Structure-Activity Relationship of Capsaicin Analogs and Transient Receptor Potential Vanilloid 1-Mediated Human Lung Epithelial Cell Toxicity

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ABSTRACT

Activation of intracellular transient receptor potential vanilloid-1 (TRPV1) in human lung cells causes endoplasmic reticulum (ER) stress, increased expression of proapoptotic GADD153 (growth arrest- and DNA damage-inducible transcript 3), and cytotoxicity. However, in cells with low TRPV1 expression, cell death is not inhibited by TRPV1 antagonists, despite preventing GADD153 induction. In this study, chemical variants of the capsaicin analog nonivamide were synthesized and used to probe the relationship between TRPV1 receptor binding, ER calcium release, GADD153 expression, and cell death in TRPV1-overexpressing BEAS-2B, normal BEAS-2B, and primary normal human bronchial epithelial lung cells. Modification of the 3-methoxy-4-hydroxybenzylamide vanilloid ring pharmacophore of nonivamide reduced the potency of the analogs and rendered several analogs mildly inhibitory.

Correlation analysis of analog-induced calcium flux, GADD153 induction, and cytotoxicity revealed a direct relationship for all three endpoints in all three lung cell types for nonivamide and N-(3,4-dihydroxybenzyl)nonanamide. However, the N-(3,4-dihydroxybenzyl)nonanamide analog also produced cytotoxicity through redox cycling/reactive oxygen species formation, shown by inhibition of cell death by N-acetylcysteine. Molecular modeling of binding interactions between the analogs and TRPV1 agreed with data for reduced potency of the analogs, and only nonivamide was predicted to form a “productive” ligand-receptor complex. This study provides vital information on the molecular interactions of capsaicinoids with TRPV1 and substantiates TRPV1-mediated ER stress as a conserved mechanism of lung cell death by prototypical TRPV1 agonists.

Introduction

The capsaicin receptor, TRPV1, is an emerging pharmacological target with many hypothesized roles in mammalian physiology, pathology, and disease (Jia and Lee, 2007; Szallasi et al., 2007). Endogenous and exogenous noxious stimuli including temperature, acid, proinflammatory mediators, and chemical irritants activate TRPV1 (Caterina et al., 1997; Tominaga et al., 1998; Szallasi et al., 2007). The prototypical agonist of TRPV1 is capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide), the pungent compound in chili peppers.

TRPV1 is expressed by sensory neurons and non-neuronal cells. Activation of TRPV1-expressing nerves in the airways elicits cough, dyspnea, and neurogenic inflammation (Geppetti et al., 2006, 2008; Jia and Lee, 2007; Nassini et al., 2010). In lung epithelial cells, coupling of TRPV1 and cellular
responses is less understood, but studies suggest that TRPV1 regulates host defenses through the regulation of proinflammatory cytokine and chemokine genes (Veronesi et al., 1999a, b; Oortgiesen et al., 2000; Veronesi and Oortgiesen, 2001; Reilly et al., 2003b, 2005).

Rats exposed to capsaicin aerosols exhibited cell loss in the conducting airways and bronchial and alveolar cell damage (Reilly et al., 2003b). In cultured human lung cells, capsaicin and other TRPV1 agonists activated TRPV1 and promoted both time- and dose-dependent cytokine release, ER stress, and cell death (Reilly et al., 2003b, 2005; Thomas et al., 2007). Increased expression of interleukin-6 and interleukin-8 were associated with activation of cell surface TRPV1, and these molecules contribute to pulmonary inflammation and neutrophilia in the lung (Reilly et al., 2005; Johansen et al., 2006, 2007). Activation of ER-associated/ or intracellular TRPV1 causes ER stress, activation of eukaryotic translation initiation factor 2 alpha kinase, induction of GADD153 mRNA and protein, and cell death (Reilly et al., 2003b, 2005; Johansen et al., 2006; Thomas et al., 2007). This latter process is proposed to be the mechanism by which capsaicin damages epithelial cells in the respiratory tract.

Previous studies of TRPV1 agonist toxicity in lung cells demonstrated that blocking TRPV1 with the cell-permeable antagonist N-(4-tert-butylbenzyl)-N’-(1-[3-fluoro-4-(methylsulfonyl)amino]-phenyl)ethylthiourea (LJO-328) ameliorated ER stress and increases in GADD153 expression, which correlated with cell death (Reilly et al., 2003b, 2005; Thomas et al., 2007). This study also provided guidance for analogs that should be prepared and evaluated in the current study.

Materials and Methods

**Chemicals.** Nonivamide (N-vanillylvanillamide or N-(4-hydroxy-3-methoxybenzyl)vanillamide), ionomycin, N-acetyllysteine (NAC), dimethyl sulfoxide, 3-hydroxy-4-methoxybenzylamine-HCl, 3,4-dihydroxybenzylamine-HBr, 3,4-dimethoxybenzylamine 4-trifluromethylbenzylamine, 3-methoxybenzylamine, nonanoyl chloride, glutathione, and sulfinpyrazone were purchased from Sigma-Aldrich (St. Louis, MO). 4-Hydroxybenzylamine was from Matrix Scientific (Columbia, SC). Nonivamide and capsaicin have indistinguishable toxicity profiles in the human lung cells used in this study, thus nonivamide was used as the prototype TRPV1 agonist because of its facile synthesis and availability of reagents to prepare variants. LJO-328 (Reilly et al., 2005) was synthesized by J.L. PCR primers for TRPV1, GADD153, and β2 macroglobulin (β2M) were synthesized at the University of Utah Core Facilities, and cell culture media were purchased from Invitrogen (Carlsbad, CA) unless otherwise stated. All other reagents were purchased form established suppliers.

**TABLE 1**

<table>
<thead>
<tr>
<th>Analog Name</th>
<th>Abbreviation</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
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<tr>
<td>N-(3-Methoxy-4-hydroxybenzyl)vanillamide</td>
<td>Nonivamide</td>
<td>OCH3</td>
<td>OH</td>
</tr>
<tr>
<td>N-Benzylvanillamide</td>
<td>Benzyl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>N-(3,4-Dimethoxybenzyl)vanillamide</td>
<td>3-MeO</td>
<td>OCH3</td>
<td>H</td>
</tr>
<tr>
<td>N-(3-Hydroxy-4-methoxybenzyl)vanillamide</td>
<td>3,4-DiMeO</td>
<td>OCH3</td>
<td>OCH3</td>
</tr>
<tr>
<td>N-(3,4-Dihydroxybenzyl)vanillamide</td>
<td>3-OH-4-MeO</td>
<td>OH</td>
<td>OCH3</td>
</tr>
<tr>
<td>N-(3-Methoxy-4-(nonamidomethyl)phenyl)</td>
<td>Sulfate</td>
<td>OCH3</td>
<td>SO4</td>
</tr>
<tr>
<td>N-(4-Trifluoromethylbenzyl)vanillamide</td>
<td>CF</td>
<td>H</td>
<td>CF2</td>
</tr>
<tr>
<td>N-(4-Hydroxybenzyl)vanillamide</td>
<td>4-OH</td>
<td>H</td>
<td>OH</td>
</tr>
</tbody>
</table>

**Fig. 1.** Annotated structure of nonivamide.
Synthesis of Capsaicinoid Analogs. All analogs, except the sulfate analog, were synthesized as described previously for N-benzylnonanamide (Thomas et al., 2007). Analogs containing a hydroxyl group were deacetylated by reacting with sodium methoxide. Product purity and structure were determined by high-performance liquid chromatography with UV detection at 230 nm, liquid chromatography/mass spectrometry, and 1H NMR. Detailed synthetic methods and NMR and mass spectrometry data of the analogs are provided as Supplemental Data Section 1.

Cell Culture. Immortalized human bronchial epithelial cells (BEAS-2B) were purchased from the American Type Culture Collection (Manassas, VA), and TRPV1 overexpressing BEAS-2B (TRPV1-OE) cells were generated as described previously (Reilly et al., 2003b). BEAS-2B and TRPV1-OE cells were cultured in LHC-9 media. NHBE cells were purchased from Lonza Walkersville, Inc. (Walkersville, MD) and cultured in bronchial epithelial cell growth medium. Culture flasks were coated with LHC basal media fortified with 30 μg/ml collagen, 10 μg/ml fibronectin, and 10 μg/ml bovine serum albumin. Cells were maintained between 30 and 90% maximum density and were subcultured every 2 to 4 days depending on cell growth rates.

Cytotoxicity/Dose-Response Assays. Cells were subcultured into 96-well plates, grown to ~90% confluence, and treated for 24 h at 37°C with increasing concentrations of chemical. Treatment solutions were prepared using LHC-9 media for BEAS-2B and TRPV1-OE cells and Opti-MEM I for NHBE cells. Treatment solutions contained <0.5% dimethyl sulfoxide. Cell viability was assessed using the Dозидо cell counting kit-8 (Dojindo Laboratories, Gaithersburg, MD).

Fluorometric Calcium Assays. TRPV1-OE cells were used to evaluate calcium flux as a consequence of TRPV1 activation. Cells were subcultured into 96-well plates and grown to approximately 90% maximum density. Cells were loaded with Fluor-4-acetoxymethyl ester (2.5 μM) (Invitrogen) for 60 min at room temperature (approximately 22°C), in the presence of 200 μM sulfipyrazone in LHC-9 media. Cells were washed twice and incubated at room temperature for an additional 20 min in sulfipyrazone containing media to permit methyl ester hydrolysis and activation of Fluor-4 in cells. Changes in cellular fluorescence were measured over 60 s using a NOVOSTar plate reader (BMG Labtech GmbH, Offenburg, Germany) with excitation at 485 nm and emission at 520 nm. Baseline readings were normalized, and the maximum fluorescence intensity was determined (n = 3 for each treatment). Ionomycin (10 μM) was also used to normalize responses.

Quantitative Real-Time PCR Analysis of Gene Expression. Cells were subcultured into six-well cell culture plates and grown to ~90% density. Cells were treated with TRPV1 agonists and antagonists for 4 h at 37°C. Total RNA was extracted from cells using the Invitrogen PureLink Micro-to-Midi Total RNA Purification kit, and 1 μg of the total RNA was transcribed into cDNA using Superscript III Master Mix from SA Biosciences (Frederick, MD) in 25-μl reaction volumes on a Chromo 4 Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA) using MJ Opticon Monitor 3 software (Bio-Rad Laboratories). The PCR program used consisted of an initial 10-min incubation at 95°C, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, then 72°C for 30 s. Experiments were performed in triplicate with a copy number standard curve for both the normalization gene (βM) and the genes of interest. Primer sequences were provided as supplemental data.

Statistical Analysis. Statistical analysis was performed using 95% confidence intervals as the limit for significance. All results are represented as mean ± S.E.M. unless otherwise indicated. For comparisons of two groups, an unpaired Student’s t test was used. To compare more than two groups, one-way ANOVA analysis with Newman-Keuls multiple comparison test for post hoc testing was used, as indicated in figure legends. Statistical analyses were performed using Prism version 4.02 for Windows (GraphPad Software Inc., San Diego, CA).

Results

Structural attributes of each analog are presented in Fig. 1 and Table 1. Synthetic methods and analytical data are provided as supplemental data.

TRPV1 mRNA was quantified using RT-qPCR in TRPV1-OE, BEAS-2B, and NHBE cells. TRPV1-OE cells expressed ~1.5 copies of TRPV1 mRNA/copy βM, which was ~10 times more than BEAS-2B cells (0.14 copies/copy βM), and ~45 times more than NHBE cells (0.03 copies/copy βM) (Fig. 2A). Correlation of the mean copy number for TRPV1 mRNA with cytotoxicity for the three cell types (Fig. 2B) produced a Pearson R value of −0.99; however, this was not significant with the limited number of samples (p = 0.1) (Supplemental Data Section 2).

The cytotoxicity of each analog was determined, and the LC50 values for each analog in TRPV1-OE, BEAS-2B, and NHBE cells are presented in Table 2. TRPV1-OE cells were most sensitive to nonivamide and N-(3,4-dihydroxybenzyl)nonanamide with LC50 values of 1 and 3.4, respectively. All other analogs produced LC50 values ~63 μM, with N-benzylnonanamide and N-(3,4-dimethoxybenzyl)nonanamide exhibiting the lowest tox-
signaling in cell death. All other analogs produced LC50 values sensitive to nonivamide, and 3-methoxy-4-(nonamidomethyl)phenyl sulfate with LC50 values of 115, 16, and 87 μM, respectively, but they were also sensitive to N-(3-methoxybenzyl)nonanamide at ~100 μM, similar to the TRPV1-OE cells (LC50 116 μM) and NHBE cells (LC50 106 μM), suggesting a reduction in TRPV1-mediated signaling in cell death. All other analogs produced LC50 values >164 μM, and N-benzylnonanamide and N-(3,4-dimethoxybenzyl)nonanamide were again the least toxic with LC50 values >250 μM. NHBE cells exhibited comparable sensitivity to the analogs as BEAS-2B cells, with the exception that they were markedly less sensitive to nonivamide and N-(3,4-dihydroxybenzyl)nonanamide. These results indicate that the abundance of TRPV1 expression is generally predictive of the relative sensitivity of lung cells to N-(3-hydroxy-4-methoxybenzyl)nonanamide, N-(3,4-dihydroxybenzyl)nonanamide, and N-(4-trifluoromethylbenzyl)nonanamide, as presented above for nonivamide, but not for the other analogs, indicating that TRPV1 binding and cytotoxicity were not directly coupled for the lowest potency analogs.

TRPV1 activation by the analogs was quantified using calcium flux (Fig. 3). TRPV1-OE cells treated with nonivamide and N-(3,4-dihydroxybenzyl)nonanamide exhibited dose-dependent, up to 25-fold, increases in cytosolic calcium content, with EC50 values of 1.4 and 10 μM, respectively. All other analogs were markedly less potent with maximum changes in fluorescence between 0 and 9 at 200 μM. The EC50 values for N-(3-hydroxy-4-methoxybenzyl)nonanamide, N-(3,4-dimethoxybenzyl)nonanamide, and 3-methoxy-4-(nonamidomethyl)phenyl sulfate were 70, 120, and 470 μM, respectively, whereas EC50 values for the other analogs could not be estimated because of little, if any, response. Comparisons of calcium flux at 2, 20, 100, and 200 μM for all of the analogs are presented in Table 3. Analogs exhibiting the greatest toxicity to cells (Table 2), having a rank order of TRPV1-OE > BEAS-2B ≥ NHBE, were also the most potent in the calcium flux assay, indicating a functional relationship for these two endpoints.

Correlation analysis between calcium flux elicited by the analogs and cytotoxicity in TRPV1-OE cells (Fig. 4 and Supplemental Data Section 3) demonstrated significant negative correlations with Pearson R values = −0.78, p = 0.01; −0.87, p = 0.003, and −0.75, p = 0.02 at low, intermediate, and high concentrations of analog, respectively. Correlation analyses for BEAS-2B and NHBE cells were not evaluated because calcium flux was not detectable.

Changes in GADD153 gene expression were quantified as a maker of ER stress and precursor to cytotoxicity (Thomas et al., 2007) (Table 4). Treatment of TRPV1-OE cells with 2 or 20 μM nonivamide for 4 h produced up to 7-fold increases in GADD153 mRNA, whereas 2 and 5-fold increases were observed for N-(3,4-dihydroxybenzyl)nonanamide. All other analogs failed to induce GADD153 at these concentrations, consistent with their low cytotoxicity and low potency (Figs. 3 and Table 3). However, at 100 μM, increases in GADD153 mRNA were observed with most analogs, again consistent with data in Tables 2 and 3, but the relationship between toxicity, potency, and GADD153 became insignificant (Fig. 5, E and F) because of an apparent cell-specific effect of the less potent analogs on GADD153 expression. BEAS-2B cells exhibited increased GADD153 mRNA after 4-h treatment with

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**TABLE 2**

<table>
<thead>
<tr>
<th>Analog</th>
<th>TRPV1-OE</th>
<th>BEAS-2B</th>
<th>NHBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonivamide</td>
<td>1.0 (0.7–1.4)</td>
<td>115 (104–127)</td>
<td>160 (156–164)</td>
</tr>
<tr>
<td>N-Benzylnonanamide</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;200</td>
</tr>
<tr>
<td>N-(3,4-Methoxybenzyl)nonanamide</td>
<td>116 (102–133)</td>
<td>101 (95–106)</td>
<td>106 (46–171)</td>
</tr>
<tr>
<td>N-(3,4-Dimethoxybenzyl)nonanamide</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;200</td>
</tr>
<tr>
<td>N-(3-Hydroxy-4-methoxybenzyl)nonanamide</td>
<td>75 (62–91)</td>
<td>217 (165–286)</td>
<td>181 (121–270)</td>
</tr>
<tr>
<td>N-(3,4-Dihydroxybenzyl)nonanamide</td>
<td>3.4 (2.6–4.3)</td>
<td>16 (13–19)</td>
<td>157 (121–203)</td>
</tr>
<tr>
<td>3-Methoxy-4-(nonamidomethyl)phenyl sulfate</td>
<td>65 (47–86)</td>
<td>87 (84–91)</td>
<td>58 (46–73)</td>
</tr>
<tr>
<td>N-(4-Trifluoromethylbenzyl)nonanamide</td>
<td>113 (85–150.0)</td>
<td>184 (145–234)</td>
<td>174 (109–277)</td>
</tr>
<tr>
<td>N-(4-Hydroxybenzyl)nonanamide</td>
<td>148 (122–180)</td>
<td>164 (135–198)</td>
<td>165 (113–242)</td>
</tr>
</tbody>
</table>

Data represent the calculated LC50 for each analog in each cell type with the 95% confidence interval (CI).
Fig. 3. Dose response curves for calcium flux in TRPV1-OE cells treated with capsaicinoid analogs: nonivamide (○), N-(3,4-dihydroxybenzyl)nonanamide (□), N-(3-hydroxy-4-methoxybenzyl)nonanamide (▲), 3-methoxy-4-(nonamidomethyl)phenyl sulfate (▽), N-benzylnonanamide (●), N-(3-methoxybenzyl)nonanamide (▲), N-(3,4-dimethoxybenzyl)nonanamide (●), and N-(4-hydroxybenzyl)nonanamide (◇). Statistically significant calcium flux, $p < 0.05$ by one-way ANOVA, was observed for nonivamide and the 3,4-DiOH analog at concentrations equal to and greater than as indicated by ◇. Data are represented as percentage of response relative to ionomycin ± S.E.M. ($n \geq 3$).

all analogs (200 μM), except N-benzylnonanamide and N-(3,4-dimethoxybenzyl)nonanamide. Significant increases in GADD153 mRNA were observed in NHBE cells with nonivamide (26-fold) and N-(3,4-dihydroxybenzyl)nonanamide (16-fold), similar to TRPV1-OE cells. Increases with other analogs were not statistically significant.

Correlation analysis between calcium flux and GADD153 expression and cytotoxicity for TRPV1-OE cells (Fig. 5, A–F and Supplemental Data Section C) demonstrated a relationship among all three phenomena. For calcium flux and GADD153 (Fig. 4, A, C, and E and supplemental data) Pearson $R$ values of $0.94, p = 0.0002$ (low dose); $0.95, p < 0.0001$ (intermediate dose); and $0.91, p = 0.001$ (high dose) were obtained. For GADD153 and cytotoxicity (Fig. 4, B, D, and F and supplemental data), Pearson $R$ values of $-0.87, p = 0.002$ (low dose); $-0.9, p = 0.001$ (intermediate dose); and $-0.5, p = 0.17$ (high dose) were obtained. For BEAS-2B and NHBE cells comparison of cell viability and GADD153 expression also demonstrated significant correlations with Pearson $R$ values of $-0.73, p = 0.02$ and $-0.81, p = 0.01$, respectively, when the sulfate analog was omitted from the NHBE data set (Fig. 5, G and H and supplemental data). Collectively these results demonstrate that TRPV1 activation/calcium flux, GADD153 expression, and cytotoxicity are coupled and stringent structural requirements exist for agonists to elicit these specific responses.

Table 2 shows that N-(3,4-dihydroxybenzyl)nonanamide exhibited similar toxicity in TRPV1-OE and BEAS-2B cells, but reduced toxicity in NHBE cells, suggesting a decrease in potency at TRPV1 and the potential involvement of parallel processes that lead to cytotoxicity. Cotreatment of TRPV1-OE cells with N-(3,4-dihydroxybenzyl)nonanamide and LJO-328 had little effect on toxicity, whereas cotreatment with NAC significantly reduced cytotoxicity, implicating redox cycling/ependinophore formation as a major factor for cytotoxicity for this analog (Fig. 6). However, similar protective results by NAC were not observed for either BEAS-2B or TRPV1-OE cells using a 25 μM dose of N-(3,4-dihydroxybenzyl)nonanamide (data not shown), indicating a limitation in NAC to attenuate toxicity, thus preventing us from completely ruling out a role for TRPV1 in cytotoxicity, as suggested by the shorter-term calcium flux and GADD153 expression studies. Likewise, 3-methoxy-4-(nonamidomethyl)phenyl sulfate, N-(3-methoxybenzyl)nonanamide, and N-(4-hydroxybenzyl)nonanamide exhibited equal cytotoxicity in the three cell types, suggesting that cell death was also partially independent of TRPV1. Bioactivation of N-(3-methoxybenzyl)nonanamide and N-(4-hydroxybenzyl)nonanamide by cytochrome P450 enzymes to either nonivamide or N-(3,4-dihydroxybenzyl)nonanamide via hydroxylation was investigated by preincubating and cotreating BEAS-2B and NHBE cells with the cytochrome P450 inhibitor 1-aminobenzotriazole. Inhibition of P450s by 1-aminobenzotriazole using the protocol of Weemaes and Yost (2010) had no effect on cytotoxicity, indicating that P450 metabolism was not a substantial factor (data not shown). The toxicity of 3-methoxy-4-(nonamidomethyl)phenyl sulfate was not investigated further, but production of nonivamide via sulfate ester hydrolysis was discounted because the cytotoxicity was not reduced by LJO-328 cotreatment in any cell type (data not shown).

The least toxic analogs were evaluated as protective agents for cytotoxicity via inhibition of TRPV1 by nonivamide. Pretreatment of TRPV1-OE cells with all analogs except N-(3,4-dihydroxybenzyl)nonanamide inhibited nonanamide-induced calcium flux, indicating competitive inhibition of TRPV1 (Fig. 7), but N-(3,4-dihydroxybenzyl)nonanamide cotreatment increased basal fluorescence consistent with the results in Fig. 3. The

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**Table 3**

<table>
<thead>
<tr>
<th>Analog</th>
<th>EC$_{50}$ (µM)</th>
<th>Calcium Flux (% Ionomycin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 µM</td>
<td>20 µM</td>
</tr>
<tr>
<td>Nonivamide</td>
<td>1.4</td>
<td>15 $\pm$ 3*</td>
</tr>
<tr>
<td>N-Benzylnonanamide</td>
<td>—</td>
<td>N.D.</td>
</tr>
<tr>
<td>N-(3-Methoxybenzyl)nonanamide</td>
<td>120</td>
<td>N.D.</td>
</tr>
<tr>
<td>N-(3,4-Dimethoxybenzyl)nonanamide</td>
<td>70</td>
<td>N.D.</td>
</tr>
<tr>
<td>N-(3,4-Dihydroxybenzyl)nonanamide</td>
<td>10.0</td>
<td>N.D.</td>
</tr>
<tr>
<td>3-Methoxy-4-(nonamidomethyl)phenyl sulfate</td>
<td>470</td>
<td>4 $\pm$ 1</td>
</tr>
<tr>
<td>N-(4-Trifluoromethylbenzyl)nonanamide</td>
<td>—</td>
<td>N.D.</td>
</tr>
<tr>
<td>N-(4-Hydroxybenzyl)nonanamide</td>
<td>—</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., no calcium flux (i.e., the deviation exceeded the mean value).

*EC$_{50}$ value could not be calculated.

* Statistical significance vs. untreated control using one-way ANOVA with Neuman-Kuels post-test ($p < 0.05$).
Materials and Methods

using a BMG Labtech NOVOstar plate reader as described under
/H9262 and 100 relative to untreated controls ( ). Analogs are arranged on the
maximum attainable calcium response ( ). IC50 values were 45, 62, 73, 78, 89, 120, and
concentrations of 50 and 100 μM, respectively. Protection (~15–
20% decrease) was also observed with N-(3-methoxybenzyl)nonanamide at 50 μM, but all other analogs failed to protect and produced additive toxicity at higher concentrations. These results were consistent with the results in Figs. 4 and 5 and Tables 2 and 3 in that these analogs failed to elicit significant calcium flux at 100 μM and were the least potent inducers of GADD153 and the least toxic.

Finally, molecular modeling was used to explore bonding interactions between capsaicinoids and TRPV1 as a basis for differential potency and toxicity. Representative poses for nonivamide (the prototype), N-(3-hydroxy-4-methoxybenzyl)nonanamide, N-(3,4-dihydroxybenzyl)nonanamide, N-(3-methoxybenzyl)nonanamide, N-(4-hydroxybenzyl)nonanamide, N-benzylnonanamide, N-(3,4-dimethoxybenzyl)nonanamide, and N-(4-trifluoromethylbenzyl)nonanamide are shown in Fig. 8. For nonivamide (Fig. 8A), the lowest energy pose (green) was most consistent with the model proposed by Gavva et al. (2004) and the presumed binding location suggested from site-directed mutagenesis studies. In this pose, the oxygen atom of the 4-OH moiety was predicted to hydrogen-bond with the hydrogen on the imidazole nitrogen of Trp549 (distance 1.9 Å), and the hydroxyl on the amide nitrogen of nonivamide was predicted to hydrogen-bond with the carboxylate group of Glu513 (2.3 Å). The alkyl terminus of nonivamide was localized ~2.4 Å from the phenol ring of Tyr511 where hydrophobic interactions probably occur and the 3-MeO group was localized in the hydrophobic “hole-A” pocket composed of Trp549, Met552, Leu553, and likely Phe516 and Leu521, as identified by Ryu et al. (2008). This “network” of bonding is consistent with a mechanism of activation for TRPV1 by capsaicinoids whereby the ligand acts as a “molecular tether” between TM3 and TM4 that induces a change in protein structure and/or protein dynamics that ultimately translate into pore opening. Analysis of N-(3-hydroxy-4-methoxybenzyl)nonanamide, N-(3-methoxybenzyl)nonanamide, N-(4-hydroxybenzyl)nonanamide, and N-benzylnonanamide failed to show similar intermolecular interactions, regardless of binding energy or occupancy. These results correspond with the low potency of these analogs in the functional assays. However, the lowest energy confirmation cluster for N-(3,4-dihydroxybenzyl)nonanamide (Fig. 8C) did exhibit similarities to nonivamide binding, in that the oxygen atom of the 3-OH moiety was predicted to be within hydrogen-bonding distance (1.9 Å) with the hydrogen of Trp549. However, hydrogen bonding was not predicted because of bond angle constraints presumably resulting from decreased ligand stabilization in the hydrophobic hole-A pocket caused by conversion of the 3-MeO moiety to the more hydrophilic 3-OH moiety. In addition, hydrogen bonding between the carboxylate of Glu513 and the amide hydrogen (distances of 3.9–5.5 Å) and the alkyl terminus with Tyr511 (~3.1–4 Å) were not predicted to be as favorable, because of an apparent “kinking” of the substrate molecule at the A/B region compared with nonivamide (Fig. 8C), supporting a decrease in potency for the diol in the functional assays. Similarly hydrogen-bonding interactions between the 4-OH and amide hydrogen of N-(4-hydroxybenzyl)nonanamide (Fig. 8E) with Trp549 and Glu513 were not observed, emphasizing the requirement for localization of the 3-MeO group in hole A for proper ligand binding and maximum potency. Finally, the most highly populated pose for N-(3,4-dihydroxybenzyl)nonanamide (Fig. 8C, purple) revealed the association of the ligand in an opposite orientation.

Fig. 4. Comparison of TRPV1-OE cell viability at 2.5 μM (A), 25 μM (B), and 100 μM (C) and analog-induced calcium flux at 2 μM (A), 20 μM (B), and 100 μM (C) analog concentration. Cell viability was assayed after 24-h treatment, and calcium flux was measured in real time over 1 min using a BMG Labtech NOVOstar plate reader as described under Materials and Methods. Data (mean ± S.E.M.) (left axis) are percentage of maximum attainable calcium response (Δ) and percentage of cell viability relative to untreated controls ( ). Analogs are arranged on the x-axis in order of lowest to highest cytotoxicity (left to right). Correlations between these two indices of TRPV1 activation are reported as the Pearson R value and two-tailed p value under Results and in the supplemental data.

IC50 values were 45, 62, 73, 78, 89, 120, and >200 μM for N-(3-methoxybenzyl)nonanamide, N-(4-hydroxybenzyl)nonanamide, N-(3-hydroxy-4-methoxybenzyl)nonanamide, N-benzylnonanamide, 3-methoxy-4-(nonamidomethyl)phenyl sulfone, N-(3,4-dimethoxybenzyl)nonanamide, and N-(4-trifluoromethylbenzyl)nonanamide, respectively. Cotreatment of TRPV1-OE cells with nonivamide and increasing concentrations of N-benzylnonanamide also demonstrated an attenuation of cell death from 53 ± 8% viability to 85 ± 10 and 78 ± 8% at
TRPV1-OE, BEAS-2B, and NHBE cells demon-
and the cytotoxicity of various nonivamide analogs
levels of TRPV1 expression. Quantification of TRPV1 mRNA
ictivity) in three different lung cell lines with vastly different
ways (TRPV1 expression, TRPV1-mediated calcium flux,
and that when this optimum configuration is present all of
the other inactive analogs, further supporting the
observed decrease in potency in the functional assays.

Discussion
Charakterization of cellular responses to TRPV1 agonists
is essential to understanding how TRPV1 regulates normal
and aberrant cellular processes. TRPV1 is expressed in many
cell types, and it is often difficult to translate results from one
model to another because of differences in its expression
level, subcellular distribution and coupling, physiological
regulation, and variability in antagonist efficacy, as observed
in previous studies of BEAS-2B and NHBE cells (Reilly et al.,
2003b, 2005; Thomas et al., 2007). Here, a set of capsaicinoid
analogos (Fig. 1 and Table 1) with minimal structural modi-
fications were developed to study the relationship between
TRPV1 binding, activation, and ER stress/GADD153 induc-
tion in lung cell death. The collective data demonstrate that
TRPV1 binding, activity, ER stress, and cytotoxicity highly
depend on the presence of a vanilloid ring pharmacophore
and that when this optimum configuration is present all of
these processes are coupled in lung cells.

The SAR for capsaicinoid analogs was evaluated in four
ways (TRPV1 expression, TRPV1-mediated calcium flux,
changes in proapoptotic GADD153 expression, and cytotox-
icity) in three different lung cell lines with vastly different
levels of TRPV1 expression. Quantification of TRPV1 mRNA
(Fig. 2) and the cytotoxicity of various nonivamide analogs
(Table 2) in TRPV1-OE, BEAS-2B, and NHBE cells demon-
strated that the relative sensitivity of lung cells to noniv-
amide, and to some extent N-(3,4-dihydroxybenzyl)nonana-
mide, were predicted by the level of TRPV1 expression.
However, for structural analogs with substantially reduced
potency at TRPV1, this relationship was less significant.
These results support a role for TRPV1 in lung cell death by
TRPV1 agonists, but also show that there is a concentration
(~200–250 μM) at which all analogs become cytotoxic inde-
dependent of TRPV1 expression or function.
This conclusion was further explored by comparing cal-
cium flux, GADD153 induction, and cytotoxicity for the
various analogs because these endpoints each represent a
step in a proposed mechanism of TRPV1-mediated cell
dearth for lung epithelial cells (Thomas et al., 2007). Anal-
ysis of TRPV1 activation using calcium flux demonstrated
that nonivamide and N-(3,4-dihydroxybenzyl)nonanamide
were most potent (Fig. 3 and Table 3). Calcium flux was
also detected with N-(3,4-dimethoxybenzyl)nonanamide,
N-(3-hydroxy-4-methoxybenzyl)nonanamide, and 3-me-
oxy-4-(nonamidomethyl)phenyl sulfate, but the magni-
tude was reduced and occurred only at concentrations >50
times the EC_{50} of nonivamide. These data agree with pre-
vious structure-activity relationship studies where the
3-MeO-4-OH vanilloid ring motif was shown to be essential
for maximum activation of TRPV1 by capsaicin-like mole-
cules (Walpole et al., 1993c). The link between calcium
flux, GADD153 expression, and cell death was subse-
quently evaluated using correlation analysis. Figures 4
and 5, A to E (and Supplemental Data Section 3) indicate
that analog-induced calcium flux was associated with in-
creases in GADD153 expression and cell death in
TRPV1-OE cells at 2 and 20 μM. However, at 100 μM, the
relationship for GADD153 and cytotoxicity became less
significant, but was still quite evident (Fig. 4, E and F).
Using analog potency data for GADD153 induction and cell
dearth, significant correlations also were found for
BEAS-2B and NHBE cells, but only when the sulfate an-
alog was omitted from the NHBE data set (Fig. 5, G and H
and Supplemental Data Section 3). These data confirm
that calcium flux, GADD153 induction, and cell death are
coupled in lung cells for TRPV1 ligands that optimally
bind TRPV1 and activate TRPV1, regardless of TRPV1
expression.
Parallel and/or alternative pathways of toxicity for
N-(3,4-dihydroxybenzyl)nonanamide, N-(4-hydroxybenzyl)
nonanamide, and N-(3-methoxybenzyl)nonanamide were
also investigated. Similar to nonivamide, N-(3,4-dihy-
droxybenzyl)nonanamide elicited significant calcium flux
in TRPV1-OE cells and induced GADD153 in all three cell
types. However, because the EC_{50} value for N-(3,4-
dihydroxybenzyl)nonanamide was ~5-fold that of noniv-
amide in TRPV1-OE cells and the LD_{50} for BEAS-2B cells
was lower than was predicted (Table 2), we hypothesized
that the diol analog toxicity was not fully TRPV1-
mediated. This notion was supported by results showing
that the TRPV1 antagonist LJO-328 did not attenuate cell
killing in TRPV1-OE cells, but that cell death was almost
completely inhibited by N-acetylcysteine (Fig. 6). It is
known that oxidative stress and binding of electrophiles to
cellular nucleophiles can trigger ER stress, eukaryotic
translation initiation factor 2α kinase 3 activation, and

<table>
<thead>
<tr>
<th>Analog</th>
<th>TRPV1-OE (2 μM)</th>
<th>TRPV1-OE (20 μM)</th>
<th>TRPV1-OE (100 μM)</th>
<th>BEAS-2B (200 μM)</th>
<th>NHBE (200 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonivamide</td>
<td>7 ± 3*</td>
<td>6 ± 3*</td>
<td>12 ± 2*</td>
<td>5 ± 2*</td>
<td>26 ± 20*</td>
</tr>
<tr>
<td>N-Benzylnonanamide</td>
<td>1.2 ± 0.8</td>
<td>1.3 ± 0.7</td>
<td>1.0 ± 0.5</td>
<td>2 ± 2</td>
<td>1.6 ± 1.0</td>
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<tr>
<td>N-(3-Methoxybenzyl)nonanamide</td>
<td>1.0 ± 0.9</td>
<td>1.1 ± 0.5</td>
<td>1.2 ± 0.6</td>
<td>5 ± 1*</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>N-(3,4-Dimethoxybenzyl)nonanamide</td>
<td>2 ± 1</td>
<td>1.1 ± 0.5</td>
<td>5 ± 2*</td>
<td>1.8 ± 0.6</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>N-(3-Hydroxy-4-methoxybenzyl)nonanamide</td>
<td>2 ± 1.0</td>
<td>2.1 ± 0.9</td>
<td>4 ± 1*</td>
<td>3 ± 1</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>N-(3,4-Dihydroxybenzyl)nonanamide</td>
<td>2.3 ± 0.8</td>
<td>5 ± 2</td>
<td>6 ± 1*</td>
<td>5 ± 2*</td>
<td>16 ± 8*</td>
</tr>
<tr>
<td>3-Methoxy-4-(nonamidomethyl)phenyl sulfate</td>
<td>1.5 ± 0.5</td>
<td>1.0 ± 0.5</td>
<td>2 ± 2</td>
<td>10 ± 4</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>N-(4-Trifluoromethylbenzyl)nonanamide</td>
<td>0.9 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>3 ± 1</td>
<td>6 ± 2*</td>
<td>10 ± 7</td>
</tr>
<tr>
<td>N-(4-Hydroxybenzyl)nonanamide</td>
<td>1.9 ± 0.4</td>
<td>2.0 ± 0.5</td>
<td>4.0 ± 0.8*</td>
<td>8 ± 1*</td>
<td>10 ± 7</td>
</tr>
</tbody>
</table>

* Statistical significance using one-way ANOVA with Neuman-Keuls post-test (p < 0.05).
GADD153 induction through activation of the Nrf2/Keap-1 pathway (Cullinan et al., 2003; Li and Kong, 2009; Copple et al., 2010). This provides a plausible explanation for the toxicity of this analog, but lack of attenuation of cell death at 25 μM in both TRPV1-OE and BEAS-2B cells by NAC suggests that TRPV1 must still be involved in some way, as suggested by the correlation studies. The substantially lower toxicity of the diol in NHBE cells also suggests a
1.25
2.25
1.75
2.50
2.00
1.50

Fig. 6. Assessment of TRPV1 dependence and non-TRPV1-mediated/redox stress-mediated toxicity for nonivamide and N-(3,4-dihydroxybenzyl)nonanamide in TRPV1-OE cells. *, statistically significant difference (p < 0.05) from untreated controls. #, statistical difference between treatment with analog alone or with LJO-328 or NAC (p < 0.05) using one-way ANOVA. Data are represented as the mean ± S.D. (n = 3).

Fig. 7. Dose response curves for inhibition of nonivamide- (2.5 µM) induced calcium flux in TRPV1-OE cells treated by capsaicinoid analogs: N-(3-hydroxy-4-methoxybenzyl)nonanamide (○), 3-methoxy-4-(nonamidomethyl)phenyl sulfate (●), N-benzyl)nonanamide (▲), N-(3-methoxybenzyl)nonanamide (▼), N-(3,4-dimethoxybenzyl)nonanamide (▲), N-(4-trifluoromethylbenzyl)nonanamide (■), and N-(4-hydroxybenzyl)nonanamide (▲). Data are normalized to nonivamide only and were fit using the sigmoidal dose response (variable slope) equation to estimate the IC_{50} value. *, concentration at which statistically significant inhibition, p < 0.05 by one-way ANOVA, was observed. Data are the mean and S.E.M. (n = 3).

Fig. 8. Images depicting predicted binding interactions between TRPV1 transmembrane helices 3 and 4 (residues Ser510–Ile569) (Gavva et al., 2004) and capsaicinoid analogs. Lowest energy (green) and most highly populated (purple) poses for nonivamide (A), N-(3-hydroxy-4-methoxybenzyl)nonanamide (B), N-(3,4-dihydroxybenzyl)nonanamide (C), N-(3-methoxybenzyl)nonanamide (D), N-(4-hydroxybenzyl)nonanamide (E), N-benzyl)nonanamide (F), N-(3,4-dimethoxybenzyl)nonanamide (G), and N-(4-trifluoromethylbenzyl)nonanamide (H) are shown. For nonivamide, the structure represents both the lowest energy and highest populated structure.

A fundamental difference in reactive oxygen species/electrophile detoxification capacity between immortalized and primary lung cells, which is important because N-(3,4-dihydroxybenzyl)nonanamide is efficiently produced by CYP1A2 and CYP2C19 (Reilly et al., 2003a; Reilly and Yost, 2006) and, thus, it may be difficult to modulate capsaicinoid toxicity in the intact lung using TRPV1 antagonists alone. Currently, an explanation for the toxicity of N-(4-hydroxybenzyl)nonanamide, 3-methoxy-4-(nonamidomethyl)phenyl sulfate, and N-(3-methoxybenzyl)nonanamide is not apparent, but metabolism by cytochromes P450 by hydroxylation to produce nonivamide or N-(3,4-dihydroxybenzyl)nonanamide does not seem to be operative.

Molecular modeling was used to evaluate TRPV1 binding by the different analogs and provide a theoretical explanation for the observed differences in agonist potency and toxicity. Using a homology model of TRPV1 transmembrane segments 3 and 4 (residues Ser510–Ile569) (Gavva et al., 2004), it was predicted that nonivamide formed a “productive” binding interaction with TRPV1 (Fig. 8A) wherein the 4-OH moiety of the vanilloid ring was hydrogen-bonded with Trp549, the carboxylate group of Glu513 hydrogen-bonded with the amide hydrogen, the 3-MeO group resided in a hydrophobic pocket composed of Trp549, Met552, Leu553, and probably Phe516 and Leu521, and the alkyl terminus interacted with Tyr511 via hydrophobic interactions. Unique from the model proposed by Jordt and Julius (2002), the alkyl terminus of nonivamide was predicted to interact with Tyr511, as shown by Gavva et al. (2004) using capsaicin. However, direct roles for Leu547 (in human TRPV1; Met in rat) and Thr550, were not indicated. Because most of the residues implicated in our model are conserved residues between both highly sensitive (humans and rats) and minimally sensitive (rabbits) species, our results suggest that changes of residues adjacent to these key residues alters the geometry of the binding site of TRPV1 such that binding is either favored or disfavored, rather than specific interactions of these residues with agonists, as discussed previously by...
Gavva et al. This conclusion is supported by showing that the propensity to form the “productive” pose of nonivamide was reduced for the other analogs. Nonivamide binding seemed to be highly driven by the combined interactions of the 4-OH and 3-MeO moieties and TM3/4 side chain residues because neither N-(3-methoxy-4-hydroxybenzyl)nonanamide (Fig. 8B), N-(3-methoxybenzyl)nonanamide (Fig. 8D), nor N-(4-hydroxybenzyl)nonanamide (Fig. 8E) formed comparable poses. Although the dihydroxy analog (Fig. 8C) was predicted to adopt a similar pose in TRPV1, hydrogen bonding between either the 3- or 4-OH groups with Thr549 was not predicted and the orientation of the A/B region was skewed relative to nonivamide, presumably caused by decreased anchoring by the 3-MeO group in the hole-A pocket, thus decreasing interaction with Glu513 and effectively reducing the length of the analog, such that interactions with Tyr511 were less favorable. However, if the alkyl terminus of the dihydroxy analog was lengthened, perhaps TRPV1 would be more potently activated, as suggested by studies with arvanil, linvanil, anandamide, and the arachidonic acid esters of acetaminophen (AM404) (Högstedt et al., 2005) and dopamine (Huang et al., 2002). The removal of the 3-MeO group in the TRPV1 antagonist capsazepine and constraint of the A/B region (Walpole et al., 1994), as well as increased potency of N-(2-benzyl-3-pivaloxypropyl) 2-[4-(methylsulfonyl)amo]nophenyl]propionamide antagonist analogs by the addition of a 4-tert-butyl group to the 2-phenylproionate moiety (Ryu et al., 2008), also support the importance of the 3-MeO and amide groups in TRPV1 activation by agonists. Finally, N-benzylnonanamide (Fig. 8F), as well as the other inactive analogs, were predicted to form essentially opposite binding orientations within TRPV1 compared with nonivamide, as predicted previously (Jordt and Julius, 2002), suggesting that such an orientation may be inhibitory, idea conjecture that was confirmed (Fig. 7) by the ability of N-benzylnonanamide and N-(3-methoxybenzyl)nonanamide to reduce cytotoxicity.

In conclusion, the data provide consistent evidence that TRPV1-mediated ER calcium release, ER stress, and GADD153 expression are major components of the chain of events that cause lung cell death by capsaicin and nonivamide, but that additional cellular processes such as biotransformation and detoxification modulate the disposition of less potnet analogs that must be considered when using these modified substances to probe TRPV1 function in different models. Finally, molecular modeling supports the functional data and previous SAR and site-directed mutagenesis studies and further emphasizes the critical relationship between agonist structure, TRPV1 function, ER stress, and cytotoxicity.

References


Authorship Contributions

Participated in research design: Thomas, Shahrokh, Sun, Yost, and Reilly.
Conducted experiments: Thomas, Ethirajan, Shahrokh, Sun, and Reilly.
Contributed new reagents or analytic tools: Ethirajan and Lee.
Performed data analysis: Thomas, Ethirajan, Shahrokh, Sun, and Reilly.
Wrote or contributed to the writing of the manuscript: Thomas, Ethirajan, Shahrokh, Sun, Cheatham, Yost, and Reilly.
Other: Lee, Cheatham, Yost, and Reilly obtained funding for the research.

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