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ORIGINAL RESEARCH

Ingredients of Vicks VapoRub inhibit rhinovirus-induced ATP release

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Abstract

Background: Over-the-counter therapies, such as Vicks VapoRub, are frequently used in the management of upper respiratory tract infection symptoms. Of these, acute cough is the most bothersome; however, the mechanisms involved have not been fully elucidated. The temperature-sensitive transient receptor potential (TRP) channels, including TRPA1, TRPV1, TRPM8 and TRPV4, are potential candidates. TRPV4 is also thought to be involved in cough through the TRPV4–ATP–P2X3 pathway. Here, we hypothesise that Vicks VapoRub ingredients (VVRIs) modulate the TRP cough channels.

Methods: Stably transfected HEK cells expressing TRP channels were challenged with VVRIs, individually or in combination, and the agonist and antagonist effects were measured using calcium signalling responses. In addition, rhinovirus serotype-16 (RV16)-infected A549 airway epithelial cells were pre-incubated with individual or combinations of VVRIs prior to hypotonic challenge and extracellular ATP release analysis.

Results: Calcium signalling reconfirmed some previously defined activation of TRP channels by specific VVRIs. The combined VVRIs containing menthol, camphor and eucalyptus oil activated TRPV1, TRPV4, TRPM8 and untransfected wild-type HEK293 cells. However, pre-incubation with VVRIs did not significantly inhibit any of the channels compared with the standard agonist responses. Pre-incubation of RV16-infected A549 cells with individual or combined VVRIs, except menthol, resulted in a 0.45–0.55-fold reduction in total ATP release following hypotonic stimulation, compared with infected cells not treated with VVRIs.

Conclusion: These findings suggest that some VVRIs may reduce symptoms associated with upper respiratory tract infection by modulating specific TRP receptors and by reducing RV16-induced ATP release.

Keywords: adenosine triphosphate, camphor, cough, eucalyptus, menthol, turpentine, upper respiratory tract infections.

Citation

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Introduction

Utilized by millions of individuals globally, Vicks VapoRub* has been a household name for over a century, being advocated for its use in managing the symptoms associated with mild upper respiratory tract infections (URTIs).[1–3] The main constituents of Vicks VapoRub are menthol, camphor, eucalyptus oil and turpentine oil in a petrolatum base.[4–7] It is worth noting that menthol, camphor and eucalyptus oil also form the main ingredients in numerous other cough and cold remedies and have therefore been the focus of a number of studies.[8–10] Nevertheless, the molecular roles of these Vicks VapoRub ingredients (VVRIs) in alleviating the symptoms of cough and cold are not fully understood.

The wide range of cough and cold remedies available on the market is not surprising owing to the annual impact of URTIs. In excess of 17 billion cases of mild-to-moderate URTIs are estimated to occur on an annual basis, having a significant impact on the global economy due to lost

* Vicks VapoRub is a registered trademark of P&G Manufacturing GmbH, Procter-&-Gamble Str. 1, 64521 Groß-Gerau - Germany

productivity, healthcare costs and exacerbations of existing respiratory diseases.[11–13] URTIs may be caused by a myriad of viruses; however, some 50% of mild URTIs are caused by human rhinovirus (hRV), with typical symptoms including nasal congestion, excess mucus production, sore throat and headaches. The most bothersome symptom reported during an URTI infection is cough.[14–18] As yet, the mechanisms behind a viral cough have not been fully elucidated.[19] Furthermore, no effective treatment exists to relieve the acute cough often experienced during URTIs.[19–22] Whilst the mechanism of a viral cough is unclear, ATP may be a crucial mediator as has been shown in chronic cough.[23] Hypotonic stimulation of transient receptor potential (TRP) vanilloid-type 4 (TRPV4) cation channels in infected airway cells leads to the release of more ATP than from uninfected cells.[24] Further exploration of alternative treatments, which could help reduce symptoms of a mild URTI, would be beneficial especially given the socio-economic impact of the large number of infections on an annual basis.

Given the multitude of viruses causing URTIs and, therefore, cough and cold symptoms, the targeting of therapeutic strategies is challenging. Consequently, amelioration of symptoms has been the main focus of research. Most notably, modulation of nociceptors, particularly of TRP channels, has been advocated. The upregulation of TRP channels in cough and airway hypersensitivity have long been of interest, with those most noticeably involved being TRPA1 (ankyrin 1), TRPM8 (melastatin 8), TRPV1 (vanilloid 1) and TRPV4.[25] This upregulation occurs when the TRP receptors are stimulated, and their activation results in the generation of an action potential in the airway sensory neurons.[26] Although TRP channels share structural similarities and mechanisms of action,[27] they differ in terms of the stimuli that activate them and include pH, irritants, osmolarity, mechanical stretch and temperature.[28] Although cough is the most bothersome of symptoms, congestion and aching muscles also feature frequently and are thus worth targeting.[29] Relief of perceived nasal congestion is provided through the activation of TRPM8 by both menthol and eucalyptus oil by the generation of a cooling sensation in the nasal passages and may also provide an antitussive effect.[9,30–35] It has been suggested that an analgesic effect may be created through the bimodal action of menthol, activating TRPA1 at low concentrations and inhibition at high concentrations. Inhibition of TRPA1 may also occur through the 1,8-cineole component of eucalyptus oil.[33,36–38] Although the modulation of TRP channels by a number of the ingredients found in Vicks VapoRub have previously been studied, there are a number of gaps within the research.[29] In particular, research relating to the combination of ingredients found in Vicks VapoRub is less extensive. The manner in which Vicks VapoRub may interact with TRP channels during a rhinovirus infection is also lacking. Here, we aim to identify potentially new interactions and reconfirm existing ones between VVRIs and the TRP receptors involved in the cough response, namely TRPA1, TRPM8, TRPV1 and TRPV4, using calcium signalling. In addition, we aim to identify whether extracellular ATP release is changed in response to the application of Vicks VapoRub in A549 alveolar epithelial cells with and without the presence of a rhinovirus serotype-16 (RV16) infection.

Materials and methods

Cells

A549, HeLa Ohio (Public Health England), HEK293 and HEK293 cells stably transfected with TRPA1, TRPM8, TRPV1, TRPV4 and TRPA1V1 (subsequently referred to as HEK TRPA1, etc.) were grown in complete growth media (DMEM) supplemented with 10% fetal calf serum, 50 U mL^{-1} penicillin, 50 µg mL^{-1} streptomycin, and 2 mM L-glutamine; 2.5 µg mL^{-1} G418 was added to all transfected HEK293 cells. Cells were grown at 37°C and 5% CO_2. A549 and HeLa Ohio were harvested in HEPES buffered saline solution EDTA (10 mM HEPES, 150 mM sodium chloride, 9.1 mM EDTA, pH 7.5). HEK293 cells transfected with TRPV1, TRPA1 and TRPM8 were produced in house, and TRP channel activity was characterized via RT-PCR, western blotting or functionally via calcium signalling as described previously.[39–42] For the HEK TRPV4 cell line, HEK293 cells were transfected with human TRPV4 (NM_021625) tagged open reading frame clone (Cambridge Bioscience) using FuGENE 6 (Promega) as per the manufacturer's instructions. Three rounds of single-cell cloning were carried out to select HEK cells permanently expressing TRPV4 and cells were screened by flow cytometry (using the green fluorescent protein tag) and calcium signalling assay (data not shown).

Vicks VapoRub ingredients

VVRIs (kindly provided by Procter & Gamble) were screened both individually and in combination. For individual screening of VVRIs, eucalyptus oil and turpentine oil were used at stock concentrations (4.54 M and 7.34 M, respectively). Menthol and camphor were diluted in absolute ethanol to a stock concentration of 5 M. For mixed screening, combinations of three or four VVRIs were utilized, and both combinations contained menthol, eucalyptus oil and camphor, with the addition of turpentine oil in four VVRIs. Mixed VVRIs were combined to make a stock concentration of 4.5 M. Subsequent dilutions of all VVRIs were made in calcium signalling buffer (1× HBSS, 4.2 mM sodium bicarbonate, 20 mM HEPES, 1.5 mM calcium chloride, pH 7.4) or DMEM. Equivalent vehicle concentrations of absolute ethanol in calcium signalling buffer or DMEM were utilized throughout as a control. Vehicle concentration for menthol and camphor were equivalent to 0.2% or 0.002% v/v ethanol, all other dilutions were equivalent to 0.02% or 0.0002% v/v ethanol for 10 mM and 100

μM, respectively. VVRIs were utilized throughout the study as both agonists and antagonists. Agonist usage aimed to identify whether the VVRIs cause any immediate activation of the TRP receptors. Antagonist usage aimed to identify whether pre-incubation with the VVRIs or individual ingredient could prevent subsequent activation of the TRP receptor when challenged with either a specific TRP receptor agonist or 33% hypotonic solution.

Calcium signalling assay

Transfected HEK cells were seeded in black, flat, clear bottomed 96-well plates, coated with 50 μg mL^{-1} poly-d-lysine at a density of 5×10^4 and incubated for 16–24 hours at 37°C, 5% CO_2. Cells were loaded with 2 μM Fluo-4AM calcium probe in phenol red free DMEM and incubated for 1 hour (30 minutes at 37°C followed by 30 minutes at room temperature) in the dark. Cells were then washed, and calcium signalling buffer was added. Cells were stimulated with the antagonist or agonist, diluted in calcium signalling buffer and intracellular calcium flux was measured. Relative fluorescence was measured at 494 nm excitation wavelength and 525 nm emission wavelength on a FlexStation 3 Multi-mode Plate Reader (Molecular Devices) every second for a maximum of 600 seconds, depending on experiment conditions. As a positive control, cells were treated with 2 μM A23187 to achieve a measurement of maximal relative fluorescence, and data were presented as a percentage of this maximum response for agonists. The concentration effect curves were normalized by subtracting the response obtained by the vehicle control (ethanol). To demonstrate any antagonist effect, responses were presented as a percentage of the known TRP channel agonist. Subsequently, measurements were converted to fold change (where vehicle was equal to one) and presented against equivalent vehicle.

VVRI agonist effect using calcium signalling assay

Transfected HEK cell lines were stimulated with individual VVRIs, three VVRIs, four VVRIs, or equivalent vehicle at 100 μM or 10 mM concentrations. Given the variation in EC_{50} values calculated through dose–response curves of individual VVRIs, these concentrations were utilized to try to encompass this variation. Intracellular calcium flux measurements of agonist effect were taken immediately after application of the VVRI, every second for 180 seconds, prior to the addition of the calcium ionophore for 60 seconds and the response rate was calculated as previously outlined.

VVRI antagonist effect using calcium signalling assay

Transfected HEK cells were incubated with individual VVRIs, three VVRIs, four VVRIs, or equivalent vehicle at 100 μM or 10 mM concentrations for 300 seconds, during which time intracellular calcium flux measurements of agonist effect were taken every second. As with EC_{50} calculations, there was a large variation in the dose–response curves for IC_{50} values; thus, the concentrations used for antagonist screening were identical to the agonist screening to ensure consistency across the data sets. Subsequently, cells were challenged with the EC_{50} of a known TRP channel agonist for each HEK TRP cell line (TRPA1 – 49 μM cinnamaldehyde, TRPV4 – 1.2 nM GSK1016790A, TRPV1 – 660 nM capsaicin, TRPM8 – 4.8 μM WS-5) and calcium signalling buffer for HEK wild-type cells. Measurements were taken for 120 seconds, prior to the addition of the calcium ionophore for 60 seconds, and the response rate was calculated as previously outlined.

RV16 infection

A549 cells were seeded in 24-well plates at a density of 6.25×10^4 and incubated for 16–24 hours at 37°C, 5% CO_2. Cells were then incubated with RV16 (infected; multiplicity of infection, 1) or ultraviolet (UV) inactivated RV16 (sham infected; UV crosslinked for 30 minutes at 1200 μJ/cm^2), at an equivalent multiplicity of infection. Basal plates were also created using DMEM at the same volume as sham and RV16 infections. All treatment types were incubated at room temperature for 1 hour with gentle agitation. The cells were subsequently washed twice with PBS, replenished with fresh infection media (DMEM supplemented with 4% FCS, 1% L-glutamine) and incubated for 72 hours at 37°C, 5% CO_2 for later ATP analysis.

ATP determination assays

A549 cells previously infected with RV16, UV-inactivated RV16 and basal treatment were incubated with 10 mM concentrations of individual VVRIs, three VVRIs, four VVRIs, DMEM (basal) or vehicle for 15 minutes, followed by stimulation with 33% hypotonic solution (HBSS; 10 mM HEPES, pH 7.4 diluted by 33% with dH_2O). Supernatant samples were taken at 15-second intervals for 5 minutes, and ATP was measured using a commercially available ATP determination kit (Invitrogen Molecular Probes ATP Determination Kit) as per the manufacturer's instructions. Duplicate measurements of luminescence were made at 560 nm wavelength using a Tecan Infinite 200 Pro plate reader, background readings were deducted and mean values were plotted. Concentrations of ATP were quantified from luminescence measurements using a standard curve. ATP measurements were expressed as peak ATP release and area under the curve (AUC) for the entire sample duration. Subsequently, measurements were converted to fold-change (where basal cells, which were not treated with VVRIs, were equal to one) and presented against basal.

Statistical analysis

Data are presented as the mean of three independent experiments with errors reported as standard error of the mean (± S.E.M.). Data were checked for normality visually using a quantile–quantile (Q-Q) plot and statistically using a Shapiro–Wilk test. Where appropriate, data were analysed using either one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* analysis for multiple comparisons or two-way ANOVA followed by Tukey's *post hoc* analysis for multiple comparisons against a control. A p value of less than 0.05 was used as statistically significant; exact p values are stated where possible.

Results

Activation of TRP channels occurs with individual VVRIs

To determine whether individual components of Vicks VapoRub had any agonist activity against different TRP channels, stably transfected HEK TRP cell lines were challenged with half log concentrations between 30 µM and 30 mM of either menthol, camphor, eucalyptus oil or turpentine oil. Changes in intracellular calcium levels were detected, normalized to vehicle control and expressed as a percentage of the maximum response (evoked by A23187).

The dose–response curves generated indicate that a number of the VVRIs act as an agonist across the TRP channels tested (Figure 1). However, it is worth noting that the dose–response curves generated through calcium signalling did not reach a plateau at higher concentrations or produce a typical sigmoid curve. Thus, accurate EC_{50} values could not be calculated. HEK TRPA1 demonstrated an agonist response to menthol, eucalyptus oil and turpentine oil (Figure 1A). HEK TRPV4 demonstrated no obvious agonist responses to any of the VVRIs (Figure 1B). HEK TRPM8 demonstrated an agonist response to eucalyptus oil and turpentine oil (Figure 1C). HEK TRPV1 produced an agonist response to eucalyptus oil (Figure 1D). HEK TRPA1V1 produced a positive agonist response to all VVRIs, with the clearest response being to menthol and camphor (Figure 1E). This is potentially a reflection of the co-expression of both TRPA1 and TRPV1 channels, which are stimulated to a differing extent by the various VVRIs. HEK wild type does not naturally express any of the TRP channels studied; as such, the agonist response to eucalyptus oil is likely a consequence of some off-target responses (Figure 1 and Table 1).

Individual VVRIs have limited ability to inhibit activation of TRP channels

To determine whether individual components of Vicks VapoRub had any antagonist activity against different TRP channels, stably transfected HEK TRP cell lines were incubated with half log concentrations between 30 µM and 30 mM of either menthol, camphor, eucalyptus oil or turpentine oil for 300 seconds prior to challenging with EC_{50} concentrations of known TRP channel agonist for each HEK TRP cell line (TRPA1: 49 µM cinnamaldehyde, TRPV4: 1.2 nM GSK1016790A, TRPV1: 660 nM capsaicin, TRPM8: 4.8 µM WS-5, which had evoked 30%, 33%, 49% and 17% of A23187, respectively; data not shown) or calcium signalling buffer for HEK wild type cells for 120 seconds. Cells were then activated with calcium ionophore for 60 seconds and the responses were calculated as a percentage response to known TRP channel agonist. The concentration effect curves showed little inhibitory effect of the VVRIs against the TRP channels tested in this overexpression system (Supplementary Figure S1; available at: https://www.drugsincontext.com/wp-content/uploads/2023/10/dic.2023-3-2-Suppl.pdf) and IC_{50} values could not be calculated.

Combined VVRIs have varying agonist effects on TRP channels involved in cough

To investigate whether combinations of the VVRIs activated the TRP channels of interest, HEK TRP cell lines were challenged with either three VVRIs or four VVRIs at 10 mM or 100 µM concentrations, and these were compared with the individual VVRIs at the same concentration. Measurements were made via calcium signalling and presented as fold-change against equivalent agonist response following vehicle pre-treatment.

When data collected from agonist-induced calcium signalling with 100 µM concentrations of VVRIs were considered, there was no significant difference between the VVRIs and vehicle control, or between individual and mixed VVRIs for any cell line that expressed a specific TRP channel (Figure 2Ai–Ei). However, though HEK wild-type cells do not naturally express the TRP channels tested here, there was a 0.42-fold and 0.35-fold increase in response when cells were challenged with menthol and camphor, respectively, compared with turpentine oil (menthol p=0.0038; camphor p=0.0305) (Figure 2Fi).

When 10 mM concentrations of VVRIs were utilized, some differences in agonist response were observed between individual VVRIs and mixed VVRIs (Supplementary Figure S2). When HEK TRPA1 cells were challenged with eucalyptus oil and turpentine oil, there was a respective 1.90-fold and 2.07-fold increase in percentage response compared with equivalent vehicle (eucalyptus oil p<0.0001; turpentine oil p<0.0001) (Figure 2Aii). When HEK TRPV4 cells were challenged with three VVRIs, there was a 1.19–1.70-fold increase in response compared with equivalent vehicle and all individual VVRIs (vehicle

Figure 1. Agonist dose-response curves of individual Vicks VapoRub ingredients (VVRIs) effect on transfected HEK cell lines. HEK TRPA1 (A), HEK TRPV4 (B), HEK TRPM8 (C), HEK TRPV1 (D), HEK TRPA1V1 (E) and HEK wild type (F) stimulated with various concentrations of menthol, eucalyptus oil, camphor or turpentine oil. Responses measured as a percentage of maximum response to control, calcium ionophore A23187. All data presented as mean ± S.E.M. of three independent experiments (*n*=3).

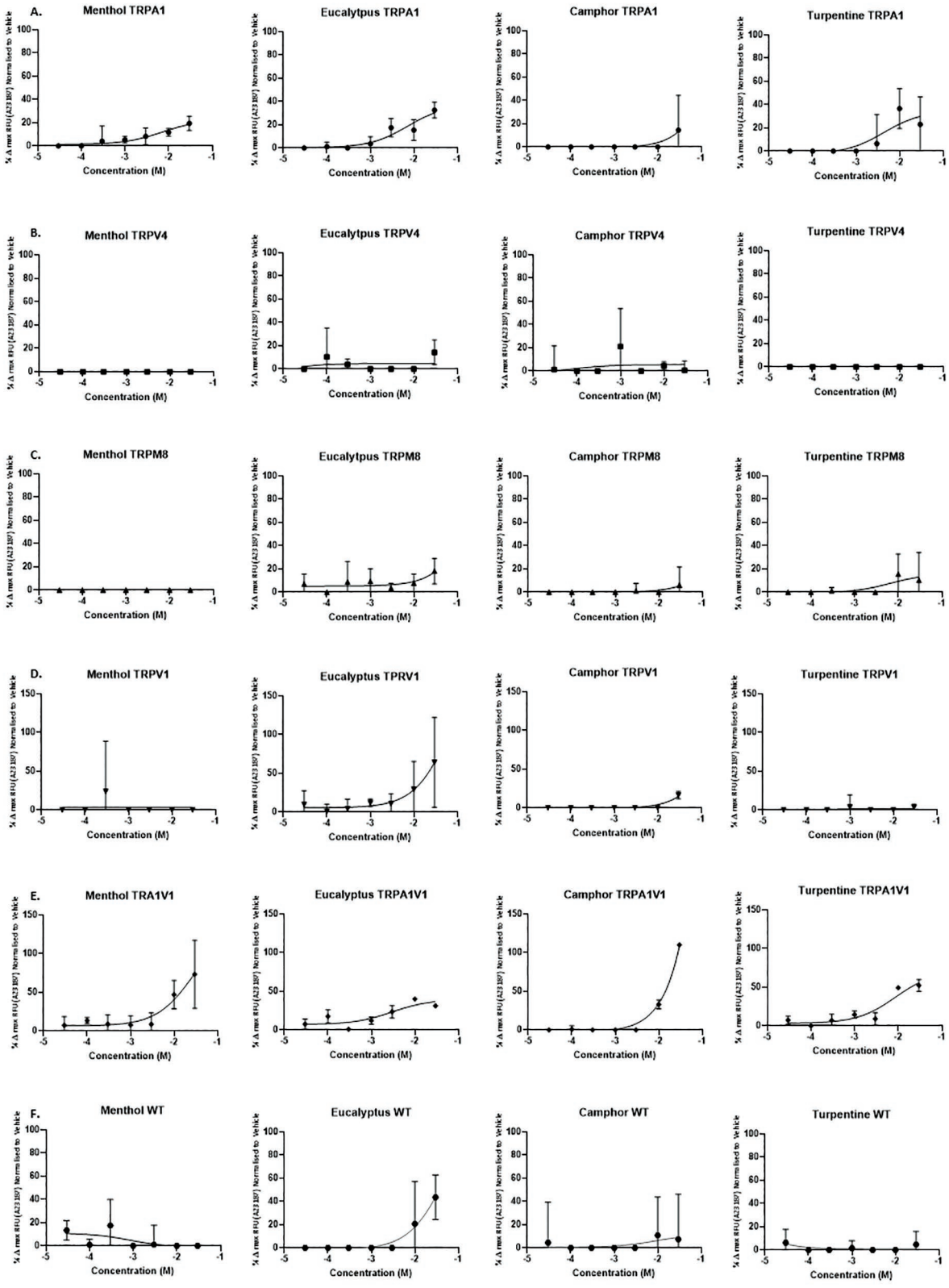


Table 1. Agonist and antagonist responses of individual Vicks VapoRub ingredients (VVRIs) on transfected HEK cell lines. Identifying tendencies of TRP receptors towards activation by specified VVRIs. The TRP receptors identified in bold are those that have not previously been shown to be activated by the specific VVRIs.

API	Channels showing significant agonist effect at 10 mM
Menthol	
Camphor	
Eucalyptus oil	TRPA1, **TRPV1**, TRPA1V1
Turpentine oil	**TRPA1**, **TRPA1V1**
3 VVRI	**TRPV4**, **TRPM8**, **TRPV1**
4 VVRI	**TRPM8**, **TRPV1**

p=0.0012; menthol p<0.0001; camphor p=0.0021; eucalyptus oil p=0.0114; turpentine oil p=0.0068) (Figure 2Bii). HEK TRPM8 cells challenged with three VVRIs resulted in a 1.49-fold change compared with equivalent vehicle (p=0.0012) and a 1.39–1.41-fold increase compared with menthol (p=0.0031) or eucalyptus oil (p=0.0026). Similarly, four VVRIs also resulted in a 1.45-fold change against equivalent vehicle (p=0.0018) and 1.35–1.37-fold change against menthol (p=0.0044) and eucalyptus oil (p=0.0037) (Figure 2Cii). When HEK TRPV1 cells were challenged with the VVRIs, there was a significant increase in response of the cells to three VVRIs compared with equivalent vehicle (1.94-fold change; p=0.00026), menthol (1.84-fold change; p=0.0005), camphor (1.50-fold change; p=0.0086) and turpentine oil (1.64-fold change; p=0.0028). Likewise, four VVRIs resulted in an increase in response compared with equivalent vehicle (1.57-fold change; p=0.0056), menthol (1.47-fold change; p=0.0106) and turpentine oil (1.27-fold change; p=0.0437). In addition, eucalyptus oil also caused an increase in response compared with equivalent vehicle (1.20-fold change; p=0.0056) and menthol (1.10-fold change; p=0.0124) (Figure 2Dii). HEK TRPA1V1 cells challenged with eucalyptus oil resulted in a 2.63–3.30-fold increase compared with equivalent vehicle and all other combinations of VVRIs except turpentine oil (all p<0.0001). Furthermore, turpentine oil also resulted in a 1.64-fold increase compared with equivalent vehicle (p=0.0075) and four VVRIs (p=0.0477) (Figure 2Eii). HEK wild-type cells do not naturally express any TRP channels studied here; however, some increases in response compared with equivalent vehicle were observed. Most noticeable is the increase in response when cells were challenged with three VVRIs compared with equivalent vehicle (2.33-fold change; p=0.0051), menthol (2.12-fold change; p=0.0134), camphor (1.96-fold change; p=0.0318) and turpentine oil (2.13-fold change; p=0.0125). In addition, there was also an increase in response when cells were challenged with eucalyptus oil compared with equivalent vehicle (1.42-fold change; p=0.0487) (Figure 2Fii). Slight non-significant increases were also seen in a number of VVRIs compared with equivalent vehicle control (Figure 2Aii–Fii).

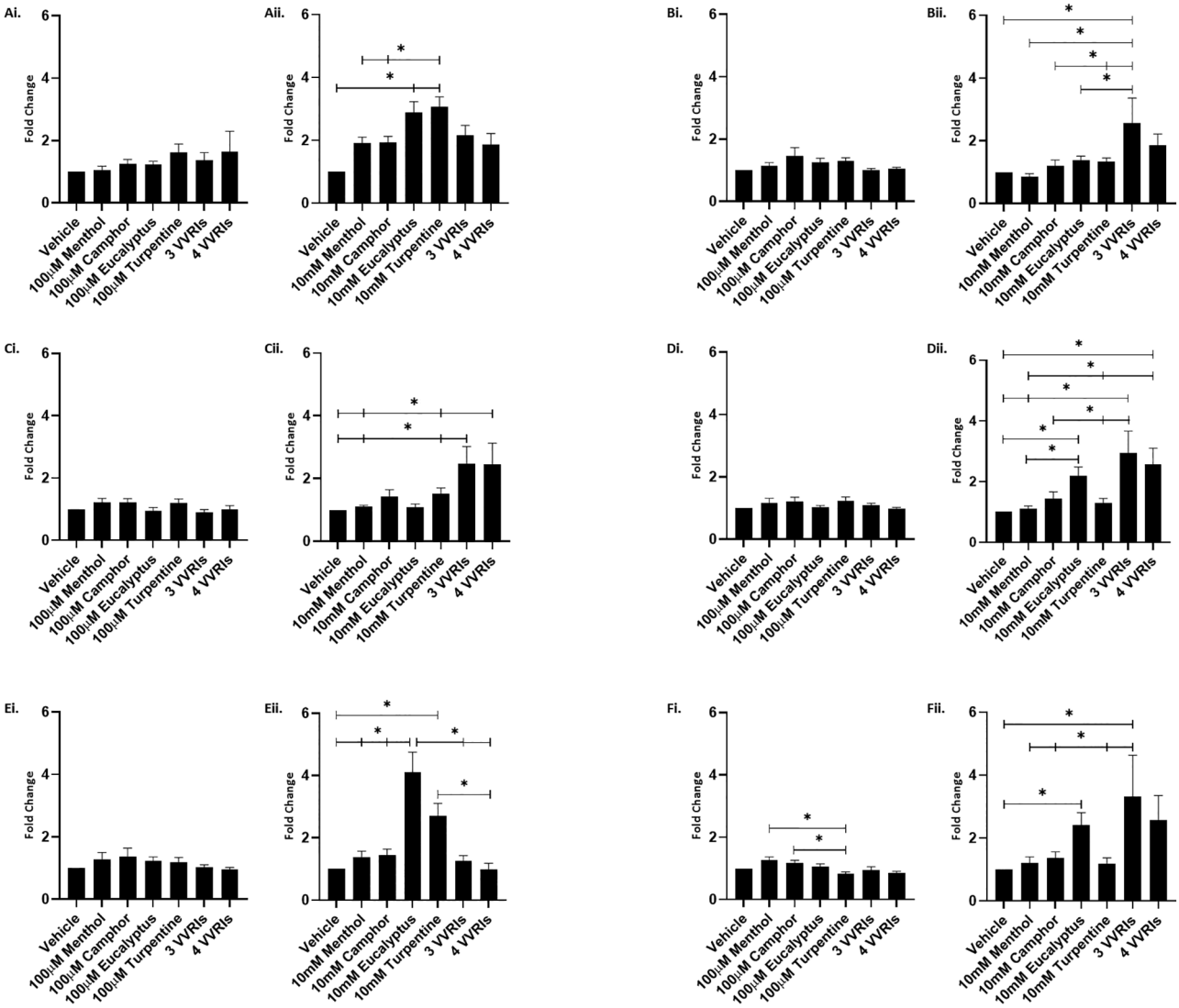
Combined VVRIs have varying antagonist effects on TRP channels involved in cough

To investigate whether combinations of the VVRIs inhibited the TRP channels of interest, HEK TRP cells were pre-incubated with either three VVRIs or four VVRIs at 10 mM or 100 µM concentrations and these were compared with the individual VVRIs at the same concentrations. Subsequently, cells were challenged with the EC_{50} of a known TRP channel agonist for each HEK TRP cell line (TRPA1 – 49 µM cinnamaldehyde, TRPV4 – 1.2 nM GSK1016790A, TRPV1 – 660 nM capsaicin, TRPM8 – 4.8 µM WS-5) and calcium signalling buffer for HEK wild type cells. Intracellular calcium responses to the known agonist were measured and responses following VVRI pre-incubation were expressed as fold-change compared with vehicle pre-treated agonist responses.

When 100 µM or 10 mM concentrations of VVRIs were utilized, no evidence of VVRIs acting as antagonists was observed in any cell line when compared with either vehicle or to individual VVRIs (Figure 3). The only significant differences were caused by an increase in response to cinnamaldehyde in TRPA1-expressing HEK cells following turpentine (100 µM) pre-incubation compared with menthol (0.86-fold, p=0.0111) and camphor (100 µM) (0.74-fold, p=0.0426). At 10 mM, pre-incubation with eucalyptus oil also caused a significant increase in response to the subsequent cinnamaldehyde activation in TRPA1 expressing HEK cells compared with all individual VVRIs (0.85–1.35-fold change).

The mechanistic assessment conducted and presented here demonstrates that VVRIs modulate a number of different TRP channels involved in the cough response. However, despite being of considerable interest in terms of the mechanistic action of Vick VapoRub on TRP channels, the stably transfected HEK cell lines utilized throughout this initial section of the study do not truly reflect cells involved in the common cold. Although HEK cells play a key role in our understanding of the interaction of the active ingredients of Vicks VapoRub with TRP channels, they are not permissible to rhinovirus, which is the leading cause of the common cold and the symptoms that Vicks VapoRub is often used to treat.

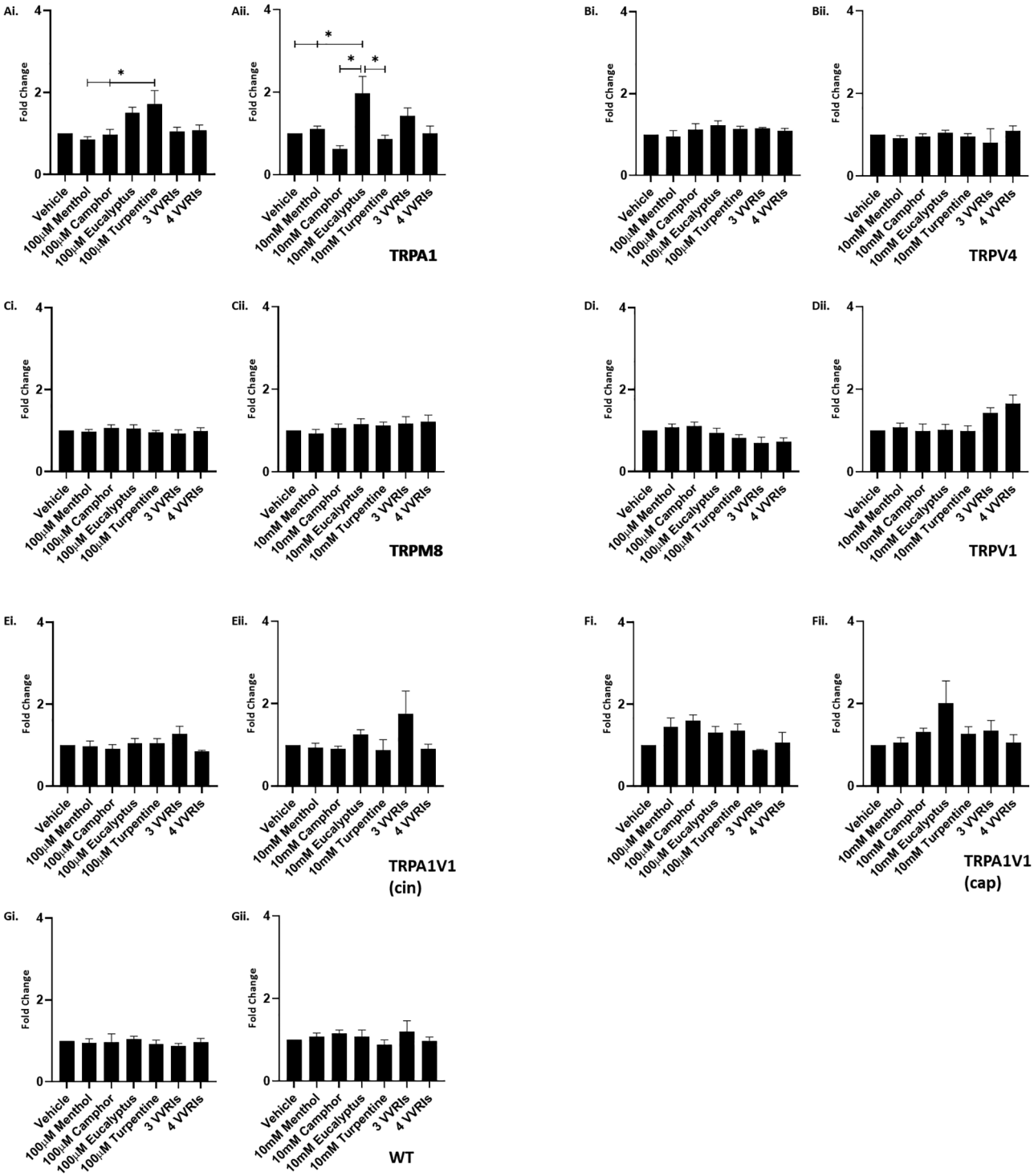
Figure 2. Agonist evoked responses of individual and mixed Vicks VapoRub ingredients (VVRIs) on transfected HEK cell lines measured using calcium signalling and presented as fold-change compared with vehicle control. HEK TRPA1 (A), HEK TRPV4 (B), HEK TRPM8 (C), HEK TRPV1 (D), HEK TRPA1V1 (E) and HEK wild type (F) stimulated with 100 µM (Ai–Fi) or 10 mM (Aii – Fii) concentrations of menthol, camphor, eucalyptus oil, turpentine oil, three VVRIs, four VVRIs or equivalent vehicle concentration. Responses measured as a percentage of maximum response to control, calcium ionophore A23187 and presented as fold-change against equivalent vehicle. All data presented as mean ± S.E.M. of six independent experiments (n=6). Statistical analysis was carried out using multiple comparison two-way mixed model ANOVA followed by Tukey's test. * p<0.05.



VVRIs have an antagonistic effect on the extracellular ATP released by rhinovirus-infected A549 airway epithelial cells

Given that Vicks VapoRub is frequently used to help alleviate symptoms of the common cold, A549 cells were infected with RV16, sham UV–inactivated RV16 or left untreated (uninfected) to determine whether there was any difference in the amount of extracellular ATP released by the cells in response to the use of the VVRIs as an antagonist. The TRPV4–ATP–P2X3 pathway is thought to play a role in the cough response.[20] Furthermore, increased ATP release has been observed in RV16-infected cells.[24] Changes to the levels of ATP released following TRPV4 activation can be measured with and without VVRI pre-treatment and RV16 infection. This can indicate the possible modulatory effects of these extracts on TRPV4 and rhinovirus-evoked cough. Following infection, cells were incubated with individual VVRIs, three VVRIs, four VVRIs, DMEM (basal) or equivalent vehicle at a concentration of 10 mM for 15 minutes, then challenged with

Figure 3. Antagonist evoked responses of individual and mixed Vicks VapoRub ingredients (VVRIs) on cells transfected with TRPA1, TRPV4, TRPM8, TRPV1 and TRPA1V1 measured using calcium signalling and presented as fold-change compared with vehicle control. HEK cell lines were incubated with 100 µM (Ai–Gi) or 10 mM (Aii–Gii) concentrations of menthol, eucalyptus oil, camphor, turpentine oil, three VVRIs, four VVRIs or equivalent vehicle concentration for 5 minutes prior to stimulation with the specific EC_{50} of the TRP channel under test; HEK TRPA1 – 49 µM cinnamaldehyde (A), HEK TRPV4 – 1.2 nM GSK1016790A (B), TRPM8 – 4.8 µM WS-5 (C), HEK TRPV1 – 660 nM capsaicin (D), HEK TRPA1V1 – 49 µM cinnamaldehyde (E) or – 660 nM capsaicin (F) and HEK wild type – calcium signalling buffer (G). The EC_{50} agonist responses evoked following pre-treatment with VVRIs are displayed and are expressed as fold change compared with vehicle pre-treated cell agonist responses. All data presented as mean ± S.E.M. of six independent experiments (n=6). Statistical analysis was carried out using multiple comparison two-way mixed model ANOVA followed by Tukey's test. *p<0.05.



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33% hypotonic solution. ATP measurements were analysed for peak ATP release and AUC for the entire sample duration. Although peak ATP highlights the maximum amount of ATP released by the cells during hypotonic challenge, it does not encompass any secondary smaller peaks or other changes in ATP release across the time course. To enable a clearer understanding of the total changes in ATP released over time, AUC was calculated for the entire 5-minute time course. This analysis aimed to provide a greater indication of whether the total amount of ATP release was changed overall when the cells were exposed to different conditions. These measurements were subsequently converted to fold-change (where basal is equal to 1) and presented against cells treated with no antagonist.

RV16-infected cells incubated with no antagonist (basal, treated with DMEM only) prior to stimulation with 33% hypotonic solution showed a 41.42 nM (±21.14 nM) increase in ATP release compared with uninfected cells (p=0.0086) (Figure 4A). When the antagonist effect on peak ATP was considered, there was minimal difference between the peak ATP released by any uninfected cells regardless of antagonist applied compared with uninfected/basal cells (cells not pre-treated with VVRIs), albeit with the exception of the mixture of four VVRIs, which resulted in a 0.60-fold reduction in ATP release (p=0.0025) (Figure 4B). When sham-infected cells were compared against sham-infected/basal cells (sham-infected cells not pre-treated with VVRIs), there was a reduction in the maximum amount of ATP released by cells treated with either individual or mixed VVRIs with a significant reduction seen with eucalyptus oil (0.63-fold reduction) and four VVRIs (0.54-fold reduction) (p=0.0199 and p=0.0054, respectively) pre-incubation (Figure 4C). A similar effect was also observed in infected cells compared with infected/basal cells (RV-infected cells not pre-treated with VVRIs). Camphor (0.64-fold reduction), turpentine oil (0.62-fold reduction) and four VVRIs (0.63-fold reduction) all resulted in a significant reduction in peak ATP release compared with infected/basal cells (p=0.0102 camphor; p=0.0444 turpentine oil and p=0.0017 4 VVRIs) (Figure 4D).

When AUC for the 5-minute sample period is considered, similar observations can be made regarding the effects of VVRIs on ATP release. As with peak ATP, when cells are incubated with no antagonist (basal, treated with DMEM only) prior to stimulation with 33% hypotonic solution, there was a significant increase in ATP released by infected (86%) and sham cells (83%) compared with uninfected (p=0.0052 and p=0.0074, respectively). However, there was no significant difference between sham-infected and infected cell ATP release (Figure 5A). Uninfected cells showed minimal difference in the total amount of ATP released by cells regardless of the antagonist utilized compared with uninfected/basal cells (cells not pre-treated with VVRIs). This is with the exception of the application of four VVRIs (0.51-fold reduction) which significantly reduced the total amount of ATP released by the cells (p=0.0018); in addition, as with peak ATP, eucalyptus oil also caused a reduction in total ATP (Figure 5B). In sham-infected cells, a significant reduction in ATP release was observed following incubation with camphor (0.52-fold reduction), eucalyptus oil (0.54-fold reduction) and four VVRIs (0.45-fold reduction) as an antagonist (p=0.0081 camphor; p=0.0068 eucalyptus oil and p=0.0045 four VVRIs) compared with sham-infected/basal cells (sham-infected cells not pre-treated with VVRIs) (Figure 5C). Similarly, in the RV-infected cells, there was a significant reduction (camphor 0.55, eucalyptus oil 0.47, turpentine oil 0.54, three VVRIs 0.45, four VVRIs 0.49-fold reduction) in total ATP released with any antagonist except menthol (p=0.0080 camphor; p=0.0232 eucalyptus oil; p=0.0089 turpentine oil; p=0.0044 three VVRIs and p=0.0024 four VVRIs) (Figure 5D).

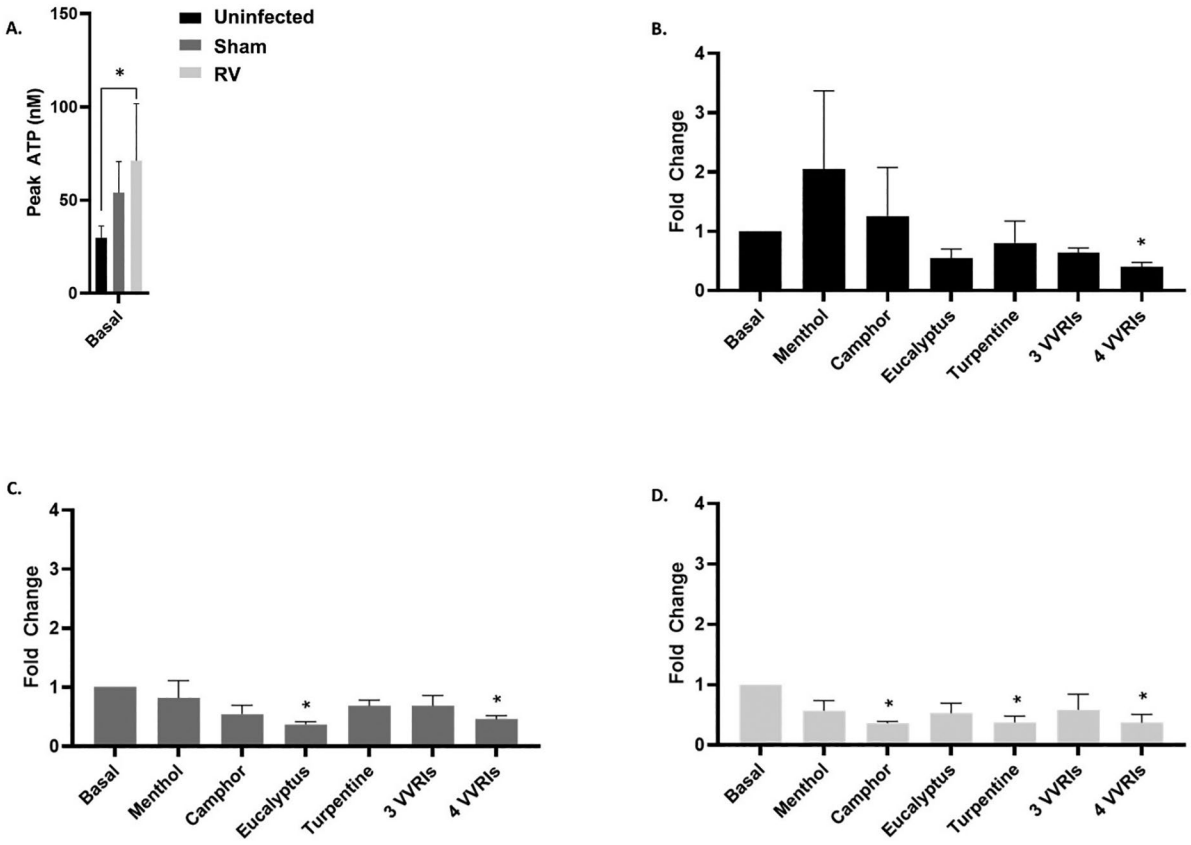
To determine whether there was any difference between the effects of the three and four VVRIs, peak ATP (Figure 6A) and total ATP release (Figure 6B) were compared. This analysis showed a slight trend toward a further reduction in both the total and peak ATP release in four VVRIs compared with three VVRIs; overall, there was no statistically significant difference between three and four VVRIs for any RV16 infection state.

In addition to the peak ATP release and AUC analysis, the ATP release traces were also plotted to enable any differences in ATP released at specific time points to be identified (Figure 7). The main differences were observed in the trace for menthol, three VVRIs and turpentine oil. In the menthol trace, there was an approximately 60 second delay in the ATP peak for both RV16 and sham infection compared with basal (Figure 7A). For turpentine oil, the peak for both RV16 and sham infection elongated compared with basal, lasting approximately 60 seconds rather than peaking at a single time point (Figure 7D). In contrast, the trace for three VVRIs had a double peak for both RV16, and sham-infected cells of approximately 45 seconds apart compared with the single peak observed in basal cells (Figure 7E).

Discussion

Vicks VapoRub is an over-the-counter cough and cold remedy utilized by consumers all over the globe. Despite its proven clinical efficacy, the molecular pharmacology of the product is not well defined.[4,43] Here, we show that VVRIs modulate the TRP cough receptors TRPA1, TRPV1, TRPM8 and TRPV4 overexpressed in HEK293 cells. Furthermore, preparations of three and four VVRIs

Figure 4. Peak ATP release presented as fold change (basal equal to 1) following hypotonic stimulation of RV16-infected cells. A549 cell line infected with RV16, UV-inactivated RV16 (sham) and uninfected incubated with menthol, camphor, eucalyptus oil, turpentine oil, three Vicks VapoRub ingredients (VVRIs), four VVRIs, or DMEM (basal) for 15 minutes, then post-treated with 33% hypotonic solution. Supernatants sampled at 15 second intervals for 5 minutes. Basal peak ATP release (A), peak ATP release as fold change compared against cells not treated with VVRIs (basal) for uninfected cells (B), sham-infected cells (C) and RV16-infected cells (D). All data presented as mean ± S.E.M. of three independent experiments (n=3). Statistical analysis was carried out using multiple comparison two-way ANOVA followed by Tukey's test. *p<0.05.

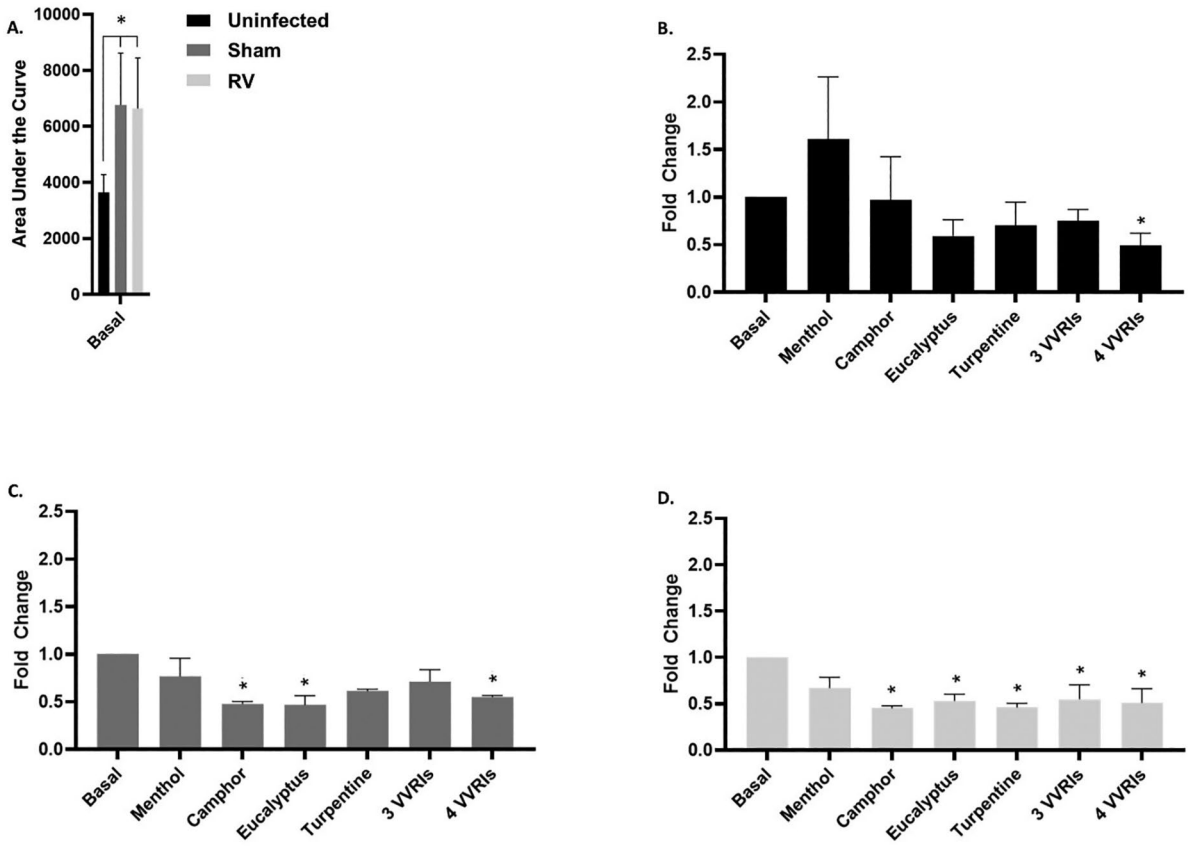


activated all TRP channels to varying extents; however, neither preparation showed any evidence of significant TRP channel inhibition. Whilst the aforementioned data showed interaction with various TRP channels, of greater interest and potentially of therapeutic benefit were the interactions that VVRIs could have with TRPV4 and the subsequent ATP release. Given that ATP release as part of the TRPV4–ATP–P2X3 pathway may play an important role in cough, including post viral cough,[24] the effect of VVRIs on ATP release during a hRV infection was therefore investigated. In rhinovirus-infected airway epithelial cells, all VVRIs either individually, with exception of menthol, or in combination resulted in reduced ATP release from cells. These results suggest that Vicks VapoRub interacts with the TRP channels linked to cough and has the potential to reduce a number of the symptoms experienced during a mild URTI.

Concentration effect curves showed that TRPA1 is activated by menthol, eucalyptus and turpentine oil and TRPV1 is activated by eucalyptus oil (Figure 1). When assessed individually at 10 mM, significant activation of TRPA1, TRPV1 and co-expressing TRPA1V1 channels was caused by eucalyptus oil (Table 1). Furthermore, turpentine oil appeared to have an agonistic effect on the TRPA1 and TRPA1V1 channels tested. Whilst the majority of VVRIs had some interaction with the TRP channels linked to cough, turpentine oil has not previously been studied in this manner. Thus, the observation that high concentrations appear to activate TRPA1 suggests that turpentine oil has only limited pharmacological activity in TRP channel modulation.

Although beneficial to developing a clearer picture of the interactions of the individual VVRIs with each TRP channel, the formulation of Vicks VapoRub is comprised

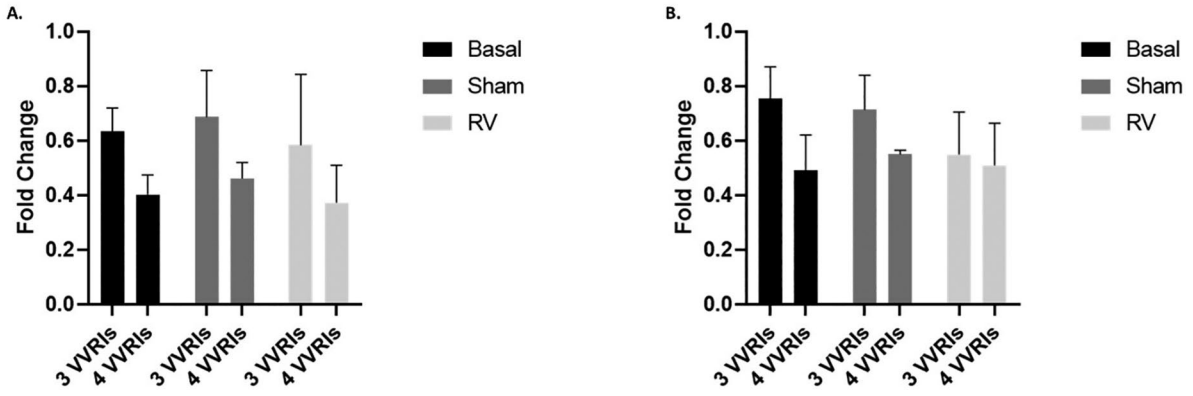
Figure 5. AUC analysis of total extracellular ATP release for full 5-minute time course presented as fold change (basal equal to 1) following hypotonic stimulation of RV16-infected cells. A549 cell line infected with RV16, UV-inactivated RV16 (sham) and basal treated incubated with menthol, camphor, eucalyptus oil, turpentine oil, three Vicks VapoRub ingredients (VVRIs), four VVRIs, or DMEM (basal) for 15 minutes, then post treated with 33% hypotonic solution. Supernatants sampled at 15 second intervals for 5 minutes. Basal AUC analysis of total extracellular ATP release (A). AUC analysis as fold change compared against cells not treated with VVRIs (basal) for uninfected cells (B), sham-infected cells (C) and RV16-infected cells (D). All data presented as mean ± S.E.M. of three independent experiments (*n*=3). Statistical analysis was carried out using multiple comparison two-way ANOVA followed by Tukey's test. **p*<0.05.



of a mixture of these VVRIs; as such, the impact these have on the extent of intracellular calcium flux as either agonists or antagonists is of interest. When considered as agonists, both three and four VVRIs at 10 mM significantly activated TRPV1 and TRPM8 compared with individual VVRIs or equivalent vehicle. Significant activation of TRPV4 and the untransfected wild type HEK293 cells was also observed following pre-incubation with three VVRIs. However, a similar effect is not observed when 100 µM concentrations are utilized. When the antagonist response is considered, no inhibitory effect is seen with either combination reducing calcium signalling response at 100 µM or 10 mM concentrations, compared with individual VVRIs.

Whilst an antagonist effect provides a more obvious way of reducing the impact of acute cough, an agonist effect could also be beneficial, whereby the activation of TRP channels could subsequently cause a desensitization effect, thus reducing the cough mechanism. The idea that a TRP channel can be desensitized following activation has been investigated previously. A study considering the role of TRPA1 in inflammation and pain in colitis demonstrated that activation by capsazepine results in a significant and sustained desensitization.[44] Similarly, TRPV1 desensitization has also been considered for use in pain management of irritable bowel syndrome, where stimulation of TRPV1 with N-palmitoyl-vanillamine showed a greater extent of desensitization compared with capsaicin; however, in both instances, treatment with the agonist prevented subsequent activation with the noxious chemical.[45] Furthermore, repeated application of menthol to HEK cells expressing TRPM8 showed a gradual desensitization to the stimulus.[46] Nevertheless,

Figure 6. Extracellular peak ATP release and AUC analysis of total extracellular ATP release for full 5-minute time course presented as fold change from basal (basal equal to 1) following hypotonic stimulation of RV16-infected cells. Comparative data for A549 cell line infected with RV16, UV-inactivated RV16 and basal-treated incubated with three Vicks VapoRub ingredients (VVRIs) or four VVRIs for 15 minutes, then post treated with 33% hypotonic solution. Supernatants sampled at 15 second intervals for 5 minutes. Extracellular peak ATP release as fold change (A) and AUC analysis of total extracellular ATP release as fold change (B). All data presented as mean ± S.E.M. of three independent experiments (*n*=3). Statistical analysis was carried out using multiple comparison two-way ANOVA followed by Tukey's test.

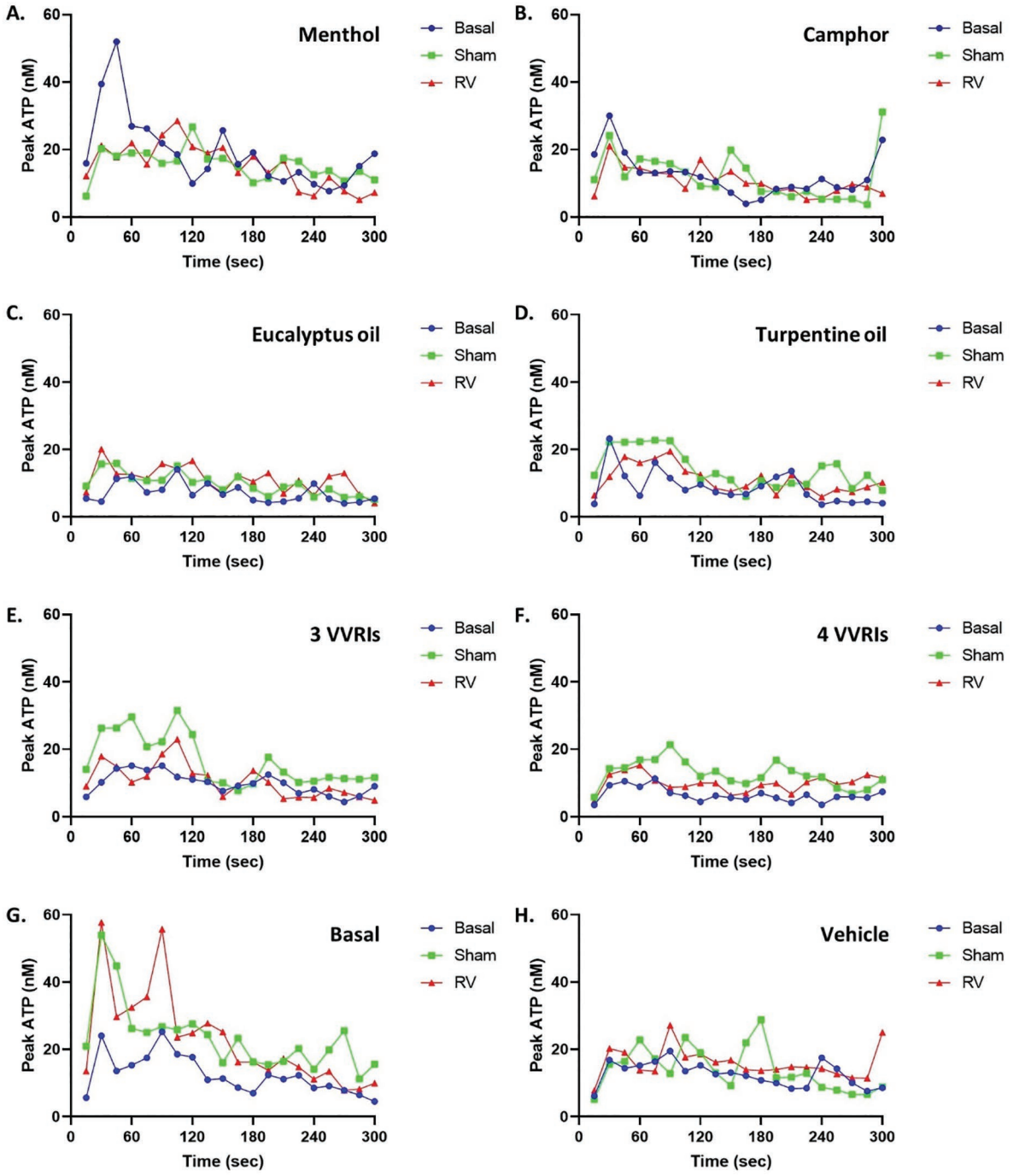


this evidence of desensitization effect is not directly related to cough. Therefore, exposure to Vicks VapoRub could cause desensitization of the channels to other stimuli.

Although the calcium signalling experiments did not show much interaction between the VVRIs and TRPV4, the role that this receptor plays in cough is currently an area of interest. The TRPV4–ATP–P2X3 pathway is a promising hypothesis in the development of our understanding of the mechanism involved in cough.[20] Thus, this potential interaction was further investigated through the measurement of ATP release. A549 airway epithelial cells were utilized for these experiments as they are permissible to rhinovirus infection without any cytopathic effect, thus enabling RV16 infections to be studied *in vitro*.[47] In addition, they endogenously express TRPV4 and pannexin 1 channels,[24] providing a mechanism for extracellular ATP release upon stimulation.[48] When no VVRIs were utilized, there was an increase in ATP released by RV16-infected A549 cells compared with uninfected cells in response to hypotonic stimulation of TRPV4 for both peak and total ATP, an observation which has been shown previously.[24] This increase in extracellular ATP release may be indicative of the RV16 infection resulting in airway epithelial cells becoming more sensitive to external stimulation.[24] Of note, the response observed in sham-infected cells when treated with no VVRIs was similar to that seen in RV16-infected cells. Inactivating a virus via UV treatment is a standard method for removing the infective element of the virus. However, this method does not remove viral particles or prevent virus binding to epithelial cells. Thus, in the case of both RV16 and UV-inactivated RV16, the viral particles bind to ICAM1 receptors on the epithelial cells causing the cells to respond in a similar manner in both cases.[47]

Although the increase in ATP release in response to RV16 infection is interesting, the changes in ATP release after treatment with the individual and mixed VVRIs provides further noteworthy responses. When RV16-infected A549 cells were treated with individual VVRIs, the resulting ATP release was significantly reduced for all VVRIs except for menthol. However, differences in peak and total ATP release were apparent depending on the VVRIs applied to RV16-infected cells. When peak ATP is considered, camphor and turpentine oil caused the most noticeable reduction in maximum ATP released. This was further reflected in the combined API results, whereby a greater reduction in ATP release was observed in four VVRIs compared with three VVRIs. This is potentially a reflection of the presence of turpentine oil in the former and its absence in the latter; thus, the combination of turpentine oil and camphor result in a more pronounced reduction in ATP release. Interestingly, when total ATP was considered, eucalyptus oil also resulted in a marked reduction in ATP release over the duration of the experiment. Thus, eucalyptus oil may have a lesser effect on the maximum amount of ATP released during an RV16 infection, instead causing a reduction over a prolonged period. This reduction in ATP release was also observed in sham-infected cells albeit to a lesser extent but was not observed when

Figure 7. Quantified ATP sampling traces of RV16-infected, UV treated (sham) or basal A549 cells treated with menthol (A), camphor (B), eucalyptus oil (C), turpentine oil (D), 3 VVRIs (menthol, camphor and eucalyptus oil) (E), 4 Vicks VapoRub ingredients (menthol, camphor, eucalyptus oil and turpentine oil) (F), basal (treated with DMEM instead of VVRIs) (G) and vehicle (0.04% v/v ethanol, only relevant for menthol and camphor) for 15 minutes prior to stimulation with 33% hypotonic solution. Graphically displayed as mean data points of three independent experiments (n=3) without error bars for visibility. Data processed and statistically analysed as seen in Figures 3–5.



uninfected cells were challenged. Interestingly, eucalyptus oil appears to have the most perceptible impact as an antagonist on TRPV4 activity in uninfected cells, causing a reduction in both peak and total ATP release compared with basal. Similarly, when mixtures of three and four VVRIs were applied to A549 cells, reductions in ATP release were also observed. Whilst both mixtures resulted in a reduced ATP release for infected cells, only four VVRIs caused a reduction in sham and uninfected cells compared with basal (no VVRI treatment). Furthermore, when the reduction in ATP as a response to the application of three or four VVRIs was compared directly,

there was no significant difference in the extent of ATP released, though four VVRIs showed a trend towards a greater reduction in ATP release. This latter observation suggests that the inclusion of turpentine oil in the formulation of Vicks VapoRub does not have a significant additive effect on the overall response to RV16 infections. Moreover, the limited change in the extent of ATP released when cells are treated with four rather than three VVRIs would suggest that the efficacy of Vicks VapoRub is a consequence of the presence of camphor and eucalyptus oil, with turpentine oil having little additional impact. Whilst the individual VVRIs have previously been shown to interact with a number of other TRP receptors linked to the cough response, with similar responses being reconfirmed through calcium signalling in this study, this was not the case with the TRPV4 receptor. The aforementioned ATP data suggest that a component of the TRPV4–pannexin 1 ATP release pathway, thought to be involved in cough,[20] is a potential target of Vicks VapoRub. However, the exact component involved needs further elucidation. Thus, application of Vicks VapoRub during a rhinovirus infection may limit the production of ATP on stimulation of TRPV4 and thus reduce activation of P2X3 and therefore the need to cough.

From a physiological perspective, the aforementioned TRP channels implicated in the cough reflex are situated in relatively close proximity to each other throughout the respiratory tract.[49] Thus, the topical application and subsequent inhalation of Vicks VapoRub is likely to result in the activation or inhibition of a range of TRP receptors within the airways. Consequently, when the observations of the calcium signalling and ATP assays are considered together, these suggest that Vicks VapoRub functions in such a way that the individual VVRIs interact with different TRP channels, resulting in a multifaceted approach to managing the multiple symptoms of the common cold. Whilst the pharmacodynamics of Vicks VapoRub has not been fully explored, the activity of the individual VVRIs is more widely recognized in relation to the TRP channels. Whilst not observed previously, ATP release from RV16-infected airway epithelial cells indicated that individual VVRIs (excluding menthol) can inhibit the release of ATP, thus potentially reducing cough response through the TRPV4–ATP–P2X3 pathway. The relatively distinct interaction of different VVRIs with different TRP channels supports the potential for multiple receptors to be targeted when Vicks VapoRub is applied during the course of a common cold, giving the greatest opportunity for the reduction of symptoms.

Study limitations

Although several novel observations have been discussed here, there are some limitations within the study, which need to be addressed. For example, the HEK293 cells do not represent cells that are found within the respiratory tract. These cells are frequently utilized as they can be readily and stably transfected, and can thus be used to express TRP channels.[40,50–52] However, these cells often overexpress the transfected receptor,[40] thus leading to the possibility that responses are different to those that would be seen in more physiologically relevant cell lines.[53] Nevertheless, these cells provide a good basis for the study of novel interactions, particularly in channels that would otherwise have low expression and may be difficult to study.[40] Additionally, the concentration–effect curves in this study were often variable, this is likely attributed to the ingredients being derived from plant extracts. Some of these plant extracts are likely to include a mixture of compounds with varying degrees of medicinal properties.[29] For example, eucalyptus oil consists of multiple different chemical compounds at different concentrations, which may have varying interactions with the TRP channels studied. Furthermore, the three and four VVRI mixtures utilized do not represent the exact concentration of each ingredient used in Vicks VapoRub. The lower concentrations used reflected the limited solubility of the VVRIs and a need to ensure vehicle concentrations were suitable for direct application to the cells. As such, the concentrations utilized in this experiment, though not strictly physiologically relevant, suggest that VVRIs, either individually or in combination, provide a potential therapeutic treatment for the acute cough associated with mild URTIs and are in line with results of previously documented clinical trials.[43]

Conclusion

Vicks VapoRub has been a household name for over a century; however, despite its proven clinical efficacy, the pharmacology of its action is not well established.[4,43] Nevertheless, it is widely used to relieve the symptoms experienced during a mild-to-moderate URTI. Here, we have shown through calcium signalling that the ingredients used in Vicks VapoRub interact with TRP channels involved in cough when used either individually or in combination. Furthermore, when airway epithelial cells are infected with hRV, there is an overall trend towards the reduction in ATP released when cells are treated with VVRIs, either individually or in combination. Except for menthol, all VVRIs reduced the total amount of ATP released by RV16-infected cells, while camphor and turpentine oil reduced the maximum amount of ATP released. However, the combination of three and four VVRIs does not further enhance any antagonistic effect observed with individual VVRIs. When considered together, these results suggest that individual but differing VVRIs act on specific TRP receptors to varying degrees, thus

maximizing the impact to reduce the multiple symptoms associated with an URTI infection. As such, these findings support the intended use of Vicks VapoRub, suggesting that the application of this treatment during an URTI may also provide a therapeutic effect and aid in the relief of the acute cough associated with mild URTIs.

Contributions: Conceptualization, AM and LS; Methodology, RS; Validation, RS, AM and LS; Formal analysis, RS; Investigation, RS and BA; Resources, LS; Data curation, RS; Writing – original draft preparation, RS; Writing – review and editing, RS, AM, BA and LS; Visualization, RS; Supervision, LS; Project administration, LS; Funding acquisition, AM and LS. All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

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