16HBE14o- cells, which suggests that naringin may help repair the damaged basal cells, and if true *in vivo* it can be used as a drug agent to protect human airways from potential damages.

In addition to the effects on ASMC relaxation and BEC proliferation, previous studies have demonstrated that naringin possesses several other therapeutic properties for the pulmonary airways, including antioxidant, anti-inflammatory, antitussive and expectorant effects, as well as protection against lipopolysaccharide (LPS)-induced acute lung injury [29–33]. These therapeutic properties combined together indicate that naringin may be a favorable bitter tastant used in asthma therapy to alleviate multiple symptoms through various mechanisms.

It should be noted that naringin and its aglycon naringenin are two main bitter flavanones and very common dietary constituents in grapefruit, citrus fruits, and many other dietary products such as beans, cherries and tomatoes of which they are responsible for the bitter taste. Although naringin and naringenin may have the same origin, we found in this study that it was naringin but not naringenin promoted the proliferation of BECs. Others have found that orally administrated naringin is hydrolyzed to its aglycon naringenin by lactase-phlorizin hydrolase and intestinal microflora, turning the BEC beneficial naringin to BEC harmful naringenin [34]. Therefore, when developing naringin-based asthma therapy, it should always be considered as preparations for nebulized inhalation instead of oral administration.

Furthermore, naringin may also promote cell proliferation through other pathways alternative to TAS2Rs signaling. For example, naringin can up-regulate the expression of the growth factors such as vessel endothelial growth factor and transforming growth factor- $\beta$  during angiogenesis and wound healing, which may stimulate the proliferation of BECs [35]. It is also reported very recently that naringin can enhance the proliferation of endothelial progenitor cells that promote endothelial tube formation via CXCR4/PI3K signaling pathway [36]. Whether these signaling pathways are involved in the naringin-enhanced proliferation of BECs remains to be investigated in future study.

## Conclusion

The present study demonstrate that different bitter tastants could induce different or even opposite consequences in BECs in terms of cell proliferation. For the majority of the bitter flavonoids, at least those tested in this study, exposure to the bitter tastant inhibited the proliferation of BECs cultured *in vitro*. In contrast, exposure to naringin showed promotive effect on the proliferation of the cells, which was dependent on TAS2R signaling. Since naringin is also capable of relaxing ASMCs, it may be considered a favorable drug agent candidate for the development of both effective and safe bronchodilator and bronchoprotector in asthma therapy.

## Declarations

## Availability of data and materials

The dataset supporting the conclusions of this article is included within the article (and its additional file).

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Competing interests

The authors declare that they have no competing interests.

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# Author contributions

M.L. and D.L. conceived and designed the experiments. M.L. and K.N. performed the experiments. K.N. analyzed the data. B.B., J.G., Y.P., J.L., and L.L. collected some data for this manuscript. M. L. and L.D. revised the manuscript.

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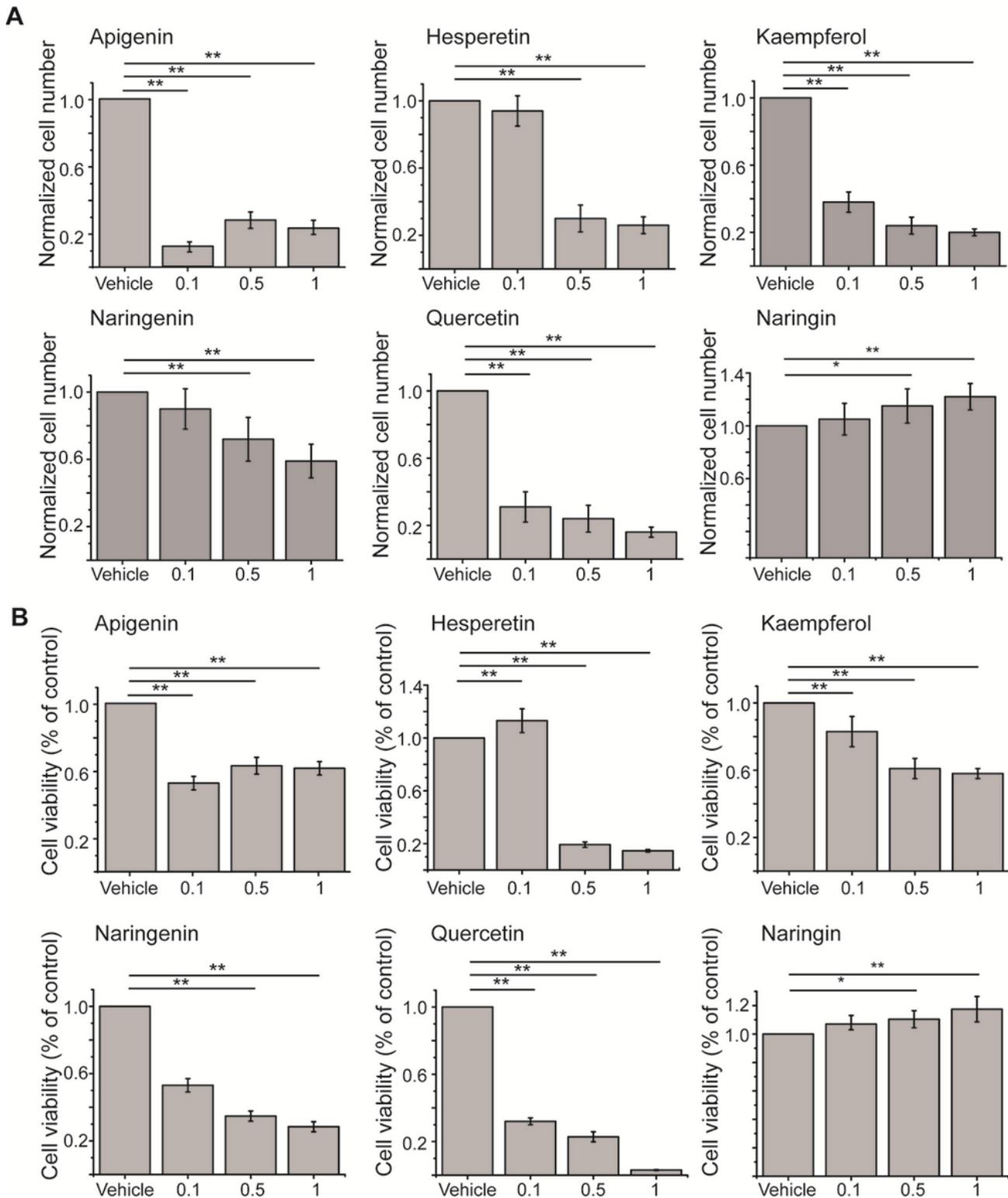
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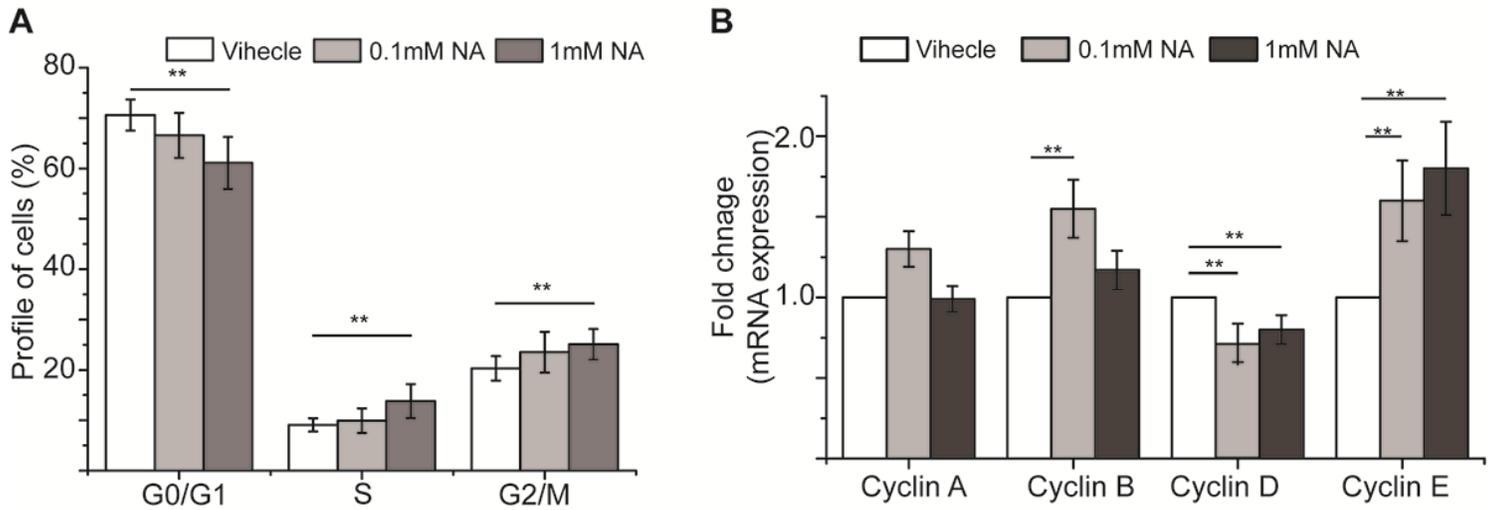
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## Figures



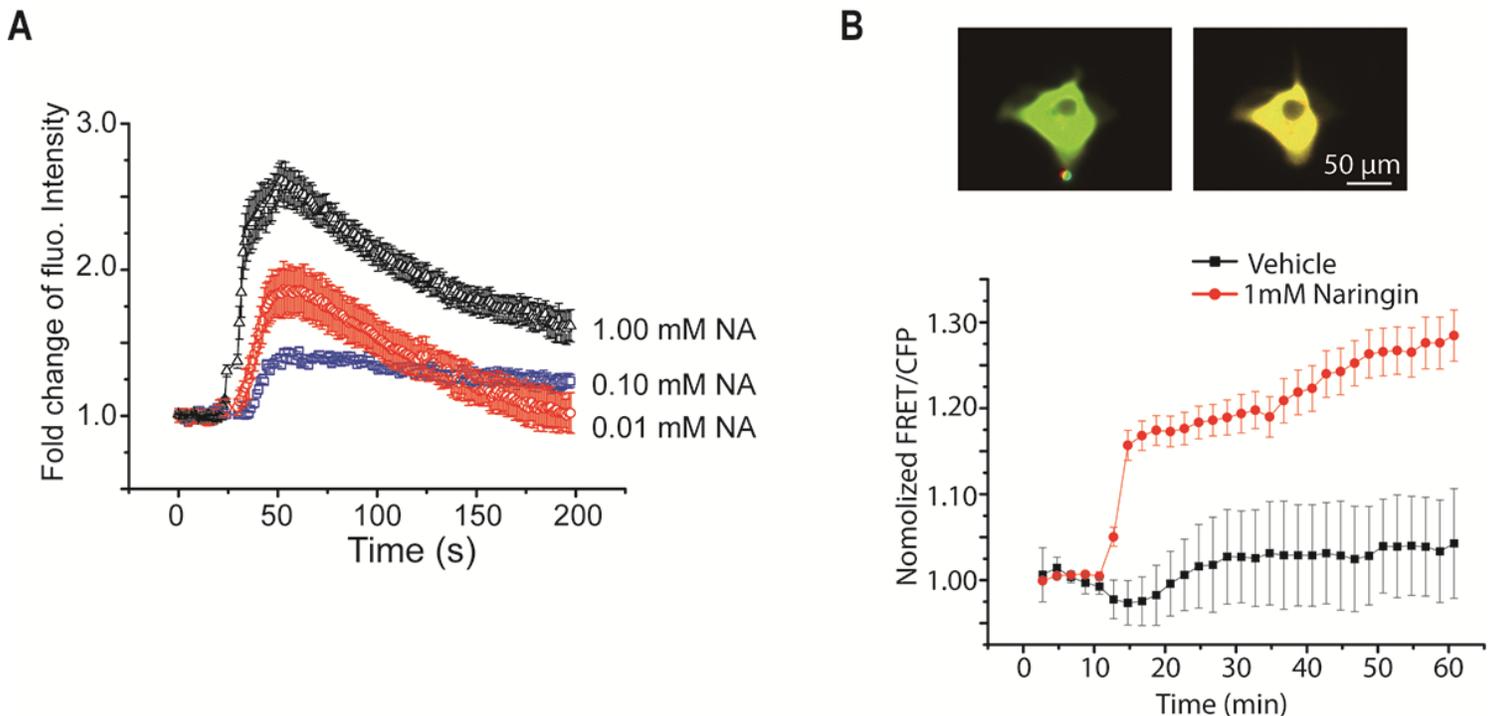
**Figure 1**

The effect Naringin promotes the proliferation of cultured 16BEC14o- cells. (A) Bar graph shows the proliferation of 16BEC14o- cells treated with or without six bitter flavonoids (0.1, 0.5, and 1 mM) for 48 h, analyzed with cell number counting. (B) Bar graph shows the cell activity of 16BEC14o- cells treated with or without six bitter flavonoids (0.1, 0.5, and 1 mM) for 48 h, analyzed with ATP luminescent assay.



**Figure 2**

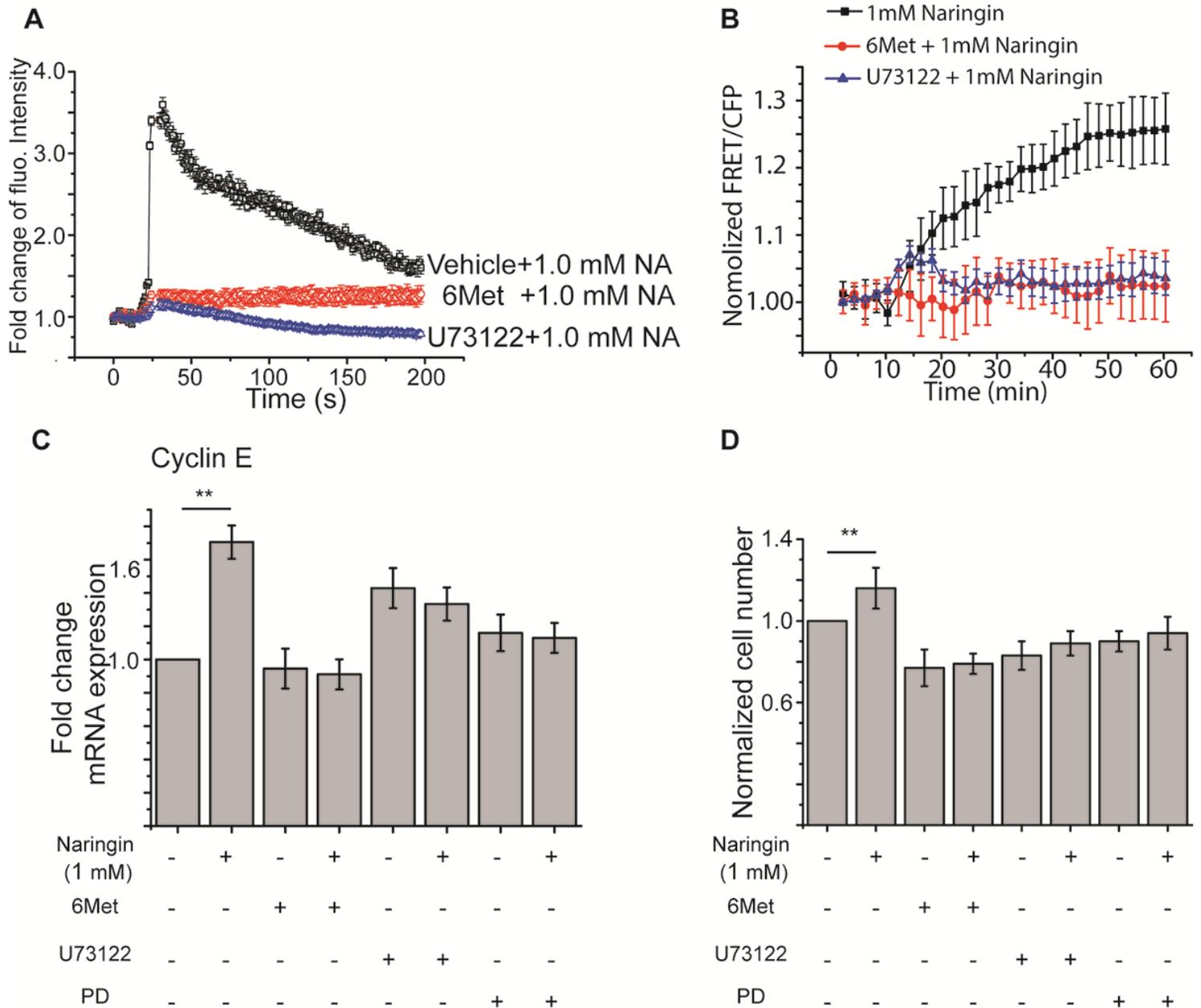
Naringin promoted G1/S cell cycle progression. (A) Bar graph shows the cell cycle profile of 16HBE14o- cells treated with or without naringin (0.1 and 1 mM) for 48 h which was performed in with a flow cytometer and analyzed with Modfit software. (B) Bar graph shows the mRNA expression of cyclin A, cyclin B, cyclin D, and cyclin E in 16HBE14o- cells analyzed with real-time quantification PCR. Data are shown as means  $\pm$  s. e. m. (n =3).



**Figure 3**

Naringin evoked calcium signaling and downstream ERK signaling in 16HBE14o- cells. (A) Time-courses of changing relative mean fluorescence intensity of Fluo-4 (normalized to time 0) in 16HBE14o- cells in response to naringin at the dose of 0.01, 0.1 and 1 mM (n=20-30 cells); To assay the  $[Ca^{2+}]_i$ , 16HBE14o-

cells were incubated with 5  $\mu$ M of Fluo-4/AM in Tyrode's solution for 30 min at 37  $^{\circ}$ C and then the images was recorded before/after the treatment of naringin. (B) Representative images of FRET/CFP rationalized figures and the dynamics of ERK activity of 16HBE14o- cells before and after treatment with 1 mM naringin; The FRET/CFP ratio of each cell was normalized to the averaged FRET/CFP value before the exposure to naringin (n=10-20 cells).



**Figure 4**

The activation of TAS2Rs mediated naringin-promoted proliferation. (A) Time course of changed fluorescent intensity of Fluo-4/AM (normalized to time 0) in 16HBE14o- cells in response to naringin, with or without blocker for TAS2R signaling (n=20-30 cells); (B) The dynamics of ERK activity of 16HBE14o- cells in response to naringin, with or without blocker for TAS2R signaling (n=10-20 cells); (C) The mRNA expression of Cyclin E in 16HBE14o- cells in response to naringin, with or without blocker for TAS2R signaling analyzed with flow cytometry (n=3). (D) The proliferation of 16HBE14o- cells exposed to

naringin with or without TAS2R signaling blockers for 48 h, analyzed with cell number counting (n=3) .  
Data are presented as means  $\pm$  s.e.m.

## Supplementary Files

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