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Nematicidal activity of two monoterpenoids and SER-2 tyramine receptor of *Caenorhabditis elegans*

Jason Lei, Michael Leser, Essam Enan *

Department of Biochemistry, Vanderbilt University School of Medicine, 635A Light Hall, Nashville, TN 37232, USA

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ABSTRACT

In vitro cultures of two nematodes (*Caenorhabditis elegans* and *Ascaris suum*) were established to study the nematicidal activity of three monoterpenoids (thymol, carvacrol and *p*-cymene). Toxicity of thymol and carvacrol was found for the two nematodes tested. The study was then aimed to address whether nematode tyramine receptor (TyrR) could interact with the two compounds by using HEK293 mammalian cells transfected with a *C. elegans* TyrR (ser-2) sequence, in hope of developing a high-throughput cell-based platform for future screening of new antihelminthic compounds. SER-2 expression and functionality in the transfected cells was first confirmed by green fluorescent protein tagging, competitive receptor binding, intracellular cyclic AMP, and intracellular calcium $[Ca^{2+}]_i$ mobilization assays. Thymol and carvacrol were then tested and demonstrated to interact with TyrR in desensitizing SER-2 for tyramine activation in $[Ca^{2+}]_i$ mobilization assay, and in translocating SER-2 from membrane to cytoplasm in receptor internalization assay. Receptor internalization activity of thymol and carvacrol was significantly blocked in cells expressing mutant SER-2 with the S210A/S214A double mutations, thus confirming specificity of the interactions. In summary, the current study showed that the nematicidal activity of thymol and carvacrol might be mediated through TyrR as the two compounds could trigger the signaling cascade downstream from the receptor in cells expressing wild-type but not a mutant SER-2. The TyrR-expressing cell system may prove to be a good screening platform for developing new antihelmintic compounds that may overcome parasite drug resistance, especially when such chemicals are used in combination with commercial drugs.

1. Introduction

Parasitic nematodes are the causal agents of many diseases in human, animals, and plants. More than two billion people are estimated to be infected and the infections are linked to deteriorated health conditions such as anemia and malnourishment even though they rarely cause death directly [1]. Infections on livestock and crops also cause severe economic losses in agriculture [2–4]. It is therefore important to develop anthelmintics that are both effective and safe to use, and can avoid or delay the development of worm resistance to the drugs.

Due to their distinctive scent or taste, many plant essential oils have been used in the food industry as food flavoring agents and in the perfume industry as fragrances [5]. Many plant essential oils have also been used in a broad range of applications. For example, plant essential oils extracted from thyme (*Thymus vulgaris* L.) and oregano (*Origanum vulgare* L.) have traditionally been used not only for the antiseptic, antispasmodic, and antitussive effects, but also for the antimicrobial, antifungal, antioxidative, and antiviral properties [6]. Plant essential oils were also found to possess insecticidal activity as well as attractant, repellent, feeding deterrent, and ovipositional stimulant activities against various insect species, suggesting an ecological role of plant essential oils [5,7–10]. In this regard, thymol and carvacrol (the two major monoterpenoid constituents of thyme and oregano essential oils) were shown to possess high insecticidal activity against the fruit fly. Judged by the loss of their insecticidal toxicity against a tyramine receptor (TyrR[1]) mutant *Drosophila melanogaster*, TyrR-^{neo30}, it was suggested that TyrR modulates the insecticidal activity of these two chemicals [5].

* Corresponding author. Tel.: +1 615 343 4282; fax: +1 615 343 0295.
E-mail address: essam.enan@vanderbilt.edu (E. Enan).

[1] The abbreviations used are: bp, base pairs; $[Ca^{2+}]_i$, intracellular calcium; cAMP, cyclic AMP; $[cAMP]_i$, intracellular cAMP; cDNA, complementary DNA; DA, dopamine; DMEM, Dulbecco's modified Eagles medium; EDTA, ethylenediamine-tetraacetic acid; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; G protein, guanine nucleotide-binding protein; HA, histamine; HEK, human embryonic kidney; His, histidine; 5-HT, serotonin; IBMX, 3-isobutyl-1-methyl-xanthine; LSD, lysergic acid diethylamide; NCS, newborn calf serum; NGM, nematode growth medium; OA, octopamine; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; TA, tyramine; TG, thapsigargin; TyrR, tyramine receptor.

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Since our current data demonstrated nematicidal activity of thymol and carvacrol against *Caenorhabditis elegans* and the pig roundworm, *Ascaris suum*, the study was then aimed to determine whether the two monoterpenoids could interact with TyrR from nematode, in an attempt to develop a cell-based platform for screening antihelminthic compounds against a new target. To address that goal, a *C. elegans* TyrR (ser-2) complementary DNA (cDNA) was cloned and used in transfection for producing the cell models. Several mutations of this TyrR were also developed by site-directed mutagenesis and cell models expressing these mutant proteins were established to study the specificity of such interactions. We demonstrate that the two plant essential oil constituent monoterpenoids, thymol and carvacrol, possessed high nematicidal activity, and that they could activate signaling cascade downstream from the receptor in cells expressing wild-type but not a mutant SER-2.

2. Materials and methods

2.1. Materials

Trizol reagent, superscript III first-strand synthesis system, platinum *Pfx* DNA polymerase, pcDNA3.1/V5-His A plasmid DNA, Dulbecco's modified Eagles medium (DMEM, Cat. No. 11885 for human embryonic kidney 293 (HEK293) cells and Cat. No. 11965 for COS-7 cells), opti-MEM I, MEM, antibiotic-antimycotic (100×), trypsin-EDTA, HEPES, and lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), newborn calf serum (NCS), thapsigargin (TG), forskolin, 3-isobutyl-1-methyl-xanthine (IBMX), and RPMI 1640 medium were purchased from, and oligonucleotide primers synthesized by Sigma–Aldrich (St. Louis, MO). DNA restriction enzymes were from New England Biolabs (Ipswich, MA). QIAquick PCR purification kit, QIAquick gel extraction kit, QIAprep spin miniprep kit, EndoFree plasmid maxi kit, and RNeasy mini kit were from Qiagen (Valencia, CA). G-418 disulfate was purchased from Research Products International Corporation (Mt. Prospect, IL). [^{3}H]-lysergic acid diethylamide (LSD) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). BD high performance calcium assay kit was from BD (Rockville, MD) and CatchPoint cAMP assay kit was from Molecular Devices (Sunnyvale, CA). Surfactant Ryoto Sugar Ester S-1570 was obtained from Kraft Foods (Northfield, IL).

2.2. Nematodes and test agents

C. elegans wild-type N2 was obtained from Caenorhabditis Genetics Center (CGC) at University of Minnesota (Minneapolis, MN). TyrR agonists and antagonists and plant essential oil compounds: tyramine hydrochloride (TA), (±)-octopamine hydrochloride (OA), dopamine hydrochloride (DA), serotonin hydrochloride (5-HT), histamine dihydrochloride (HA), yohimbine hydrochloride, phentolamine hydrochloride, chlorpromazine hydrochloride, mianserin hydrochloride, *p*-cymene (1-methyl-4-(1-methylethyl) benzene), 3-hydroxy *p*-cymene (thymol), and 2-hydroxy *p*-cymene (carvacrol) were purchased from Sigma–Aldrich. Chemical structures of TA, *p*-cymene, thymol, and carvacrol are depicted in Fig. 1. Concentrate (100 mg/ml) stocks of the plant essential oil compounds were made in pure acetone. For testing, they were further diluted to desired working concentrations in PBS containing 0.006% Ryoto Sugar Ester S-1570 surfactant (PBS/surfactant). Mebendazole (Anti-ox^{TM} by Janssen Pharmaceutica, Beerse, Belgium) was used as a standard anthelmintic for comparison.

2.3. Toxicity against C. elegans

After culturing on NGM [11] plates for six days, worms were collected by washing from the plates three times, each with 2 ml PBS/surfactant. At a titer of 200–300 worms/ml, they were then treated in triplicates with compounds at a final concentration of 0.1 mg/ml, which is equivalent to 0.67 mM for thymol and carvacrol, and 0.75 mM for *p*-cymene. A vehicle buffer treatment was run in parallel as a negative control. For comparison, the standard antihelmintic, mebendazole, was tested at a final concentration of 0.68 mM (0.2 mg/ml) or 169 mM (50 mg/ml), where 169 mM is the prescribed dosage recommended by the manufacturer. The nematodes were incubated at 22–25 °C for 18–24 h. Percent mortality (mean ± SD) was then determined from a 1-ml sample for each treatment. For comparison, two-tail unpaired *t*-tests were performed by using the statistical analysis software GraphPad Prism. An asterisk (*) on top of a histogram column indicates that the mortality rate of a particular treatment was statistically significantly different at $p < 0.05$ when compared against the mortality rate induced by mebendazole at 169 mM.

2.4. Toxicity against A. suum

A total of 35 worms without regard to sex were collected from pigs and randomly divided into seven groups with five worms each in a 150-cm^2 cell culture flask containing 200 ml RPMI 1640 medium with 1× antibiotic-antimycotic. Following a 60-min equilibration at 37 °C under 5% CO_2, test compounds were added to each flask and mixed thoroughly to achieve a final concentration of 0.05 mg/ml (0.33 mM for thymol and carvacrol or 0.37 mM for *p*-cymene). A total of 10 worms were used for each chemical treatment whereas five worms were treated with vehicle buffer only and used as a control group for comparison. Worms were monitored and mortality determined 0.5, 2, 5, and 24 h post-treatment.

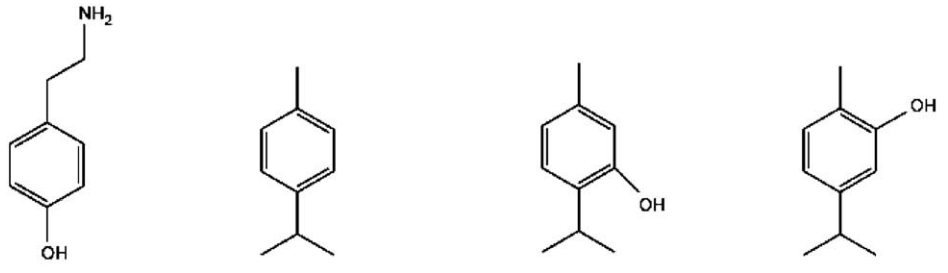

Fig. 1. Chemical structures of tyramine and three plant essential oil monoterpenoids: *p*-cymene, thymol, and carvacrol.

2.5. RNA isolation, reverse transcriptase-polymerase chain reaction (RT-PCR), cloning, and sequencing

The gene-specific primers designed based on the published ser-2 sequences (GenBank accession nos. AF539813 and AF539814) consisted of the sense PCR primer: 5'-cggggtaccgccaccATGGT GTTACGAGCCATCGACT-3' (Ser2-F) and antisense PCR primers: 5'-ccgccgcctcgagCTAAGGTTGCGCACTCATTCG-3' (Ser2-R, with the in-frame stop codon) and 5'-ccgccgcctcgagAGGTTGCGCACT-CATTCG-3' (Ser2-NS-R, without the in-frame stop codon for fusion with a 6×-His tag or the enhanced green fluorescent protein (EGFP) sequence). The capitalized nucleotides match the ser-2 gene sequence. The underlined nucleotides represent the recognition site for Kpn I and Xho I restriction enzyme in the sense and antisense primer, respectively. For translational enhancement, the sense primer also contained a Kozak sequence [12,13], as indicated by the italicized nucleotides (*gccacc*).

Total RNA was isolated from N2 wild-type *C. elegans* of mixed stages by using the trizol reagent according to manufacturer's instruction. For RT-PCR, the isolated total RNA was first primed with random hexamers and/or oligo$(dT)_{20}$ to make cDNA with superscript III first-strand synthesis system. PCR was then performed using *Pfx* DNA polymerase and the following thermo-cycling profile: 94 °C, 5 min for one cycle; 94 °C, 15 s, 68–56 °C, 15 s, and 68 °C, 1 min 30 s for 13 cycles (touchdown steps [14]); 94 °C, 15 s, 55 °C, 15 s, and 68 °C, 1 min 30 s for 35 cycles; 68 °C, 10 min for one cycle. Standard molecular techniques were followed for producing wild-type TyrR clones in vector pcDNA3.1/V5-His A or vector pcDNA3.1-cGFP (which was created by inserting an EGFP sequence in the pcDNA3.1/V5-His A vector) with the sequences confirmed by automated sequencing (Vanderbilt University DNA Sequencing Facility).

To develop mutant clones by site-directed mutagenesis, overlap-extension PCR similar to that described by Ho et al. [15] was performed with primers containing desired mutated bases in the overlapping region. Briefly, clones with a single mutation for amino acid residue 127 (GAC → GCC for D127A) were generated with primers 5′-TCACCACTGCTGCCATTTTATTA TGCA-3′ (Ser2-D127A-F, which was paired with primer Ser2-NS-R in the initial PCR) and 5′-TGCATAATAAAATGGCAGCAGTGGTGA-3′ (Ser2-D127A-R, which was paired with primer Ser2-F in the initial PCR), whereas clones with double mutations for amino acid residues 210 and 214 (TCT → GCT for S210A and TCA → GCA for S214A) were generated with primers 5′-TTGTTGTCTTCGCTGCTGCCGGTGCATTCTTCCTC-3′ (Ser2-S210A-S214A-F, which was paired with primer Ser2-NS-R in the initial PCR) and 5′-GAGGAAGAATGCACCGGCAGCA GCGAAGA-CAACAA-3′ (Ser2-S210A-S214A-R, which was paired with primer Ser2-F in the initial PCR), where the mutated bases are underlined. After PCR was performed by conditions as described above, the fragments were isolated and combined together for extension reactions to generate full-length products solely from templates with the overlapping regions (i.e., without using any PCR primers). The intended mutations for D127A and S210A/S214A were confirmed by automated DNA sequencing.

For determination of ser-2 expression in transfected mammalian cells, total RNA was isolated from transfected cells and treated with DNase I to remove any contaminant genomic DNA by using Qiagen's RNeasy mini kit according to manufacturer's instruction. RT-PCR was then performed as described above except control reactions without RT were also performed.

2.6. Cell culture and transfection

HEK293 and COS-7 cells were from American Type Culture Collection (Manassas, VA). HEK293 cells were grown in DMEM containing 5% FBS and 5% NCS whereas COS-7 cells were grown in DMEM containing 10% FBS, at 37 °C under 5% CO_2. Both types of media were supplemented with 1× antibiotic-antimycotic except during transfection with lipofectamine 2000.

COS-7 cells were transfected for transient expression as described previously [16] with some modifications. Briefly, cells were plated at 6×10^6 cells per 150-cm^2 plate in 15 ml growth medium without antibiotics the day before transfection. On the next day, the spent growth medium was replaced with 10 ml fresh medium without antibiotics. Lipofectamine 2000 (80 μl) and plasmid DNA (36 μg), each diluted in 3 ml opti-MEM I medium, were incubated separately at room temperature for 5 min, mixed together, and then incubated at room temperature for another 30 min. The mixture was then added to each dish to transfect cells. Cells were harvested for preparation of membrane fractions 48 h following transfection. Stably transfected HEK293 cell lines were established exactly as described previously [16].

2.7. Membrane preparation and radioligand binding assay

Membrane preparation was performed similarly as previously described [16]. Briefly, cells were harvested, washed with PBS, suspended in ice-cold hypotonic buffer (10 mM Tris–Cl, pH 7.4) and then lysed by using an electric homogenizer (Glas-Col, Terre Haute, IN) at 2000 rpm. After the nuclear pellet fraction was removed by centrifugation at $600 \times g$ for 10 min, the remaining supernatant was centrifuged again ($30\,000 \times g$ for 30 min) to obtain the crude membrane fraction. The membrane fraction was then suspended in binding buffer (50 mM Tris–Cl, 5 mM $MgCl_2$, pH 7.4) and protein concentration determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Membranes were snap frozen in liquid nitrogen and stored in aliquots at −75 °C until use.

Radioligand binding assay was performed as previously described [5], except 10 μg membrane protein in 500 μl binding buffer was used. The total and non-specific binding values for each [^{3}H]-LSD concentration (ranging from 0 to 10 nM) were determined in the absence and presence of 100 μM TA, respectively. The specific binding value was then obtained by subtracting the non-specific binding value from the total binding value for each [^{3}H]-LSD concentration to determine the K_d and B_{max} values. To determine the K_i values for different ligands, 5 nM [^{3}H]-LSD was used with a range of competitor concentrations giving 0–100% competition. All binding assays were performed with samples in duplicates.

2.8. Intracellular cyclic AMP [cAMP]$_i$ assay

HEK293 cells were plated in wells of a 96-well plate at 2.5×10^4 cells per well in 100 μl DMEM containing 5% FBS, 5% NCS, 1× antibiotic-antimycotic, and 0.8 mg/ml G-418. After overnight incubation at 28 °C under 3% CO_2, the medium was aspirated and cells treated with 100 μl PBS containing 800 μM IBMX (per manual of the CatchPoint cAMP fluorescent assay kit) and 5 μM forskolin with TA at 0, 0.01 and 1 μM in triplicates. Treatment with 800 μM IBMX alone (i.e., without forskolin or TA) was also performed to establish the [cAMP]$_i$ baseline. The [cAMP]$_i$ levels were determined with the CatchPoint cAMP fluorescent assay kit by following the manufacturer's protocol. Data collected on FlexStation II (Molecular Devices) was analyzed with Softmax Pro v5 software (Molecular Devices). For comparing the [cAMP]$_i$ levels, one-tail unpaired *t*-tests were performed by using the statistical analysis software GraphPad Prism. Statistical significance was set at $p < 0.05$.

2.9. Intracellular calcium [Ca^{2+}]$_i$ mobilization assay

HEK293 cells (7.5×10^4/well) suspended in 100 μl DMEM containing 5% FBS, 5% NCS, 1× antibiotic-antimycotic, and

0.8 mg/ml G-418 were plated in wells of a 96-well BD Biocoat black/clear plate (BD Labware, Bedford, MA) and incubated overnight at 28 °C under 3% CO_2. On the following day, the medium was aspirated and BD calcium assay kit fluorescent dye diluted in 1× HBSS containing 20 mM HEPES was then added to each well (100 μl/well) for cell dye-loading at 27 °C for 1 h. Test compounds plated in a 96-well Costar polystyrene plate (Corning Inc., Corning, NY) were automatically pipeted to cell wells for treatment. $[Ca^{2+}]_i$ mobilization was monitored by FlexStation II with excitation and emission wavelengths set at 485 nm and 525 nm, respectively. Fluorescent intensity was monitored for a period of 120 s, with test compounds added at 20 s and the first 20-s trace used as the pretreatment rest-state baseline.

2.10. Confocal laser scanning microscopy

To ensure good cell attachment after plating, a MatTek glass-bottom 96-well plate (MatTek Corporation, Ashland, MA) was pretreated with poly-D-lysine (2.5 μg/ml in MEM) by incubating for 3 h at room temperature. The plate was then rinsed with MEM and stored at 4 °C before use. For confocal laser scanning microscopy, HEK293 cells (3.5×10^4 cells/well) stably expressing SER-2b/EGFP fusion protein were plated into the wells of the pretreated plate in DMEM containing 5% FBS, 5% NCS, 1× antibiotic-antimycotic, and 0.8 mg/ml G-418, and incubated overnight at 37 °C under 5% CO_2. On the following day, cells were starved for 3–4 h by replacing the medium with MEM containing 20 mM HEPES, and then treated with compounds (100 μM TA or 0.1 mg/ml each of thymol, carvacrol, and *p*-cymene) for 2–3 h. Confocal microscopic imaging was performed using a Zeiss LSM 510 laser scanning microscope with a Zeiss Plan-Apochromat 63 × 1.4 numeric aperture oil-immersion objective (Vanderbilt University Cell Imaging Shared Resource). The EGFP was excited using a 488 nm argon laser and fluorescence detected with a 505 nm long-pass filter.

3. Results

3.1. Toxicity against C. elegans and A. suum

Thymol and carvacrol have been shown to have insecticidal activity against *D. melanogaster* [5]. To see if they also possess nematicidal activity, the free-living (*C. elegans*) and pig parasitic (*A. suum*) nematodes were used for treatment. Testing results showed that thymol and carvacrol are more effective than *p*-cymene as nematicides. In fact, thymol and carvacrol at 0.67 mM caused a mortality rate of 99 ± 1% and 100 ± 0%, respectively, whereas *p*-cymene at 0.75 mM caused a mortality rate of 16 ± 1% and the vehicle buffer caused a mortality rate of 1 ± 1% (Fig. 2). Compared with the standard antihelmintic, both thymol and carvacrol at 0.67 mM (0.1 mg/ml) were slightly more effective, though not statistically significant, than mebendazole at the prescribed dosage (169 mM or 50 mg/ml), which caused a 95 ± 3% mortality rate (Fig. 2). Interestingly, mebendazole at a concentration (0.68 mM) similar to that of thymol and carvacrol (0.67 mM) caused a much lower mortality rate at 10 ± 2%. Morphologically, worms killed by thymol or carvacrol had the characteristic bent and/or bursting bodies as shown in Fig. 3. The fact that thymol and carvacrol are more effective than *p*-cymene is also supported by the testing results with *A. suum*, where both thymol and carvacrol at 0.33 mM caused a mortality rate of 80% whereas *p*-cymene at 0.37 mM caused a mortality rate of 20%, as compared with the control vehicle buffer that killed no treated worms (Table 1). It is noteworthy that both thymol and carvacrol were also more efficient than *p*-cymene in terms of time required to kill at least one worm. In fact, some worms treated with thymol or carvacrol were dead within 2 h whereas no worm treated with *p*-cymene was dead within 5 h (Table 1).

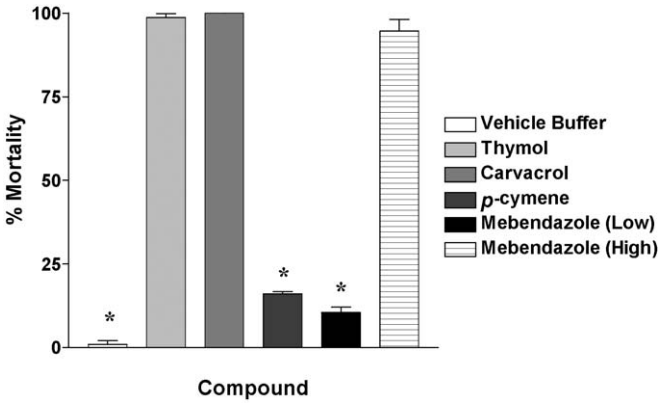

Fig. 2. Nematicidal activity of three plant essential oil monoterpenoids and a standard antihelmintic (mebendazole) against *C. elegans*. Worms were treated in triplicates with compounds at a final concentration of 0.1 mg/ml, which is equivalent to 0.67 mM for thymol and carvacrol, and 0.75 mM for *p*-cymene. The standard antihelmintic, mebendazole, was tested at a final concentration of 0.68 mM (0.2 mg/ml) or 169 mM (50 mg/ml), where 169 mM is the recommended full-strength dosage. Treatment with PBS/surfactant vehicle buffer was conducted in parallel and used as a negative control. Histogram columns for the mean mortality rates are shown with SD error bars. An asterisk (*) on top of a column indicates that the mortality rate of a particular treatment was statistically significantly different at $p < 0.05$ when compared against the mortality rate induced by mebendazole at 169 mM.

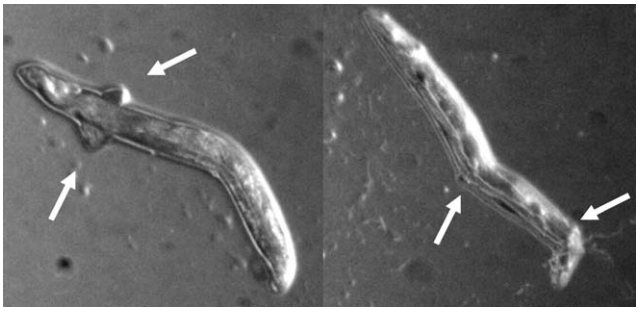

Fig. 3. Nematicidal effects of thymol on *C. elegans*. Shown are two worms with the characteristic bent and/or bursting (indicated by arrows) bodies after overnight treatment with 0.17 mM thymol.

To examine whether thymol and carvacrol can interact with nematode TyrR and develop a cell-based system for high-throughput compound screening, mammalian cells were transfected with a cloned TyrR sequence (ser-2 from *C. elegans*) and used for studies as described in the following sections.

3.2. Characterization of ser-2 cDNA and the expressed SER-2 receptor

3.2.1. Cloning of wild-type ser-2 cDNA

RT-PCR with *C. elegans* total RNA produced a product around the expected size (~1.3 kb). After restriction with Kpn I and Xho I enzymes, the PCR product was ligated into pcDNA3.1/V5-His A

Table 1
Antihelminthic activity of three plant essential oil monoterpenoids against the pig roundworm (*A. suum*).

Chemical	Post-treatment mortality at			
	0.5 h	2 h	5 h	24 h
p-Cymene	0/10	0/10	0/10	2/10
Thymol	0/10	1/10	4/10	8/10
Carvacrol	0/10	3/10	5/10	8/10
PBS/surfactant	0/5	0/5	0/5	0/5

A final concentration of 0.05 mg/ml was used for all three compounds (equivalent to 0.37 mM for *p*-cymene and 0.33 mM for thymol and carvacrol).

vector digested with the same enzymes. Restriction analysis of miniprep plasmid DNA isolated from randomly selected clones showed that there were two different sizes in the ser-2 population. Sequence analysis showed that in the coding sequence, the smaller clones had an in-frame 69-bp deletion, which is identical to that of the previously published SER-2a sequence (GenBank accession no. AF539814 [17,18]). In contrast, the larger clones had a sequence identical to the previously published SER-2 sequence (GenBank accession no. AF539813 [17,18]) and did not have any deletion. Interestingly, whereas the two shorter isoforms had mismatches at residue 60 (T vs. A) and residue 257 (D vs. V), the shorter isoform from the current study was identical to the longer isoform at both positions. Due to its unique sequence, the shorter isoform from the current study was named as SER-2b and its sequence data can be found in Genbank and other databases.[2]

3.2.2. Expression of isoform SER-2b

HEK293 cells stably transfected with constructs for SER-2 and SER-2b were established after selection with G-418 at 0.8 mg/ml for four weeks. Since the latter cell line produced higher response to TA in the initial $[Ca^{2+}]_i$ mobilization assays, only constructs and cell lines derived from the SER-2b construct were used for subsequent experiments. Expression of the transfected gene in HEK293 cells was confirmed by RT-PCR (Fig. 4A) using gene-specific primers Ser2-F and Ser2-R. Expression was further investigated by subcloning the ser-2b sequence into the pcDNA3.1-cGFP vector so that the EGFP sequence is located downstream of the receptor sequence (i.e., at C-terminus of SER-2b). As revealed by confocal microscopy with laser scanning, the EGFP fluorescence was localized on the cell surface (Fig. 4B), suggesting that the SER-2b receptor was properly synthesized and trafficked to plasma membrane like a functional G protein-coupled receptor (GPCR).

3.2.3. Pharmacological analysis of the expressed SER-2b receptor

Ligand binding affinity and capacity of the SER-2b receptor expressed in mammalian cells was determined by constructing a saturation curve using [^{3}H]-LSD at a range of concentrations giving 0–100% specific binding to the receptor on the membrane fraction isolated from COS-7 cells transiently transfected with the ser-2b sequence without any tag sequences. The K_d and B_{max} values were determined to be 14.0 nM and 9.4 pmol/mg protein, respectively.

The pharmacological profile was determined using various agonists and antagonists to compete with [^{3}H]-LSD for binding to the receptor. Order of the affinity for the expressed SER-2b receptor was determined to be: TA > DA > OA > 5-HT > HA for biogenic amines and chlorpromazine > phlentomine > yohimbine > masar-masarine for other ligands (Table 2).

3.2.4. Effect on $[cAMP]_i$ levels by TA through the expressed SER-2b receptor

SER-2 receptor has been shown to be associated with $G_{\alpha i}$ in HEK293 cells [17] as after binding to TA, it could reduce the forskolin-stimulated $[cAMP]_i$ level. TA was used to test if HEK293 cells expressing the SER-2b had the same effect. The results showed that TA at 1 μM did reduce the forskolin-stimulated $[cAMP]_i$ levels statistically significantly by 56% (Fig. 5). TA at 0.01 μM also caused slight reduction (8%) even though the reduction was not statistically significant (Fig. 5). In contrast, neither of the two TA concentrations reduced the $[cAMP]_i$ levels significantly in HEK293 cells transfected with the control empty vector (data not shown), suggesting that it was the expressed SER-2b receptor that mediated the TA inhibition on forskolin-stimulated $[cAMP]_i$ production.

[2] Sequence data reported in this paper are available in the GenBank, EMBL and DDBJ databases under the accession nos. GQ217520 and GQ217521.

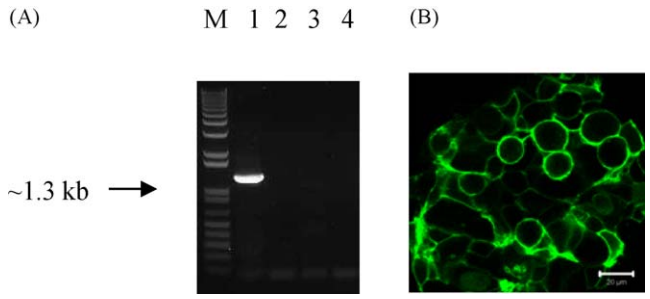

Fig. 4. Expression of SER-2b in HEK293 cells as revealed by (A) RT-PCR. M: Invitrogen 1 kb plus DNA ladder; 1, 2: HEK293 cells transfected with pcDNA3.1/V5-His A-Ser-2b DNA for SER-2b expression; 3, 4: HEK293 cells transfected with pcDNA3.1/V5-His A DNA for empty vector control; 1, 3: reactions with RT; 2, 4: reactions without RT. Position of the expected size of the RT-PCR product (~1.3 kb) was indicated with an arrow to the left of the agarose electrophoregram. (B) Confocal laser scanning microscopy. Images of HEK293 cells expressing SER-2b/EGFP fusion protein were taken by using a Zeiss LSM 510 laser scanning microscope with a Zeiss Plan-Apochromat 63 × 1.4 numeric aperture oil-immersion objective. Cells were excited using a 488 nm argon laser and the emitted fluorescence was detected with a 505 nm long-pass filter. Bar = 20 μm.

Table 2
Pharmacological profiles of the cloned SER-2b receptor.

Chemical	K_i (μM)
Biogenic amines	
Tyramine (TA)	0.61 ± 0.12
Dopamine (DA)	9.36 ± 1.69
Octopamine (OA)	34.07 ± 5.54
Serotonin (5-HT)	146.80 ± 28.24
Histamine (HA)	488.40 ± 100.80
Other ligands	
Chlorpromazine	0.003 ± 0.001
Phentolamine	0.008 ± 0.002
Yohimbine	0.827 ± 0.097
Mianserin	1.600 ± 0.231

Based on the saturation curve constructed by using [^{3}H]-LSD as the radioligand, K_d and B_{max} were determined to be 14 nM and 9.4 pmol/mg protein, respectively.

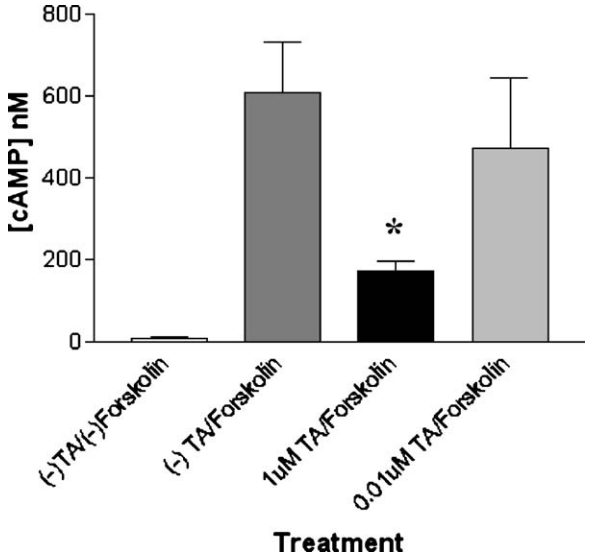

Fig. 5. TA-dependent attenuation of forskolin-stimulated $[cAMP]_i$ levels in HEK293 cells expressing SER-2b. Cells were treated with PBS containing 800 μM IBMX and 5 μM forskolin, with or without TA for 15 min at 37 °C. Treatment with 800 μM IBMX alone in PBS (i.e., without forskolin or TA) was also performed to establish the $[cAMP]_i$ baseline. The cells were then lysed and the $[cAMP]_i$ levels detected by using CatchPoint cAMP fluorescent assay kit from Molecular Devices. Data expressed as the mean ± SD are representative of two independent experiments performed in triplicates. An asterisk (*) on top of a bar indicates that reduction in the $[cAMP]_i$ level by TA treatment was statistically significant at $p < 0.05$ when compared against the forskolin-stimulated group without TA treatment.

3.2.5. Effect on $[Ca^{2+}]_i$ mobilization by TA through the expressed SER-2b receptor

Functioning of the expressed SER-2b receptor was also examined in HEK293 cells by $[Ca^{2+}]_i$ mobilization assay. Fig. 6A shows that TA (as low as 0.01 μM), but not the PBS negative control, increased the $[Ca^{2+}]_i$ beyond the baseline level in cells expressing SER-2b. In contrast, neither TA nor PBS induced similar calcium increase in cells transfected with the control empty vector (Fig. 6A). Other biogenic amines (OA, DA, 5-HT, and HA) were at least 100 fold less potent than TA in the $[Ca^{2+}]_i$ mobilization assays (data not shown), confirming that TA is the primary agonist of this receptor.

The calcium source used by the SER-2b receptor in HEK293 cells was also examined by treating cells with TG, a naturally occurring sesquiterpene lactone derived from *Thapsia garganica*, which can selectively deplete the endoplasmic reticulum (ER) calcium stores by inhibiting the ER Ca^{2+}-ATPase [19–21]. Treatment of cells expressing SER-2b with 1 μM TG released high level of Ca^{2+} into cytoplasm (Fig. 6B) and this Ca^{2+} depletion completely abolished the TA-triggered $[Ca^{2+}]_i$ mobilization (Fig. 6C) as compared with cells not pretreated with TG (Fig. 6A), suggesting that ER calcium stores are the Ca^{2+} source for the SER-2b receptor.

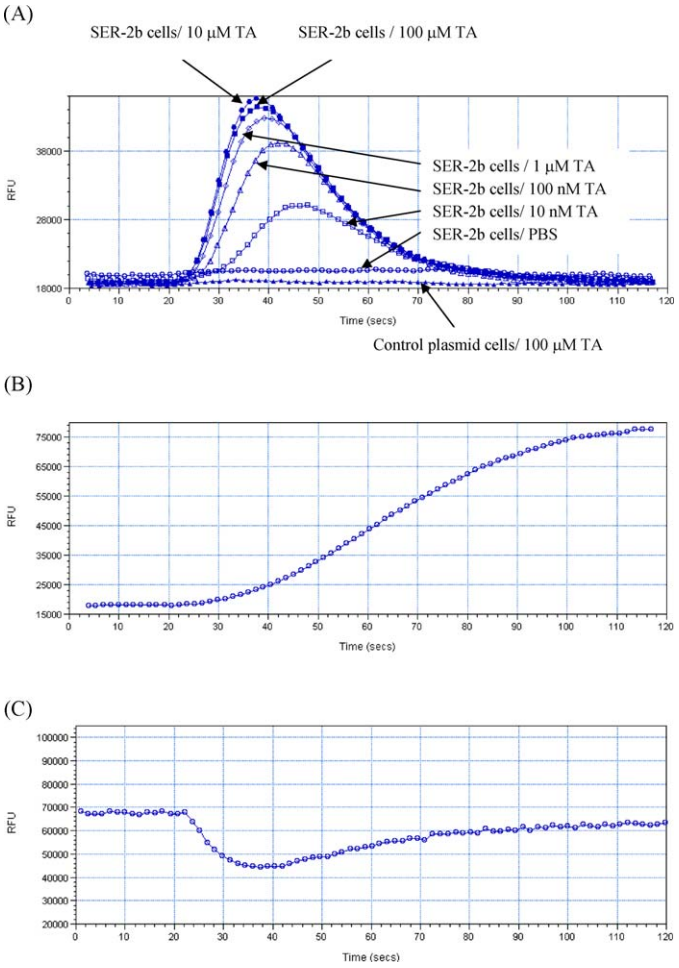

Fig. 6. Effect of TA and TG on $[Ca^{2+}]_i$ mobilization in HEK293 cells expressing SER-2b. (A) $[Ca^{2+}]_i$ mobilization by cells after treatment with PBS or TA at indicated concentrations. SER-2b cells: HEK293 cells expressing the SER-2b receptor; control plasmid cells: HEK293 cells transfected with the pcDNA3.1/V5-His A empty vector DNA. (B) $[Ca^{2+}]_i$ mobilization by cells expressing SER-2b after treatment with 1 μM TG. (C) Abolishment of TA-induced $[Ca^{2+}]_i$ mobilization by TG. Cells were pretreated with 1 μM TG (as shown in B) and then 30 min later with 100 μM TA. Diagrams shown are representative calcium fluorescent tracings from one of three independent experiments. RFU: relative fluorescence unit.

Together with the results from the RT-PCR, EGFP localization, pharmacological profiling, and cAMP assays, the calcium assay data indicate that the SER-2b receptor in the current study was properly expressed and functioned in the same way as expected of a SER-2 TyrR in the heterologous HEK293 cells.

3.3. Interactions of the expressed SER-2b receptor with thymol and carvacrol

The insecticidal activity of thymol and carvacrol was linked to a TyrR from *D. melanogaster* [5]. It would be interesting to see if the nematicidal activity of the two monoterpenoids can also be linked to nematode TyrR. As a model system, interactions between the cloned SER-2b receptor and thymol/carvacrol was investigated by $[Ca^{2+}]_i$ mobilization and receptor internalization assays in the HEK293 cell system. This heterologous cell-based system was also adopted in an attempt to develop a high-throughput system for future screening of new nematicidal compounds.

3.3.1. $[Ca^{2+}]_i$ mobilization assay

$[Ca^{2+}]_i$ mobilization assay was performed to see if thymol and carvacrol could act like TA and induce calcium mobilization in HEK293 cells expressing SER-2b. While the two monoterpenoids triggered an extremely high levels of $[Ca^{2+}]_i$ mobilization in cells expressing SER-2b, they also induced similar levels of $[Ca^{2+}]_i$ mobilization in cells transfected with the control empty vector (data not shown), thus making it difficult to examine specific effects of the two monoterpenoids on the SER-2 receptor.

To meet this challenge, cell response was recorded after SER-2b HEK293 cells were treated with thymol in a range of concentrations from 0 to 500 μg/ml in $[Ca^{2+}]_i$ mobilization assay. Cell response was recorded again for the thymol-treated cells after 10 μM TA was applied to the cells 30 min after the thymol treatment. The results showed that thymol at all concentrations tested (including the lowest concentration at 5 μg/ml) reduced or completely abolished the TA-induced $[Ca^{2+}]_i$ mobilization as compared with the control treatment with PBS/surfactant vehicle buffer only (i.e., no thymol pretreatment) (Fig. 7B). At the same concentrations, whereas carvacrol produced similar effects, *p*-cymene had no effect at all on the TA-induced $[Ca^{2+}]_i$ mobilization (data not shown), indicating that thymol and carvacrol, but not *p*-cymene, could interact with SER-2 and make the receptor stop responding fully to the activation by TA. It is noteworthy that since thymol and carvacrol did not elicit any significant calcium release at the low concentrations tested (Fig. 7A), such inhibitory effects on TA-induced $[Ca^{2+}]_i$ mobilization could not be due to depletion of the ER calcium stores like that induced by TG (Fig. 6B).

3.3.2. Receptor internalization assay

Receptor internalization is one of the mechanisms for GPCR desensitization, wherein the receptor is translocated from cell surface to cytoplasm upon agonist activation [22]. With other trafficking proteins also involved, receptor internalization is mainly mediated by β-arrestins and GPCR kinases (GRKs) [22–24]. Fluorescent proteins such as EGFP have been utilized for monitoring specific agonist-induced GPCR trafficking and for screening GPCR-interacting compounds [25]. To see if thymol/carvacrol can induce internalization of the SER-2 receptor, HEK293 cells expressing the EGFP-tagged SER-2b were examined after compound treatment by confocal laser scanning microscopy. The results showed that some receptors were indeed translocated into the cytoplasm compartment after treatment with TA, thymol, or carvacrol (Fig. 8), suggesting that all the three chemicals could activate this SER-2 receptor. In contrast, almost all receptor-associated fluorescence from cells treated with the vehicle buffer or *p*-cymene remained on the cell surface, i.e., not internalized

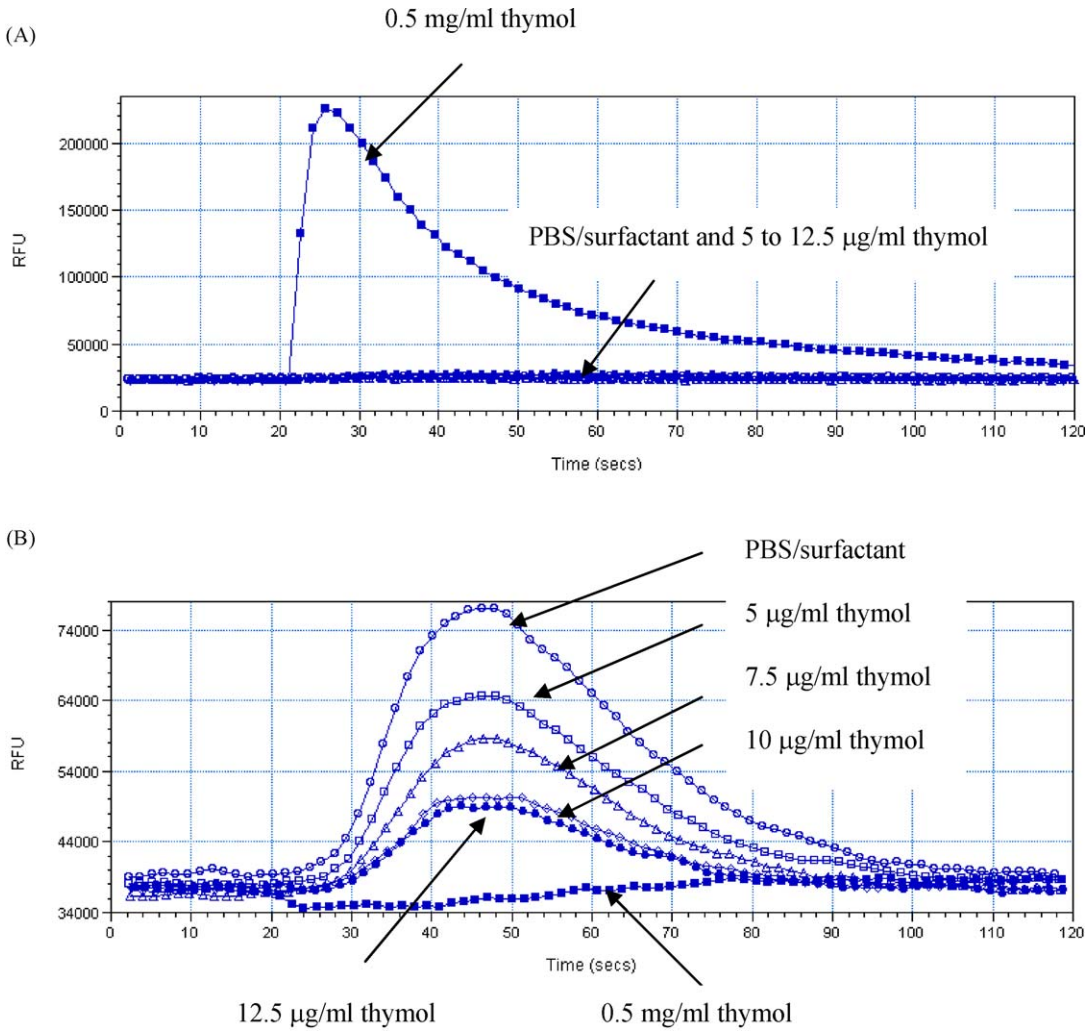

Fig. 7. Partial and complete inhibition of TA-induced $[Ca^{2+}]_i$ mobilization in SER-2b-expressing HEK293 cells by thymol. Cells expressing the SER-2b receptor were first treated with thymol at a range of concentrations (0, i.e., PBS/surfactant vehicle buffer control, 5, 7.5, 10, 12.5, and 500 μg/ml) and then 30 min later with 10 μM TA. (A) $[Ca^{2+}]_i$ mobilization after the first (i.e., thymol) treatment. (B) $[Ca^{2+}]_i$ mobilization after the second (i.e., TA) treatment. Thymol concentrations used in the first treatment are as indicated. Diagrams shown are representative calcium fluorescent tracings from one of three independent experiments. RFU: relative fluorescence unit.

(Fig. 8), suggesting that they could not activate this receptor. It is interesting that in the receptor internalization studies, the cell surface retained a significant amount of fluorescence even in cells treated with TA or thymol/carvacrol. This might result from the quick receptor turnover after internalization even though other factors, such as inefficient activation and/or internalization, cannot be ruled out.

To further study specificity of the interactions, two SER-2b mutants with changes on residues that are critical for TA binding of a *Bombyx mori* TyrR [26], were created by overlap-extension PCR: one with the D127A single mutation and the other with the S210A/S214A double mutation. $[Ca^{2+}]_i$ mobilization assay showed that response of the receptor to TA activation was either abolished (response of the D127A and S210A/S214A mutants to 1 μM TA and response of the D127A mutant to 100 μM TA) or significantly reduced (response of the S210A/S214A mutant to 100 μM TA) (Fig. 9), confirming that these residues are indeed critical for interaction between the SER-2b receptor and its cognate agonist, TA.

Cells expressing the wild-type or mutant SER-2b receptor tagged with EGFP was then tested against thymol and carvacrol in receptor internalization assays. The results showed that treatment with the PBS/surfactant vehicle buffer did not induce any significant internalization events in all cell lines tested (data not shown). On the other hand, the wild-type and D127A mutant cells did respond strongly to thymol or carvacrol treatment and produced profound receptor internalization events (Fig. 10), suggesting that the aspartate residue at position 127, while critical for TA binding, is dispensable for binding of thymol or carvacrol to the SER-2b receptor. This is probably not surprising as thymol and carvacrol do not have an amine group on the phenyl ring as TA to interact with the carboxyl group of the aspartate residue. In contrast to the D127A mutant cells, the S210A/S214A mutant cells did not produce or produced much less conspicuous receptor internalization events with either compound (Fig. 10), suggesting that the serine residues at positions 210 and 214 are critical for the SER-2b receptor to interact with these two plant essential oil monoterpenoids, probably through the hydroxyl groups on the TyrR serine residues and on the compounds.

4. Discussion

Nematode parasites have posed serious epidemiological problems in many countries around the world. Developing effective and yet safe antihelmintic agents is thus paramount in controlling the diseases or disorders. Plant essential oils have been shown to possess insecticidal and insect behavior-modifying activities [5–10]. In this study we demonstrated that two plant

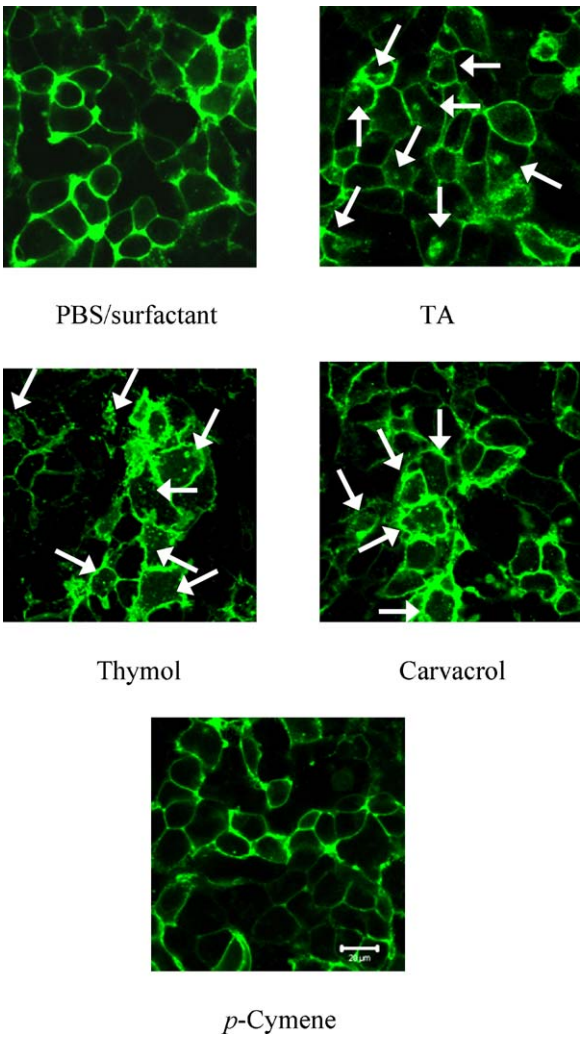

Fig. 8. Receptor internalization assay with HEK293 cells expressing the wild-type SER-2b fused with EGFP. Cells were treated with compounds as indicated (TA at 10 μM and plant essential oil compounds at 0.1 mg/ml in PBS/surfactant) for ~2 h. Images were then taken from the treated cells by using a Zeiss LSM 510 laser scanning microscope with a Zeiss Plan-Apochromat 63 × 1.4 numeric aperture oil-immersion objective. The EGFP was excited using a 488 nm argon laser and the emitted fluorescence was detected with a 505 nm long-pass filter. Cells with possible internalization events are indicated by arrows. Bar = 20 μm.

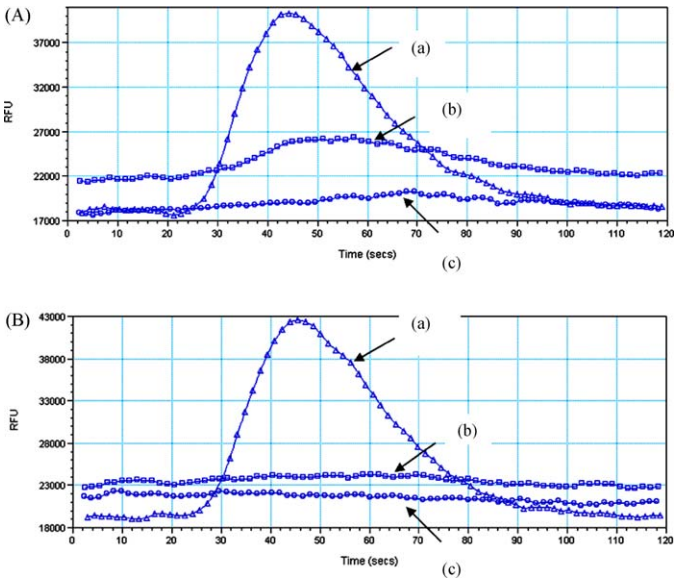

Fig. 9. $[Ca^{2+}]_i$ mobilization assay with HEK293 cells expressing the wild-type, D127A single mutant, or S210/S214 double mutant SER-2b receptor. (A) $[Ca^{2+}]_i$ mobilization after 100 μM TA treatment. (B) $[Ca^{2+}]_i$ mobilization after 1 μM TA treatment. (a) Wild-type; (b) S210/S214 double mutant; (c) D127A single mutant in both (A) and (B). RFU: relative fluorescence unit.

essential oil monoterpenoids, thymol and carvacrol, are also effective in killing nematodes, including the free-living *C. elegans* (Fig. 2) and the pig roundworm, *A. suum* (Table 1). Indeed, after the current study was initiated, thymol [27,28] and carvacrol [28] were also demonstrated to have strong nematicidal activity against other nematodes, such as the sheep gastrointestinal nematode, *Haemonchus contortus* [27], and the pine wilt nematode, *Bursaphelenchus xylophilus* [28].

As a first step toward elucidating nematicidal mechanisms of the two monoterpenoids, and also in the hopes of developing a cell-based system for future high-throughput screening of new nematicidal compounds, we cloned a SER-2 receptor from *C. elegans* and used it as a model system for studying potential interactions between the two monoterpenoids and nematode TyrR. SER-2 was selected because the previous findings showed that insecticidal activity of thymol and carvacrol could be linked to a *Drosophila* TyrR, as demonstrated by using an insect strain ($TyrR^{neo30}$) with a TyrR mutation [5]. SER-2 also has higher homology to this Drosophila TyrR than the other two *C. elegans* TyrRs, TYRA-2 [29] and TYRA-3 [30]. Furthermore, both thymol and carvacrol resemble TA in structure as like TA, they also have a hydroxyl group bonded to the phenyl ring (Fig. 1). They may thus be able to trigger a signaling cascade that might lead to death of nematodes by interacting with a TyrR like SER-2.

Consistent with characterization of the previously published SER-2 receptors [17,18], SER-2b receptor in the current study responded to TA activation by reducing forskolin-stimulated $[cAMP]_i$ levels (Fig. 5) and by triggering $[Ca^{2+}]_i$ mobilization (Fig. 6A) in HEK293 cells, indicating that the expressed receptor was functioning as a bona-fide TyrR. The notion was also supported by the results of pharmacology profiling (Table 2), where TA had the highest affinity among the biogenic amines tested. For calcium signaling, SER-2 receptor was further characterized in this study by using the ER-specific calcium store inhibitor, TG. It was shown that the ER calcium stores were the calcium source for SER-2 as response of this receptor to TA activation was blocked by this ER Ca^{2+}-ATPase inhibitor (Fig. 6C).

If TyrR is a target of thymol and carvacrol, the two compounds should be able to interact with the receptor and trigger a cascade of responses characteristic of TyrR or GPCR in general. Thymol and carvacrol can elicit calcium release in many different tissues and cell types and are agonists for many different ion channels and receptors [31–36]. Therefore, while it is not surprising that thymol or carvacrol could induce a remarkable elevation in $[Ca^{2+}]_i$ levels both in transfected and untransfected HEK293 cells, it is difficult to directly reveal thymol/carvacrol-induced response specifically mediated through SER-2 in the $[Ca^{2+}]_i$ mobilization assays. To work around this challenge, cells expressing SER-2b were sequentially treated with thymol/carvacrol and TA in $[Ca^{2+}]_i$ mobilization assays. The result that thymol (Fig. 7B) and carvacrol, but not *p*-cymene, could reduce or block the TA-triggered $[Ca^{2+}]_i$ mobilization indicates that thymol and carvacrol could desensitize the receptor. Judging from the finding that compounds triggered no or extremely low levels of $[Ca^{2+}]_i$ mobilization at low concentrations (as exemplified by the results with 5–12.5 μM thymol in Fig. 7A), such compound effect on the TA-triggered $[Ca^{2+}]_i$ mobilization is not very likely a result from depletion of the calcium stores. Whether such phenomenon was due to homologous desensitization, a

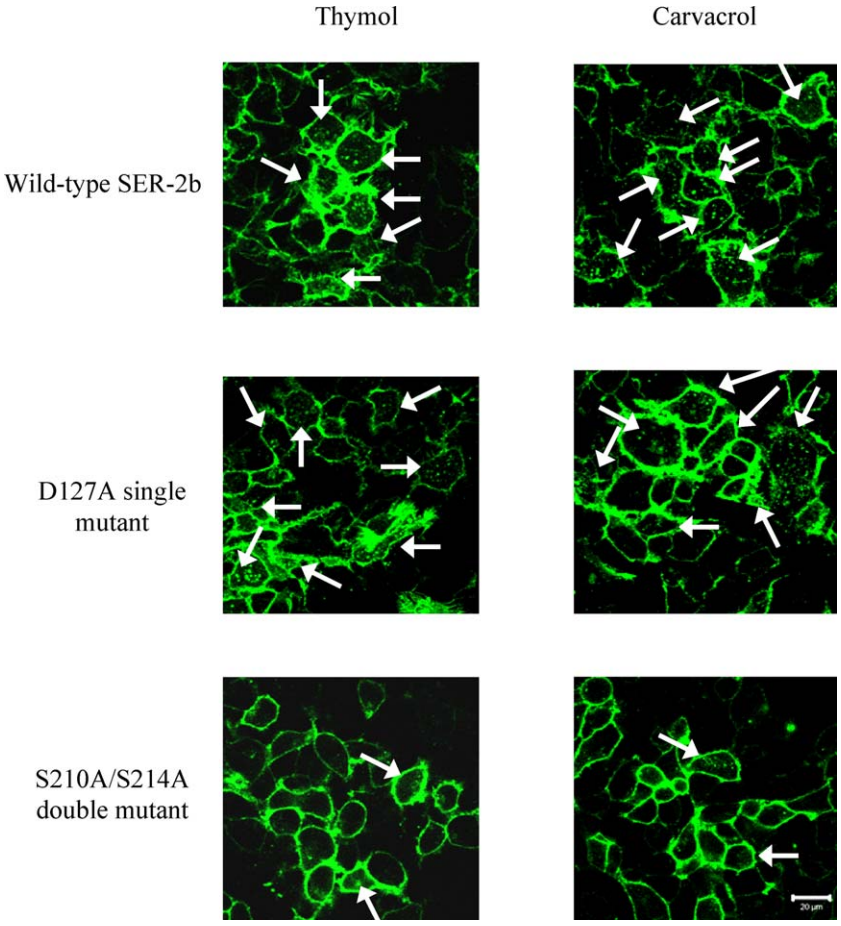

Fig. 10. Receptor internalization assay with HEK293 cells expressing the wild-type, D127A single mutant, or S210A/S214A double mutant SER-2b fused with EGFP. Wild-type (top row), D127A single mutant (middle row), and S210A/S214A double mutant (bottom row) SER-2b-expressing HEK293 cells were treated with thymol (left column) or carvacrol (right column) at 0.1 mg/ml in PBS/surfactant for ~3 h. Images were taken from the treated cells by using a Zeiss LSM 510 laser scanning microscope with a Zeiss Plan-Apochromat 63 × 1.4 numeric aperture oil-immersion objective. The EGFP was excited using a 488 nm argon laser and the emitted fluorescence was detected with a 505 nm long-pass filter. Cells with possible internalization events are indicated by arrows. Bar = 20 μm.

result triggered by GRK-mediated receptor phosphorylation upon binding by its cognate agonist [24,37], or heterologous desensitization, a result mediated through activation of another receptor [37,38], the results demonstrate that the two monoterpenoids could interact with SER-2.

Receptor internalization upon agonist activation is a mechanism of receptor desensitization [22–24]. When the receptor is fused with a fluorescent protein such as EGFP, receptor internalization assay can be used to study specific agonist-induced intracellular trafficking of GPCR. The positive receptor internalization assay results with cells expressing SER-2b/EGFP fusion protein (Fig. 8) further demonstrate that thymol and carvacrol could interact with SER-2 and have effects on functions of the receptor. Studies by other approaches are also required but interactions as revealed in the current study may have implications on the nematicidal activity of the two plant essential oil compounds.

As mentioned above, thymol and carvacrol resemble TA in structure as all the three compounds have a hydroxyl group on the phenyl ring (Fig. 1). Differing only at the position of the hydroxyl group on the phenyl ring, both thymol and carvacrol are derivatives of *p*-cymene [39,40], which lacks the phenyl ring-bonded hydroxyl group. Such lacking may explain why *p*-cymene is not as effective as thymol or carvacrol in killing nematodes (Table 1 and Fig. 2), a result consistent with the previous finding with Drosophila for its low insecticidal activity [5]. It is thus likely that the phenyl ring-bonded hydroxyl group plays an important role in binding to TyrR and triggering the downstream signaling cascades. Supporting this is that when two SER-2 serine residues, which are critical for interacting with the phenyl ring-bonded hydroxyl group of TA (Fig. 9 and [26]), were mutated in the S210A/S214A double mutant, the cells stopped responding well to thymol or carvacrol and produced no or significantly less receptor translocation, when compared with the wild-type cells receiving the same treatments (Fig. 10).

In summary, we have shown that two plant essential oil monoterpenoids, thymol and carvacrol, have good potential as effective nematicides. Since many plant essential oils and their derived compounds, such as thymol, are generally regarded as safe (GRAS) by US Food and Drug Administration (FDA) (21 CFR 182.20 and 21 CFR 172.515), it may be an appealing strategy to incorporate antihelmintic plant essential oil compounds in food and use the food as a convenient means for prevention of parasite infections. We have also demonstrated in this study that the two monoterpenoids could interact with a nematode TyrR, SER-2b. It is thus possible to use cells transfected with nematode TyrR, such as SER-2b, as a platform for high-throughput compound screening to develop more drugs against nematode parasites. Due to the potentially unique mode of action on the new target, the resulting antihelmintic compounds, especially when used together with drugs targeting other proteins or receptors, may play an important role in delaying or even preventing nematodes from developing drug resistance, a serious problem with many drugs currently used for helminthes controls [41–43].

Acknowledgements

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