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Research article

Discovery of (−)-epigallocatechin gallate, a novel olfactory receptor 2AT4 agonist that regulates proliferation and apoptosis in leukemia cells

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ABSTRACT

Olfactory receptors (ORs), the largest family of G protein-coupled receptors (GPCRs), are ectopically expressed in cancer cells and are involved in cellular physiological processes, but their function as anticancer targets is still potential. OR2AT4 is expressed in leukemia cells, influencing the proliferation and apoptosis, yet the limited number of known OR2AT4 agonists makes it challenging to fully generalize the receptor's function. In this study, we aimed to identify new ligands for OR2AT4 and to investigate their functions and mechanisms in K562 leukemia cells. After producing the recombinant OR2AT4 protein, immobilizing it on a surface plasmon resonance chip, and conducting screening to confirm binding activity using 258 chemicals, five novel OR2AT4 ligands were discovered. As a result of examining changes in intracellular calcium by five ligands in OR2AT4-expressing cells and K562 cells, (−)-epigallocatechin gallate (EGCG) was identified as an OR2AT4 agonist in both cells. EGCG reduced the viability of K562 cells and induced apoptosis in K562 cells. EGCG increased the expression of cleaved caspase 3/8 and had no effect on the expression of Bax and Bcl-2, indicating that it induced apoptosis through the extrinsic pathway. Additionally, the initiation of the extrinsic apoptosis pathway in EGCG-induced K562 cells was due to the activation of OR2AT4, using an OR2AT4 antagonist. This study highlights the potential of EGCG as an anti-cancer agent against leukemia and OR2AT4 as a target, making it a new anti-cancer drug.

1. Introduction

Olfactory receptors (ORs) are the largest family of the seven-transmembrane domain G protein-coupled receptors (GPCRs); they are highly expressed in the olfactory neurons of the nasal olfactory epithelium [1]. ORs are also ectopically expressed in various organs of the body, including the liver, adipose tissue, colon, brain, and skin [2–6]. Ectopic ORs play a critical role in cellular physiological processes, including cell proliferation, neurotransmission, development, and apoptosis, in humans and other mammals, but the function of ectopic ORs is only partially studied [5,7,8]. OR2AT4 expressed in the epidermis, including keratinocytes and hair follicles,

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contributes to wound healing by inducing cell proliferation and migration of keratinocytes and also contributes to hair loss therapy by activating the growth phase of hair follicle [9]. OR51E2 expressed in human epidermal melanocytes reduces cell proliferation and enhances melanogenesis and dendritic formation through activation [10]. OR10J5 in human hepatocytes reduces intracellular lipid contents by activation [11], and Olfr734 accelerates the hepatic glucose production, a critical determinant in the onset and progression of type 2 diabetes mellitus and related metabolic abnormalities [12]. Olfr78 and OR51E2 are related to intestinal inflammation, with notably reduced Olfr78 expression observed in mouse colitis models [13]. OR51B4 expressed in colon cancer cells induced apoptosis by regulating proliferation and migration [5]. Changes in the expression of ORs in the brain have been noted in various neurodegenerative conditions, including sporadic Creutzfeldt-Jakob disease, Alzheimer's disease, and Parkinson's disease [14]. Therefore, understanding the molecular mechanisms of ORs is important not only for elucidating their biological applications, but also for the potential development of innovative therapeutic approaches for diseases and disorders in which these receptors may have important effects.

ORs are expressed in various cancer cells and tissues, and ORs expressed in leukemia cells are associated with cancer progression in humans [15,16]. Leukemia is a cancer of blood-forming tissues characterized by the somatic acquisition of genetic and epigenetic alterations in the hematopoietic progenitor cells [17]. Acute myeloid leukemia (AML) is an aggressive cancer of the hematopoietic progenitor cells that results in uncontrolled clonal proliferation of immature myeloid blast cells [18]. ORs are highly expressed in AML samples, with 19 ORs co-expressed with cancer marker genes [19]. In particular, OR2AT4 and OR51B5 were expressed in the K562 cell line and in the white blood cells of patients with AML [3,20]. OR2AT4-and OR51B5-mediated signaling in chronic myeloid leukemia (CML) and AML regulates cell proliferation, apoptosis, and differentiation. However, because these results were obtained using only two ligands, sandalore and phenirat, the only two known ligands of OR2AT4 and OR51B5, there is a limit to generalizing the function of ORs. To enhance the physiological understanding of how the protein is regulated and operates in diverse environments, and to comprehend the various functions and physiological regulatory mechanisms of the protein, further research needs to be conducted using new ligands.

Research on the biological function of ORs must begin with discovering the ligands of the target ORs, because only 10 % of 400 ORs are known for use in functional studies [21,22]. To identify a OR ligand, OR proteins, a chemical library, and equipment that can observe protein-chemical interactions are required. OR protein production is challenging due to their low expression levels in heterologous systems and the instability of purified ORs [23,24]. A few OR proteins, such as OR1A2 and OR2AG1 proteins, were obtained by expression and purification from bacteria and their functions were characterized [25,26]. Additionally, appropriate experimental method must be established for large-scale screening of chemical libraries to identify various ligand-binding interactions with OR proteins. Surface plasmon resonance (SPR) analysis, commonly used in biomedical applications, is particularly suitable for studying the interactions between odorants and membrane proteins such as ORs [27,28]. The novel ligands for OR6M1 were discovered by observing the interactions between 108 chemicals and OR6M1 membrane fragments immobilized on a SPR chip [29]. OR3A1 proteins in liposomes stably immobilized on the SPR chip showed a dose-dependent response to helional [30].

Here, we aim to discover a new ligand for OR2AT4 and determine the function of OR2AT4 in K562 cells using the discovered ligand. We established the conditions for recombinant protein expression of OR2AT4 and discovered novel ligands of OR2AT4 by screening of 258 chemicals using recombinant OR2AT4 protein in a SPR-based assay. Using cells transiently expressing OR2AT4, agonists were identified among novel ligands and their effects on cell death in K562 cells were observed. These finding suggest that novel OR2AT4 ligands may be candidate drugs for novel cancer treatments, and furthermore will contribute to expanding our understanding of the ectopic olfactory role of ORs.

2. Materials and methods

2.1. Reagents

Ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), dimethyl sulfoxide (DMSO), Tris-HCl, sodium dodecyl sulfate (SDS), ethylenediamine tetraacetic acid (EDTA), dithiothreitol (DTT), sandalwood oil, berbamine, celastrol, EGCG, rosmarinic acid, perifosine, and SB203580 were purchased from Sigma–Aldrich (St Louis, MO, USA). Sandalore was purchased from Toronto Research Chemicals (North York, Canada). Phenirat was purchased from Tokyo Chemical Industry (Tokyo, Japan). Natural Product Libraries were provided by Selleck Chemicals (Houston, TX, USA). Dulbecco's phosphate-buffered saline (DPBS) was from Welgene (Daegu, Korea). HBS-EP buffer was purchased from Cytiva Life Sciences (MA, USA). His Capture Kit were purchased from iCluebio (Seongnam, Korea). The antibody against OR2AT4 was purchased from Invitrogen (Carlsbad, CA, USA). The antibodies against the following proteins were purchased from Cell signaling Technology (Beverly, USA): caspase-3, cleaved-caspase-3, Bcl-2, Bax, caspase-8, cleaved-caspase-8, phosphor-Akt, Akt, phosphor-p38 mitogen-activated protein kinase (MAPK), p38 MAPK, phosphor-signal transducers and activators of transcription 5 (STAT5), STAT5, β-actin, and horseradish peroxidase (HRP)-conjugated anti-mouse/rabbit IgG antibody.

2.2. Cloning of OR2AT4 gene

The construct for the OR2AT4 was designed with N-terminal Rho tag. OR2AT4 gene was amplified by polymerase chain reaction (PCR) with primers (5′- ACAATTCCCCTCTAGA ATGGATGCCACAGCCTGT-3′, 5′- GGTGATGATGACCGGT AATGCTCCTGTCACAGCCT-3′). The PCR products were inserted into a pET-DEST42 vector (Invitrogen, Carlsbad, USA) by using a gateway cloning system. A recognition site for the *Xba*I restriction enzyme was appended to the 5′ end of the gene while a recognition sequence for the *Age*I enzyme was added to the 3′ end of the gene. The pET-DEST42 was designed to produce a C-terminal fusion protein with 6xHis tag and

V5 epitope tag for purification and detection. The constructed plasmid is referred to as pET-DEST42-Rho-OR2AT4-6xHis. The expression vector sequence was confirmed by DNA sequencing.

2.3. Expression and purification of OR2AT4 protein

BL21 *E. coli* strains (RBC Bioscience, Taipei, Taiwan) were transformed with the pET-DEST42-Rho-OR2AT4-6xHis plasmid and incubated in Luria-Bertani (LB) broth (BD Difco, Sparks, MD, USA) containing 100 μg/mL ampicillin. The cells were grown at 37 °C with shaking at 200 rpm until the optical density at 600 nm reached to 0.6–0.8. Protein expression was induced with 1 mM IPTG at 16 °C for 16 h at 200 rpm. Cells were harvested at 6000×*g* for 10 min and lysed by sonication for 20 min (5 s on/off). Homogenized cells were centrifuged (12,000×*g*, 30 min) and separated into soluble/insoluble fractions. Insoluble fraction was solubilized in solubilization buffer (0.1 M Tris-HCl, 20 mM SDS, 1 mM EDTA, and 100 mM DTT) and dialysis by 10 kDa MWCO Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific, Waltham, MA, USA) in 0.1 M sodium phosphate buffer (pH 8.0) containing 10 mM SDS for 4 h. Solubilized proteins were incubated with cobalt resin (Thermo Fisher Scientific) or Ni-NTA resin (Qiagen, Crawley, UK) for 4 h at room temperature for shaking. His-tagged protein was passed through filter column (Bio-rad, Richmond, CA, USA) and eluted from the 0.1 M sodium phosphate buffer (pH 6.0) with 10 mM SDS by gravity flow. Eluted protein was performed to western blotting and Coomassie blue staining.

2.4. SPR analysis

SPR analysis were conducted using iMeasy300 (iCluebio, Seongnam, Korea). Surface of the NTA sensor chip (iCluebio) was saturated with a $NiCl_2$ solution (0.5 mM), and OR2AT4-6xHis protein was immobilized on the sensor chip. Then, 100 nM protein was injected into flow cell (FC2) and immobilized. The reference flow cell (FC1) was also activated by $NiCl_2$ solution. The compounds were diluted in HBS-EP buffer (pH 8.0) and binding affinity analysis was performed by 0.1 % DMSO in HBS-EP buffer as running buffer. All binding affinities between protein and compounds were analyzed as response unit (RU) and calculated as signal versus value difference between to protein (FC2) and reference (FC1).

2.5. Cell culture and transfection

HEK293T and K562 (human myelogenous leukemia cell line) cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Another leukemia cell line, Molt-4, was purchased from Korea Cell Line Bank (Seoul, Korea). HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Waltham, MA, USA) with 10 % fetal bovine serum (FBS; Gibco) and 1 % Penicillin/Streptomycin (P/S; Gibco). K562 and Molt-4 cells were cultured in RPMI 1640 (Gibco) with 10 % FBS and 1 % P/S. All cells were cultured at 37 °C in an atmosphere of 5 % CO_2.

OR2AT4-expressing cells were constructed by transfecting OR2AT4 (Origene, Rockville, MD, USA) and Gα15 into HEK293T cells with Lipofectamine 3000 (Invitrogen). The group transfected with the empty vector was used as a control (Mock).

To silencing OR2AT, K562 cells were seeded in 6 well plates and transfected with OR2AT4 siRNA (Santa Cruz Biotechnology, California, CA, USA) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Briefly, OR2AT4 siRNA and RNAiMAX are incubated in Opti-MEM reduced-serum medium (Gibco). After 5 min incubation, the complexes were transfected to the K562 cells for 24 h. To verify transfection, K562 cells were evaluated by PCR.

2.6. Reverse transcription (RT)-PCR

The total RNA was extracted from K562 and Molt-4 cells using the TRIzol reagent (Invitrogen), and complementary DNA (cDNA) was synthesized using the SuperScript RT kit (Invitrogen) according to the manufacturer's instructions. The cDNA was amplified with PCR using the following sequences: OR2AT4 Forward: 5′-TGACCGCTATGTGGCTATCTGC-3′, Reverse: 5′-ATGCCATCTGGGAGGTCCTTAC-3′, GAPDH Forward: 5′-CGAGATCCCTCCAAAATCAA-3′ and Reverse: 5′-GGTGCTAAGCAGTTGGTGGT-3′.

2.7. Isolation of membrane protein

Cell membrane protein was isolated using the MEM-PER Plus membrane protein extraction kit (Thermo Fisher Scientific) as previously described (Choi et al., 2021). Briefly, K562 cells were harvested and then centrifuged at 300×*g* for 5 min. The pellet was washed to 3 times with the Cell Wash Solution, resuspended in the Permeabilization Buffer, and incubated at 4 °C for 15 min. The permeabilized cells were centrifuged at 16,000×*g* for 15 min, then pellet was resuspended in the Solubilization Buffer and incubated at 4 °C for 30 min with constant mixing. Finally, the solubilized cells were centrifuged at 16,000×*g* for 15 min, and the supernatant containing solubilized membranes was collected.

2.8. SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting

Total proteins extracted using RIPA buffer (Thermo Fisher Scientific) from the cells and membrane proteins were quantified using a bicinchoninic acid protein assay kit. SDS-PAGE was performed using a 10 % polyacrylamide gel. Equal volumes of the protein were loaded and separated by electrophoresis. Gel was stained with Coomassie brilliant blue R-250 (Bio-rad) for 2 h and destained with

destaining solution for 2 h. For western blotting, separated protein gel transferred onto polyvinylidene difluoride membranes (PVDF; Bio-rad). The membranes were blocked with 5 % bovine serum albumin (BSA; Sigma) in Tris-buffered saline with 0.05 % Tween 20 (TBST) for 1 h and then incubated with primary antibodies against OR2AT4, caspase-3, cleaved caspase-3, Bax, Bcl-2, cleaved caspase-8, caspase-8, Akt, phosphor-Akt, p38 MAPK, phosphor-p38 MAPK, STAT5, phosphor-STAT5, and β-actin at 4 °C overnight. After being washed with TBST, the membranes were incubated with HRP-conjugated anti-rabbit/mouse IgG secondary antibodies for 2 h. For detection was carried out using the EZ-Western Lumi Pico ECL solution kit (DoGen-Bio, Seoul, Korea) and detected by EZ-Capture MG (Atto Co., Tokyo, Japan).

2.9. Calcium imaging

OR2AT4-expressing cells, K562 cells, or Molt-4 cells were seeded in a 96-well black plate. Cells were loaded with 5 μM Fura-2AM (Molecular Probes, Sunnyvale, CA, USA) and incubated for 30 min. Then, cells were washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, 2 mM $CaCl_2$, 145 mM NaCl, 10 mM D-glucose, 5 mM KCl, and 1 mM $MgSO_4$, adjusted to pH 7.4). Calcium images were obtained using a Zeiss inverted microscope (Jena, Germany) coupled with the DG4 system (Sutter Instrument Company, Novato, CA, USA) and displayed with the Metafluor software (Molecular Device, Sunnyvale, CA, USA). The Fura-2 ratiometric fluorescence (F_{340}/F_{380}) indicated relative changes in the intracellular calcium concentrations.

2.10. Cell viability assay

K562 and Molt-4 cells were incubated in a 96-well plate for 24 h and then treated with time-, dose-dependent EGCG. Also, EGCG was co-treated with phenirat 200 μM for 48 h on K562 cells. Then, the cell viability was evaluated by incubating the plates with the Cell Counting Kit-8 (CCK-8; Dojindo, Tyoko, Japan) solution for 2 h and measuring the absorbance at 450 nm.

2.11. Apoptosis assay

The K562 cells were cultured in 6 well plates at density of 1×10^5 cells/well. The cells were then treated with different concentrations of EGCG for 24, 48, and 72 h. The cells were harvested at 2000 rpm for 2 min, and then resuspended in 100 μL medium by incubation with 100 μL of Muse® Annexin V & Dead cell kit (Luminex Corporation, Austin, TX, USA) reagent n the dark. The

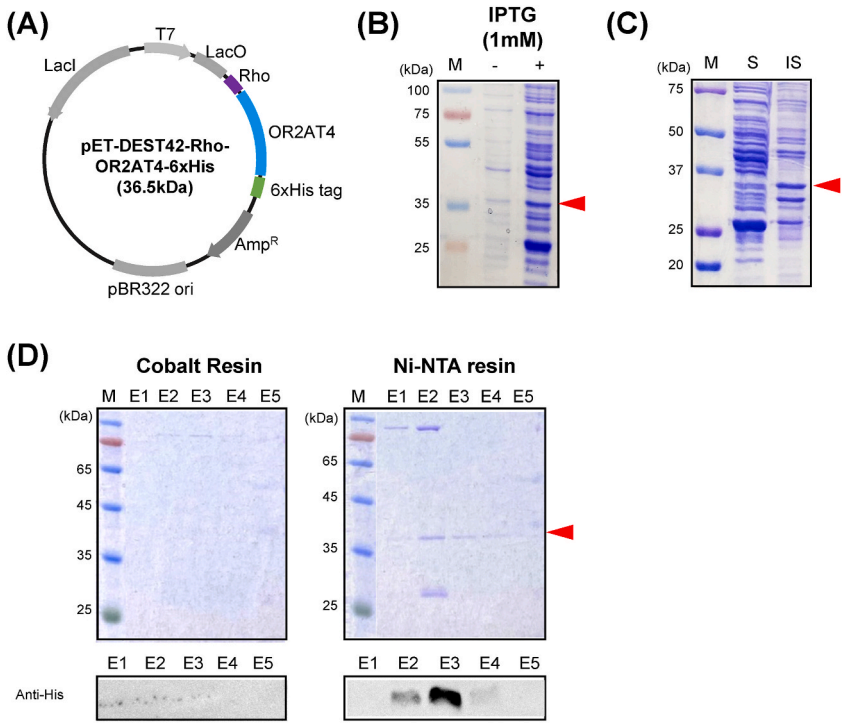

Fig. 1. Optimization of expression and purification of OR2AT4-6xHis. (A) Schematic representation of pET-DEST42-Rho-OR2AT4-6xHis plasmid. (B) Expression of OR2AT4-6xHis induced with 1 mM IPTG by Coomassie staining of SDS-PAGE gel. (C) Separation of soluble (supernatant) and insoluble (pellet) fractions of OR2AT4-6xHis protein. (D) Comparison of OR2AT4-6xHis protein purification using cobalt and Ni-NTA resin. Detection of OR2AT4 protein by Coomassie staining (upper panel) and western blotting (lower panel) with anti-His. M, protein marker; (−), without IPTG; (+), induced protein; S, soluble fraction; IS, insoluble fraction; E, elution fraction.

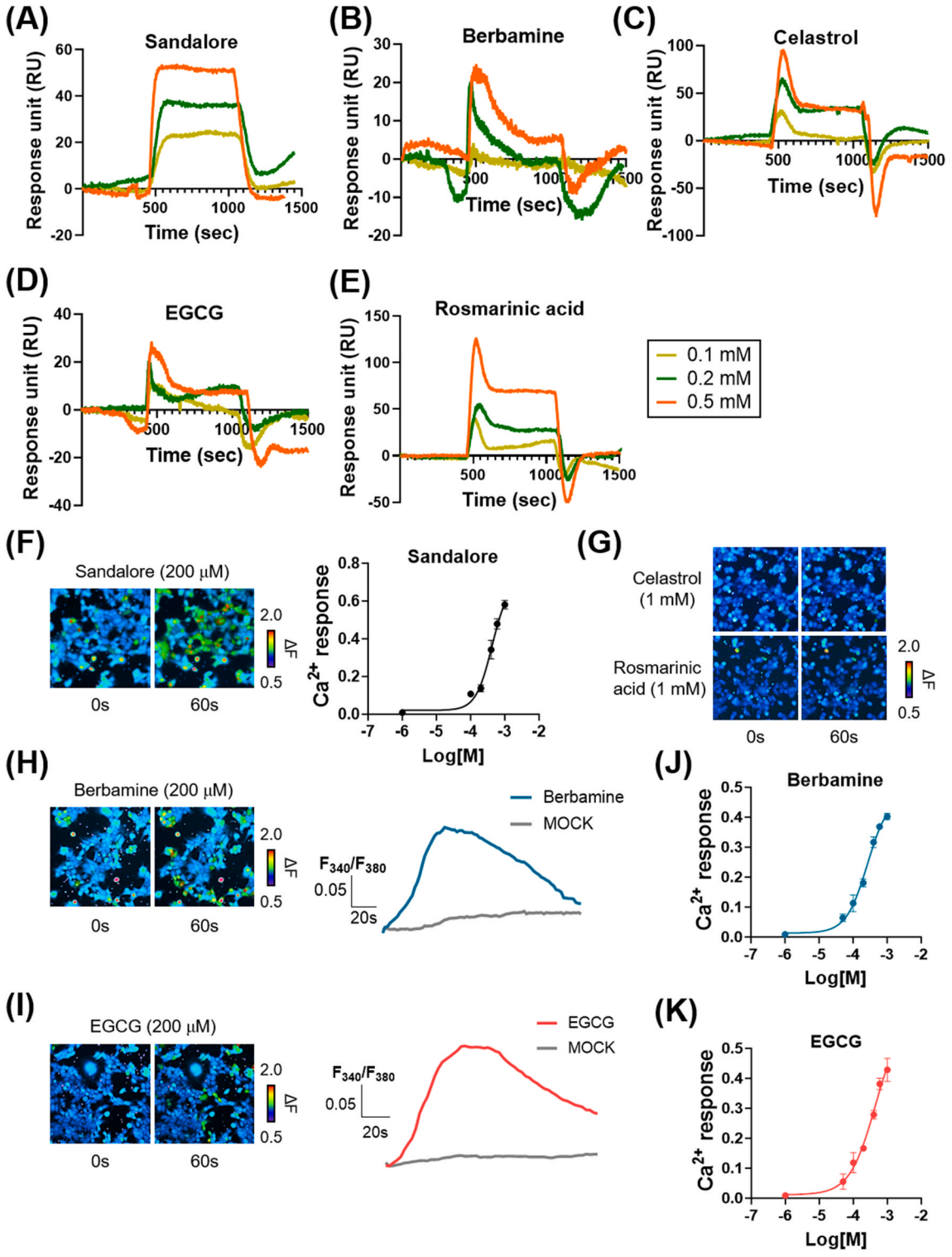

Fig. 2. Discovery of novel OR2AT4 ligands using SPR and calcium imaging. (A) Sensorgram of OR2AT4 agonist, sandalore (100, 200, and 500 μM). (B–E) Sensorgram of berbamine (B), celastrol (C), EGCG (D), and rosmarinic acid (E). (F) Fluorescence image of intracellular calcium response of sandalore (200 μM), and dose-response curve of 0–1 mM sandalore in OR2AT4-expressing cells. (G) Fluorescence image of calcium response of celastrol and rosmarinic acid in OR2AT4-expressing cells. (H,I) Intracellular calcium response of concentration at 200 μM of berbamine (H) and EGCG (I). (J,K) Dose-response curve of calcium response of berbamine (J) and EGCG (K) in OR2AT4-expressing cells. EGCG, (−)-Epigallocatechin gallate.

percentage of live, apoptotic, and dead cells was counted with the Muse Cell Analyzer (Luminex). Data were analyzed by using Muse 1.9 Analysis software (Luminex).

2.12. Statistical analysis

All experiments were tested in triplicate, and statistical analysis was performed using the GraphPad Prism 9.0 (GraphPad Software Inc., CA, USA), presented as the mean ± standard deviation (SD). One-way ANOVA was used for comparisons between groups, followed by Dunnett's post-test. In all figures, the significance of differences is represented as follow: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3. Results

3.1. Optimization of expression and purification of OR2AT4-6xHis proteins

The OR2AT4 recombinant protein was constructed using the pET-DEST42-Rho-OR2AT4-6xHis plasmid (Fig. 1A) and confirmed the purification level of the OR2AT4 recombinant protein using the Ni-NTA resin/cobalt resin method. The expression of OR2AT4 protein was confirmed in the insoluble fraction of the gel at approximately 36.5 kDa (Fig. 1B and C). Using Ni-NTA resin and cobalt resin methods, which specifically bind and purify His-tagged proteins, the conditions were established to increase purification and elution of OR2AT4 recombinant protein (Fig. 1D). His-tagged OR2AT4 was distinguishable in the elution lanes after purification with Ni-NTA resin, but was not detected when cobalt resin was used. This indicates that the target protein was successfully purified on Ni-NTA resin from the complex *E. coli* lysate. These data confirm the expression and purification of the OR2AT4 recombinant protein.

3.2. Discovery of novel OR2AT4 ligands using SPR-based high-throughput screening

Next, we investigated the novel OR2AT4 ligands that bind to the purified OR2AT4 recombinant protein using SPR techniques to study protein-ligand interactions. The success of OR2AT4 recombinant protein synthesis was judged by confirming the reactivity with sandalore (100 μM), a known OR2AT4 ligand, and sandalwood oil (44.4 μg/mL) (Fig. 2A and Fig. S1A). Based on this, 258 compounds were screened for identifying OR2AT4 ligands; this included several compounds such as berbamine, celastrol, EGCG, and rosmarinic acid (Fig. S1B). The sensor data showed the response unit (RU) values of the four strongly binding candidates: 0.89 ΔRU for berbamine; 0.84 ΔRU for celastrol; 1.67 ΔRU for EGCG; and 0.86 ΔRU for rosmarinic acid (Fig. S1C). Furthermore, we confirmed concentration-dependent reactivity for four chemicals (Fig. 2B–E). The RU values of all four chemicals increased in a concentration-dependent manner at concentrations of 100, 200, and 500 μM. Therefore, berbamine, celastrol, EGCG, and rosmarinic acid could serve as novel OR2AT4 ligand candidates.

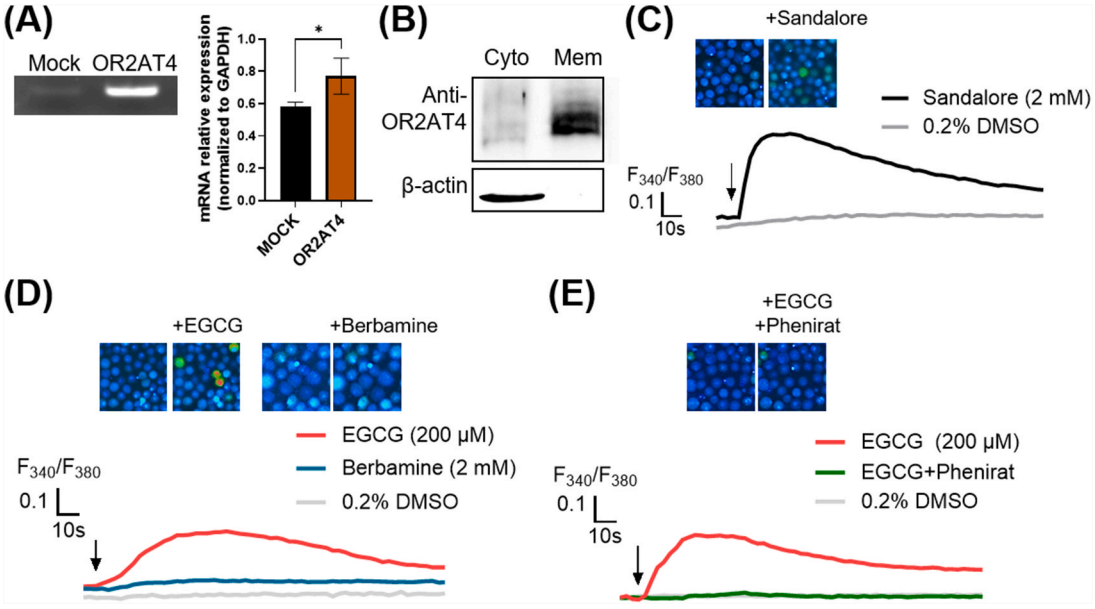

Fig. 3. Effect of OR2AT4 agonists on K562 cells. (A) mRNA expression of OR2AT4 in K562 cells (143 bp). (B) Protein expression of OR2AT4 in membrane protein of K562 cells. (C) Intracellular calcium response of OR2AT4 agonist sandalore in K562 cells. (D) Intracellular calcium response of berbamine and EGCG in K562 cells. (E) Intracellular calcium levels following co-treatment with EGCG (200 μM) and OR2AT4 antagonist phenirat (200 μM). Mock, no RNA; Cyto, cytosolic protein; Mem, membrane protein.

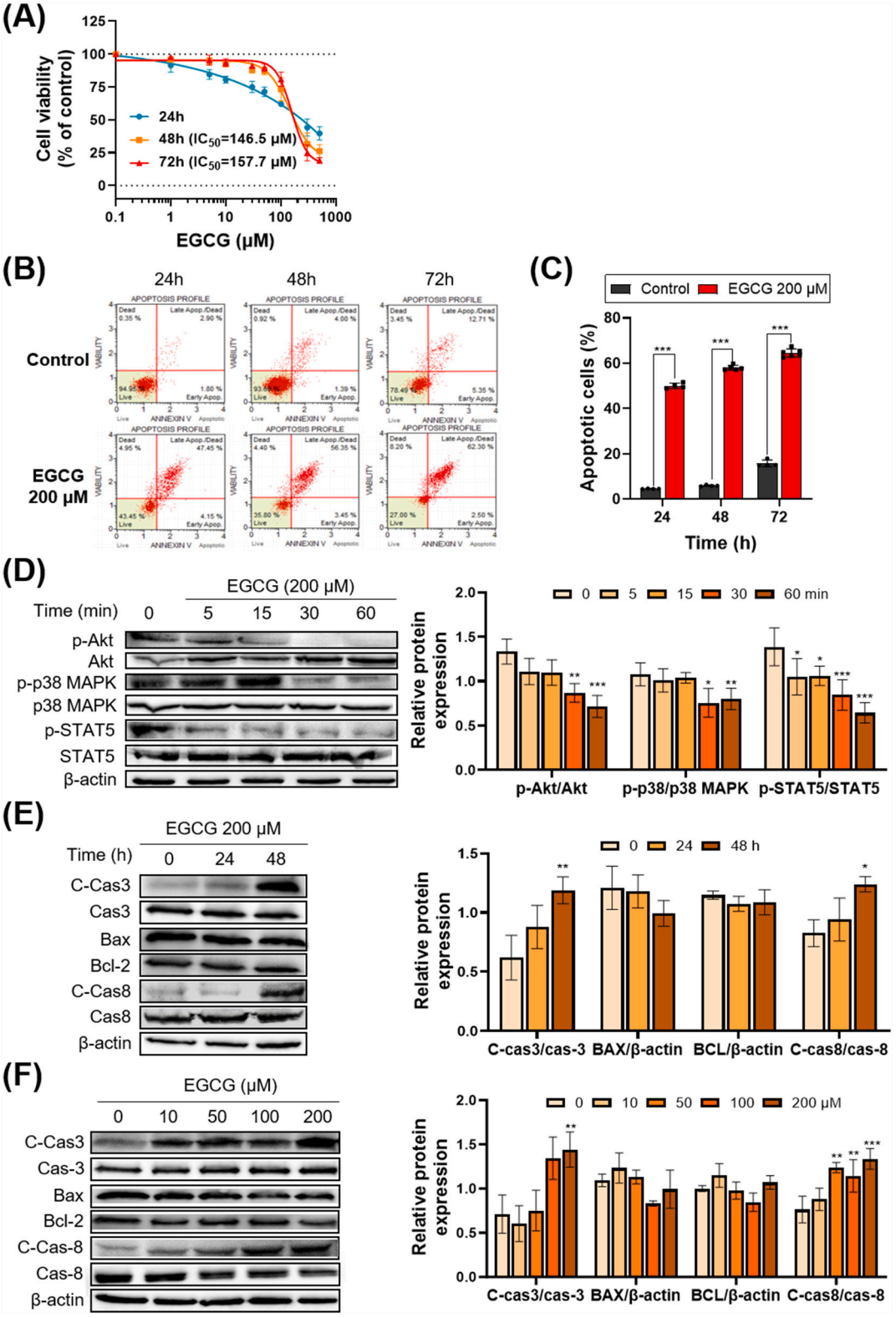

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Fig. 4. Effect of EGCG on cell proliferation and apoptosis in K562 cells (A) Cell viability of EGCG at different concentrations for 24, 48 (IC_{50} = 146.5 μM), and 72 (IC_{50} = 157.7 μM) hours. (B) Flow cytometric analysis of cell apoptosis with Annexin V & Dead cell assay in the absence or presence of EGCG (200 μM) for 24, 48, and 72 h. (C) Quantification of total apoptotic cells (%) in time-dependent. (D) Protein expression of p-Akt, Akt, p-p38 MAPK, p-38 MAPK, p-STAT5, and STAT5 following treatment with EGCG (200 μM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to 0 min. (E) Protein expression of apoptosis-related markers, cleaved caspase-3, caspase-3, Bax, Bcl-2, cleaved caspase-8 and capase-8 following treatment with EGCG (200 μM) for 24 and 48 h *$p < 0.05$, **$p < 0.01$ when compared to 0 h. (F) Protein expression of apoptosis-related markers following treated with EGCG dose-dependent for 48 h **$p < 0.01$, ***$p < 0.001$ when compared to 0 μM. Data are expressed as the mean ± SD (n ≥ 3). C-Cas3, cleaved caspase-3; Cas-3, caspase-3; C-Cas8, cleaved caspase-8; Cas-8, caspase-8.

3.3. Identification of OR2AT4 agonists using calcium response in OR2AT4-expressing cells

To elucidate the function of OR2AT4 ligands in OR2AT4-expressing cells, we assessed the intracellular calcium responses to sandalore in OR2AT4-expressing cells, using the Fura-2AM fluorescence ratio (340/380 nm) (Fig. 2F). Sandalore increased calcium response in a concentration-dependent manner from 0 to 1 mM. Subsequently, the calcium response to berbamine, celastrol, EGCG, and rosmarinic acid was demonstrated (Fig. 2G–K). Increased -intracellular calcium signals were detected under stimulation with berbamine and EGCG but not with celastrol and rosmarinic acid compared to that with the mock (empty vector transfected, data not shown) (Fig. 2G–I). Especially, berbamine and EGCG increased intracellular calcium levels in a concentration-dependent manner from 0 to 1 mM (Fig. 2J and K). These data indicated that berbamine and EGCG are OR2AT4 agonists.

3.4. Effect of OR2AT4 agonist on K562 cells

To confirm the physiological function of the OR2AT4 agonist, the expression of OR2AT4 in K562 (leukemia) cells was confirmed using RT-PCR and Western blot analysis. Our results confirmed the gene expression of OR2AT4 in K562 cells (Fig. 3A) and protein expression in on K562 cell membrane using an anti-OR2AT4 antibody (Fig. 3B). To confirm that the OR2AT4 agonist could activate calcium responses through OR2AT4 in K562 cells, we performed calcium imaging with 2 mM sandalore. Sandalore increased

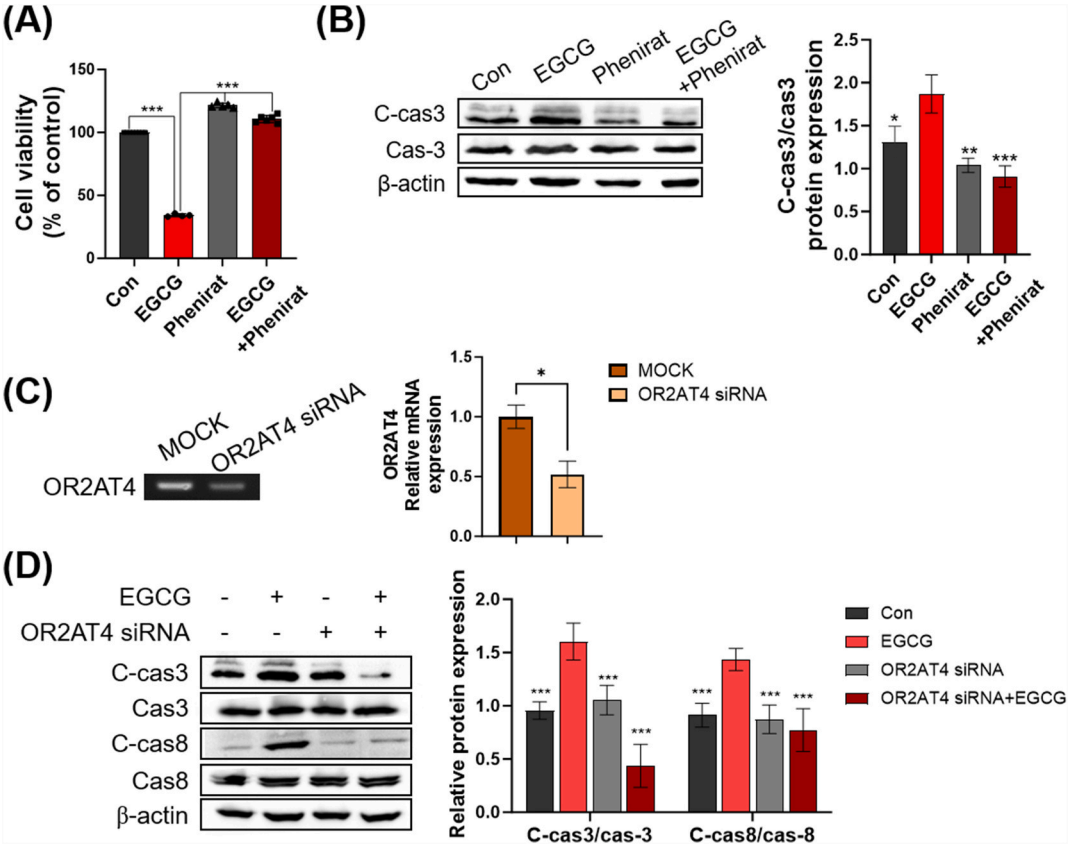

Fig. 5. EGCG efficacy mediated by OR2AT4 in K562 cells. (A, B) Cell viability of EGCG (200 μM) co-treated with OR2AT4 antagonist, phenirat (200 μM) for 48 h. (B) Protein expression of cleaved caspase-3 and caspase-3 after simultaneous treatment with phenirat (200 μM) and EGCG (200 μM) for 48 h. (C) Silencing of OR2AT4 by siRNA in K562 cells. (D) Effect of OR2AT4 knockdown on the apoptosis of K562 cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to control.

intracellular Ca^{2+} levels in K562 cells (Fig. 3C). Next, we investigated the effect of the OR2AT4 agonists, berbamine and EGCG, on K562 cells using calcium response; 0.2 mM EGCG, but not 2 mM berbamine, elevated the calcium response in K562 cells (Fig. 3D). In addition, the calcium response induced by EGCG (200 μM) was reduced in K562 cells co-treated with phenirat (200 μM) (Fig. 3E). Therefore, EGCG acts as an OR2AT4 agonist in K562 cells.

3.5. Stimulation of EGCG-induced apoptosis by inhibiting cell proliferation in K562 cells

Then, we examined the effect of EGCG on K562 cells. As a result of measuring cell viability by treating EGCG at various times and concentrations, EGCG treatment decreased cell viability in a time- (24, 48, and 72 h) and concentration-dependent manner (0–500 μM) (Fig. 4A). The IC_{50} values of EGCG at 48 and 72 h were 146.5 and 157.7 μM, respectively. Sandalore, a known agonist of OR2AT4, also decreased cell viability in a concentration-dependent manner in K562 cells (Fig. S2). To investigate whether EGCG can induce apoptosis, we examined in K562 cells with or without EGCG (200 μM) for 24, 48, and 72 h using the Annexin-V & Dead cell staining assay (Fig. 4B). Early apoptosis, late apoptosis, and cell death were characterized and EGCG increased the percentage of late apoptotic cells in a time-dependent manner. The percentage of total apoptotic cells increased from 4.56 %, 5.94 %, and 15.84 % (untreated groups) to 50 %, 58.03 %, and 64.52 % (EGCG-treated groups) for 24, 48, and 72 h, respectively (Fig. 4C). Thus, these results indicated that EGCG has an anti-cancer effect by promoting apoptosis in K562 cells. In this condition, cell proliferation-related factors including AKT, p38 MARK, and STAT5 proteins were also regulated. The protein levels of phosphor-AKT and phosphor-p38 MARK were downregulated 30 min after EGCG treatment and the expression of phosphor-STAT5 also began to decrease starting from 15 min (Fig. 4D). The levels of cleaved caspase-3, an apoptosis marker, increased in a time-dependent manner upon treatment with 200 μM

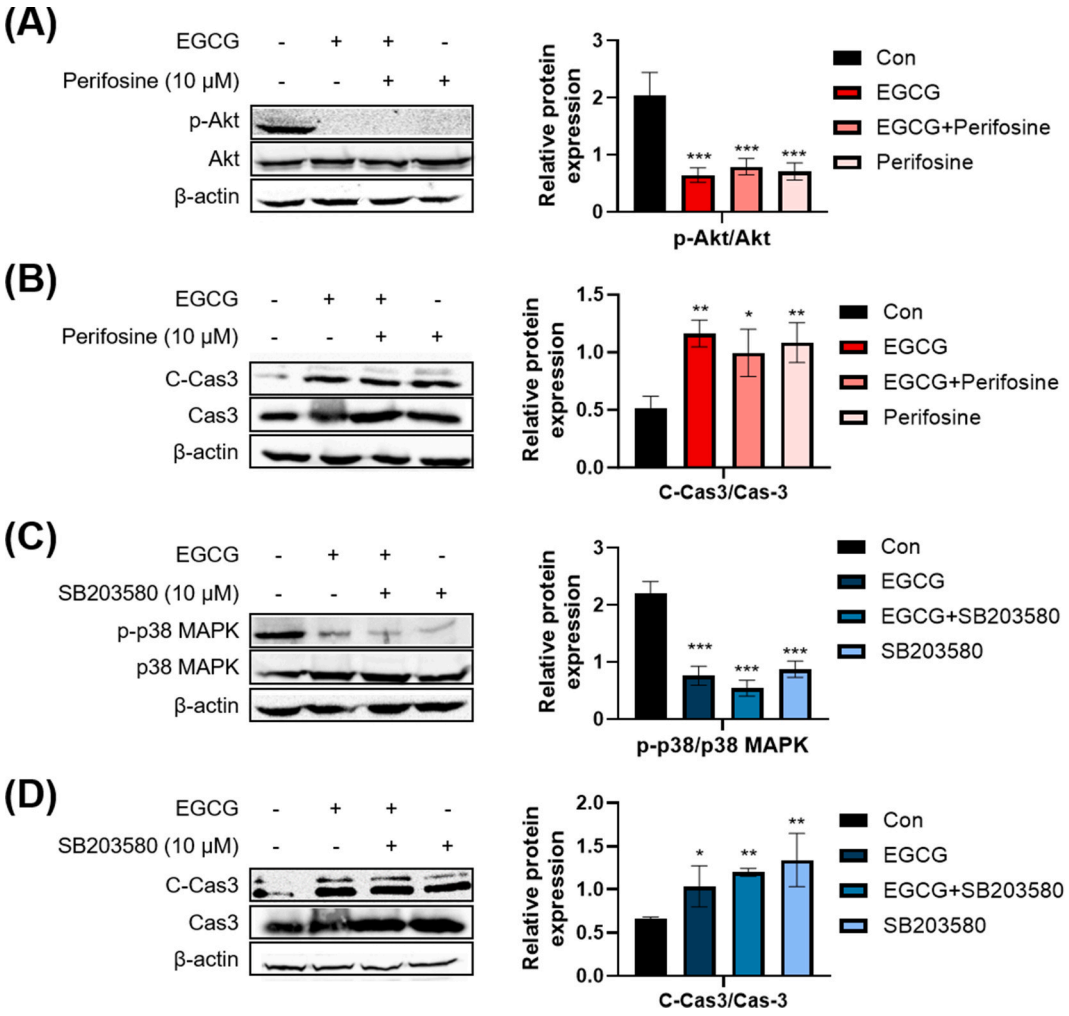

Fig. 6. Elimination of EGCG effect by Akt and p38 AMPK inhibitors in K562 cells. (A,B) Protein expression of *p*-Akt, Akt, cleaved caspase-3, and caspase-3 after treatment with perifosine, Akt inhibitor. (C,D) Protein expression of p-p38, p38 MAPK, cleaved caspase-3, and caspase-3 after treatment with SB203580, p38 MAPK inhibitor. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to control. Data are expressed as the mean ± SD (n ≥ 3).

EGCG and in a concentration-dependent manner (10–200 μM) of EGCG at 48 h (Fig. 4E and F). The expression levels of cleaved caspase-8, the extrinsic apoptosis marker, were upregulated following EGCG treatment, in a time- and concentration-dependent manner. In contrast, there was no significant difference in the expression of Bax and Bcl-2, the intrinsic apoptosis markers.

3.6. OR2AT4-mediated apoptosis in K562 cells induced by EGCG

To confirm that EGCG-induced apoptosis occurs through OR2AT4, cell viability and the levels of caspase-3 were evaluated after co-treatment with EGCG (200 μM) and phenirat (200 μM), the OR2AT4 antagonist, for 48 h. Cell viability, which was reduced by EGCG treatment alone, was restored by co-treatment with phenirat (Fig. 5A). The protein level of cleaved caspase-3 in the co-treatment group was similar to that in the control group (Fig. 5B). OR2AT4-mediated EGCG effect was also confirmed using siRNA (Fig. 5C). EGCG treatment in OR2AT4 siRNA-transfected K562 cells significantly reduced the cleaved caspase-3 and caspase-8 (Fig. 5D). Furthermore, it was confirmed that OR2AT4-mediated signal caused apoptosis by inhibiting Akt and p38 MAPK pathway using Akt inhibitor (perifosine) and p38 MAPK inhibitor (SB203580) (Fig. 6). Perifosine and SB203580 treatment induced downregulation of p-Akt and p-p38 MAPK (Fig. 6A,C), respectively, and finally upregulation of cleaved caspase-3, similar to EGCG treatment (Fig. 6B,D). Therefore, inhibition of Akt and p38 MAPK pathway may be involved in actual apoptosis induction. Overall, OR2AT4 may regulate the proliferation and apoptosis of leukemia cells by EGCG.

3.7. OR2AT4-mediated response to EGCG exclusive to K562 cells

To confirm anti-leukemia activity of EGCG, other leukemia cell line, Molt-4 cells were selected and effect of EGCG was monitored. There was no gene expression of OR2AT4 in Molt-4 (Fig. 7A), and there was no immediate calcium response to sandalore, an agonist of OR2AT4, and EGCG (Fig. 7B). Additionally, unlike K562 cells, where cell viability gradually decreases with the concentration of EGCG, Molt-4 cells showed a sudden onset of cell death at concentrations above 100 μM (Fig. 7C). Therefore, it was evident that although EGCG induced cell death in Molt-4, it did not occur via OR2AT4 mediation.

4. Discussion

Epigallocatechin-3-gallate (EGCG), the principal constituent of catching in green tea, exhibits anti-proliferative, anti-inflammatory, anti-mutagenic, anti-oxidative, and anti-cancer activities [31–35]. Specifically, EGCG has antitumor activities through engendering apoptosis and inhibit growth in several types of cancers, including colon, kidney, breast, and brain cancers as well as leukemia, as demonstrated by in vivo and in vitro research [36–39]. Much of the cancer chemopreventive properties of EGCG are mediated by altering the expression of cell cycle regulatory proteins and activating apoptotic genes, thereby inducing apoptosis and promoting cell growth arrest [40]. EGCG has great potential in treatment of cancer, but its relationship as a ligand with OR is unknown.

ORs play essential roles in cell proliferation, apoptosis, migration, and development, but their abnormal expression is closely related to the proceeding of cancer [5,8,41]. However, its function remains largely unknown. Therefore, research on the anti-cancer effect of OR activation through OR ligands is essential. For these purpose, this study confirmed the purification and expression of the OR2AT4 recombinant protein and demonstrated the function of a novel OR2AT4 ligand agonist in the regulation of cellular homeostasis in leukemia cells.

ORs are a large family of class A GPCRs that are currently targeted by 34 % of FDA-approved drugs because they play a critical role in small-molecule signaling [42]. Therefore, ligands for ORs are critical for understanding the olfactory system. However, OR ligands

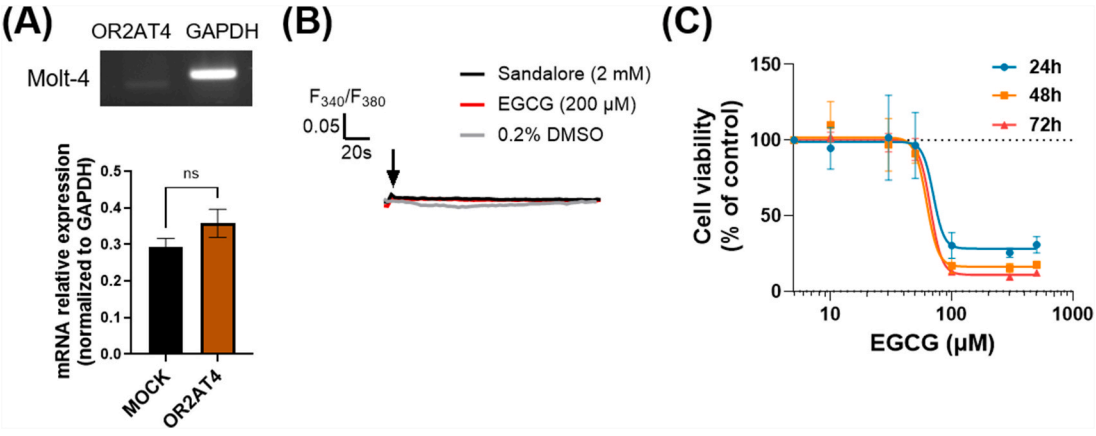

Fig. 7. Effect of EGCG on Molt-4 cells. (A) mRNA expression of OR2AT4 in Molt-4 cells. (B) Intracellular calcium response of sandalore (2 mM) and EGCG (200 μM) in Molt-4 cells. (C) Cell viability of EGCG at different concentrations for 24, 48, and 72 h. Data are expressed as the mean ± SD (n ≥ 3).

have been reported for only <6 % of human ORs [43]. This lack of data is due to the difficulties in functionally expressing ORs in heterologous systems. GPCRs are usually difficult to express, solubilize, and purify because of their complicated structures and strong hydrophobicity, making it difficult to obtain sufficiently large amounts of purified GPCRs [44]. In the case of other GPCRs, reconstitution of proteins using liposomes or detergents their use as sensing materials in biosensor systems [45]. GST-tagged OR2AG1 was successfully expressed in an *E. coli* system and an optimized method for protein purification was developed [26]. Here, we expressed OR2AT4 recombinant protein in a bacterial system and purified it. However, the vast majority are orphan receptors, and the ligands that activate them remain unknown. High-throughput SPR has emerged as a promising experimental approach for identifying ligands for these orphan receptors [28]. In this study, we identified OR2AT4 ligands using a recombinant OR2AT4 protein-based SPR system. Previous studies, hOR3A1 was expressed in the *E. coli* expression system, and liposomes containing hOR3A1 were selectively stimulated by helional, for evaluation using the SPR system [30]. OR6M1 membrane fragments demonstrated structural stability, and they contributed to the discovery of novel ligands owing to their reactivity with anthraquinone, using the SPR method [29]. Rat odorant-binding protein 3 and its mutants immobilized on the SPR chip interacted with volatile organic compounds, such as *β*-ionone [46]. Our study confirmed that OR2AT4 protein was successfully purified by confirming the reactivity with sandalore and sandalwood oil, an OR2TA4 agonist, using SPR. Sandalore is a synthetic sandalwood odorant with odor in some ways similar to sandalwood. Therefore, the low response unit in sandalwood oil is thought to have detected a lower signal than sandalore because sandalwood oil contains various component. We then discovered novel OR2AT4 ligands, such as berbamine, celastrol, EGCG, and rosmarinic acid. Additionally, agonists for OR2AT4 were selected by confirming calcium response to four compounds in OR2AT4-expressing cells. A previous study characterized the OR2AT4 agonists, sandalore and brahmanol and the antagonist, phenirat in transfected HEK293 cells and a HaCaT skin cell line, respectively [47]. Busse et al. cloned and functionally expressed the cutaneous OR, OR2AT4 and identified sandalore, a synthetic sandalwood odorant, as an agonist of this receptor. Therefore, we confirmed the intracellular calcium reactivity of sandalore, in OR2AT4-expressing cells, and identified berbamine and EGCG as agonists among these four compounds. We identified a novel OR2AT4 agonist by integrating SPR-based ligand screening with cell-based assays, suggesting its potential as a novel drug target.

ORs in various cancers regulate cancer cell invasiveness, metastasis, and differentiation, and are associated with cellular signaling, proliferation, and apoptosis [15,48]. Among the 19 types of ORs, OR2AE1, OR52B6, and OR2L3 are predominantly expressed in AML samples [19]. K562 is an immortalized human myeloid leukemia cell line. According to the prior studies, stimulation of OR2AT4 with its ligand sandalore reduces cell proliferation and induces apoptosis in myelogenous leukemia cells [3]. OR51B5 stimulation by isononyl alcohol inhibited cell proliferation in both AML and CML patients [20]. We confirmed that the expression of OR2AT4 in K562 cells and membrane proteins, and demonstrated OR2AT4 agonist EGCG increased Ca^{2+} levels in K562 cells, similar to sandalore stimulation. EGCG also induced sudden death of Molt-4 cells, an acute lymphoblastic leukemia cell line, but it does not occur via OR2AT4 mediation. EGCG, a major active polyphenol extracted from green tea, has variously effect such as anti-tumor activities, anti-obesity, anti-diabetes, and anti-inflammatory [33,38,39]. EGCG is involved in the regulation of several cellular signaling including reactive oxygen species, NF-κB, Akt, vascular endothelial growth factor, peroxisome proliferator-activated receptor, Bcl-2, and MAPKs activity [49–52]. Thus, through regulating these signaling, EGCG can inhibit oncogenic activity, proliferation, angiogenesis, migration, invasion and tumorigenesis, and induce apoptosis [53]. Also, EGCG induces apoptosis in chronic myelogenous leukemia cells [53,54]. Our data show stimulation with EGCG activated OR2AT4, reducing the proliferation and increasing the number of apoptotic cells for 24, 48, and 72 h in K562 cells. In particular, sandalore reduces the proliferation of K562 cells by preventing p38-MAPK phosphorylation [3]. In addition, isononyl alcohol inhibits cell proliferation by decreasing p-p38 MAPK via the regulation of OR51B5 in K562 cells [20]. This indicates that the involvement of MAPKs in regulating physiological mechanisms varies depending on the cellular system. Reduction in the levels of proteins that regulate cell proliferation and apoptosis in leukemia cells, is consistent with our results. Additionally, a hallmark feature of CML is the production of the BCR-ABL fusion protein. The BCR-ABL protein dysregulates cellular signaling pathways, including the activation of STAT through JAK/STAT pathway in CML. Especially, STAT5 leads to dysregulated cell proliferation, reduced apoptosis, and enhanced survival of leukemic cells. This results in the accumulation of immature white blood cells characteristic of CML [55,56]. Apoptosis or programmed cell death plays an essential role in protecting against carcinogenesis by eliminating genetically damaged cells, initiated cells, or cells that progress to a malignant phenotype [57]. Thus, the induction of apoptosis is a highly desirable chemotherapeutic and chemopreventive strategy for cancer control. The clinical effects of EGCG have been reported in hematologic malignancies such as chronic lymphocytic leukemia [58]. EGCG induces apoptosis in K562 cells by activating Fas-associated receptors and caspase-8 [59]. Our results also indicated that EGCG induces apoptosis by increasing caspase-8 expression in K562 cells. Taken together, stimulation with EGCG reduced proliferation and induced extrinsic apoptosis by activating OR2AT4 in K562 cells; both are desirable effects of anti-leukemic drugs.

In this study, we developed a recombinant OR2AT4 protein, purified it, and identified novel OR2AT4 ligands using SPR. In addition, OR2AT4 agonists were tested, and an agonist other than sandalore, EGCG, was identified. EGCG downregulates growth-related proteins such as AKT and p38 MAPK and upregulates extrinsic apoptosis-related proteins. Therefore, EGCG exhibits an OR2AT4 activating effect in the K562 leukemia cell line. This discovery suggests a new treatment strategy for leukemia by identifying a novel OR2AT4 ligand through the SPR method and presenting a new target for drug development by activating OR2AT4 using this ligand. But this study has some limitations. This study primarily focused on K562 cells in vitro. Analysis of the effect on the proliferation of CML-derived K562 cells is an effective way to explore new drugs for the treatment of CML, but in order to apply them as new drugs, there is a need to verify their efficacy in vivo. And although the efficacy of EGCG in leukemia cell lines was clearly discovered, K562 cells were the only ones mediated by OR2AT4. In other words, because OR2AT4 acts as a therapeutic target in some leukemia cell lines, it cannot be a drug target for general anti-leukemic activity. However, since there are reports that OR2AT4 is expressed in both AML and CML patients, it is believed that this can be overcome when using actual patient samples.

Data availability statement

All data supporting the findings of this study are available within this article and its Supplementary Information.

CRediT authorship contribution statement

Yae Rim Choi: Writing – original draft, Methodology, Formal analysis, Data curation. **Hyun-Jin Na:** Writing – original draft, Data curation. **Jin-Ah Lee:** Methodology, Formal analysis, Data curation. **Yiseul Kim:** Formal analysis, Data curation. **Young-Suk Kim:** Writing – review & editing. **Min Jung Kim:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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