Capsaicin Derivatives Containing Indole and Nitroindole for Improved Anti-Inflammatory Activity

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Abstract

In this investigation, capsaicin derivatives which containing indole or nitroindole in the tail region and a nitro group on the 4-hydroxybenzyl residue at the head region, were designed to mimic the benzyl residue at the terminal part of the daphnane diterpenoid moiety in resiniferatoxin (RTX). The novel capsaicin derivatives were readily synthesized using the peptide coupling reaction between heterocyclic acetic acid derivatives and benzylamine derivatives with moderate yield. Furthermore, novel capsaicin derivatives were evaluated for their ability to inhibit the production of tumor necrosis factor-alpha (TNF-α, one of the pro-inflammatory cytokines, by lipopolysaccharides (LPS)-stimulated human peripheral blood mononuclear cells (PBMCs) in which the transient receptor potential cation channel, subfamily V, member 1 (TRPV1) channel is present. It was found that capsaicin derivatives containing nitroindole in the tail region and a nitro group on the 4-hydroxybenzyl residue at the head region exhibited the highest activity for inhibition of TNF-α production, in comparison with capsaicin, giving reductions from 47.65%-51.95% and basis for this significant enhancement of the anti-inflammatory activity could potentially originate from the promotion of binding with TRPV1 on PBMCs through π-π stacking interactions provided from the nitrobenzylic and nitroindole residues at both ends.

Keywords: Capsaicin, TNF-α, TRPV1, anti-inflammatory activity

Introduction

Capsaicin, an abundant active compound found in chilis, was first isolated in 1876 by Thresh et al. (Thresh, 1876) and its structure was determined in 1919 by Nelson et al. (Nelson., 1919). The hot and pungent properties are the prominent characteristic of capsaicin and generally cause irritation and burning sensation of the exposed area. However, capsaicin remarkably possessed various interesting medical applications. For example, alleviation of neuropathic pain (Maihofner & Heskamp, 2013) (Anand & Bley, 2011), utilization as antitumor agent (Sharma et al., 2013), lowering the risk of degenerative diseases and obesity (Leung, 2014), treatment for cardiovascular and gastrointestinal diseases (Sharma et al., 2013). Additionally, capsaicin has received much attention especially for analgesic and inflammation treatment (Kim & Lee, 2014; Devesa et al., 2011; Kim et al., 2003; Winter et al., 1995), which involves the interaction between capsaicin and the transient receptor potential cation channel, subfamily V, member 1 (TRPV1)(Tsuji & Aono, 2012).

TPRV1, the protein found in humans and mammals, is a lipophilic tetramer. Each monomer protein is made from six transmembrane domains where domains S1, S2, S3, and S4 from the four monomer proteins are

arranged in four-folded symmetrical structure around ion-permeable channel. Furthermore, the S4-S5 linker located between domains S4 and S5 acts as the allosteric regulator of channel gating. Finally, domains S5 and S6 together with their linker, which forms the selectivity filter in other ion channels, form the ion channel and are involved in its functioning (Figure 1). This receptor, which acts as nociceptor and non-selective cation channel, can be activated by capsaicin, heat (43 °C), and acid (protons).

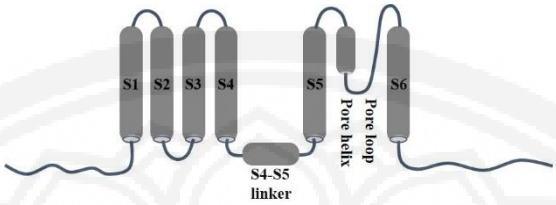

Figure 1 Representation of TRPV1 subunit structure, a lipophilic monomer, composed of S1 to S4 domain, S4-S5 linker and S5-pore-S6 domain. (Liao et al., 2013)

In 2013, the 3D-structure of ratTRPV1 (rTRPV1), which shares 85.7% similarity with humanTRPV1 (hTRPV1), was initially revealed using cryo-EM technique to observe the rTRPV1 structure with 3.4 Å resolution. This study has visualized important information about the interactions inside the rTRPV1 structure. For example, the S3 and S4 domains contain clusters of tyrosine and phenylalanine residues causing the S1-S4 domains to become relatively rigid, and leading to their conformation being unchanged during the channel activation (Liao et al., 2013). Even though the cryo-EM of rTRPV1 provided significant amount of detail for *de novo* atomic model construction, it still could not provide accurate information of the specific ligand-rTRPV1 interactions due to scattering around small ligands such as capsaicin or resiniferatoxin (RTX)(Figure 2).

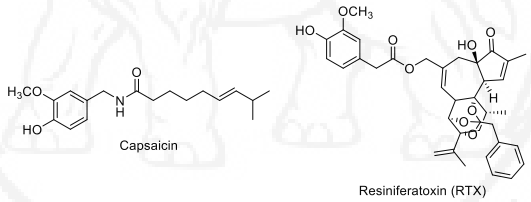

Figure 2 Chemical structures of vanilloid-containing compounds of natural origin capsaicin from; capsaicin and resiniferatoxin

Using combined structural computational approaches and functional analyses of cryo-EM data to rectify the ambiguous information, it was revealed that the vanillyl group of bound ligands (e.g. capsaicin, resiniferatoxin) is pointed downward towards the glutamic acid (E571) of the S4-S5 linker and is involved in hydrogen bonding with the protein (Yang et al., 2015). Furthermore, the amide bond showed a strong interaction with threonine (T511) and this was identified as the main contribution to capsaicin-rTRPV1 binding during the activation stage. Moreover, the long hydrocarbon (HC) chain was pointed upward establishing non-specific Van der Waal interactions. Interestingly, the HC chain of capsaicin was the first part that came in contact with rTRPV1 during the activation process before the amide bond and vanillyl residue forming the hydrogen bonding with rTRPV1 (Yang et al., 2015). The activation mechanism of rTRPV1 by capsaicin was proposed to involve dual hydrogen bonding formations. Once the hydrogen bonding between threonine (T511) and the amide bond of capsaicin is established, the hydroxyl group of vanillyl residue subsequently forms hydrogen bonding with glutamic acid (E571), which causes the S6 domain to move away from the S5 domain causing the ion-permeable gate to be opened.

By employing the interpolated elastic network modeling (iENM), the authors found out that the phenylalanine (F544) is the residue juxtaposed to the terminal part of the long HC chain of capsaicin. Additionally, it was mentioned that the sterically demanding daphnane diterpenoid at the terminal part of RTX significantly assisted its tight-binding during the gate opening. (Yang et al., 2015) Therefore, it was envisaged that the manipulation of the terminal region of the HC chain in capsaicin should confer improved binding interaction with TRPV1 and consequently enhancement of biological activities in areas such as anti-pain and anti-inflammatory.

In this work, we proposed the synthesis of novel capsaicin derivatives by installation indole and nitroindole moieties in the tail part of the HC chain. The effect of the length (4 or 5 atoms) between the amide bond and the heterocyclic residues was also to be investigated. The novel capsaicin derivatives were then to be evaluated for their anti-inflammatory activities using a TNF-α production assay, which can be considered and indirect but simple and quick method for preliminary screening to provide the evidence of TRPV1 interaction for potential novel ligands.

Methods and Materials

Materials and Apparatus

All reagents were analytical grade with > 97% purity and used without purification unless otherwise mentioned. For the syntheses, the following compounds were used: 4-hydroxybenzaldehyde, hydroxylamine hydrochloride, indole, 5-nitroindole, ethyl 5-bromovalerate, and ethyl 6-bromohexanoate (Sigma-Aldrich, Missouri, United States); 10% Palladium on activated carbon and 1-hydroxy benzotriazole (HOBt) (Acros organic, Geel, Belgium); *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC.HCl) (GL Biochem, Shanghai, China). For anti-inflammatory assay, the following compounds and materials were used: lipopolysaccharides from Escherichia coli (Sigma-Aldrich, Missouri, United States), RPMI 1640 without L-glutamine, trypan blue, fetal bovine serum (FBS) (Thermo Fisher Scientific, Massachusetts, United States); Lymphoprep, penicillin streptomycin (pen strep), and phosphate buffer saline (PBS) (Biotech and Scientific, Bangkok, Thailand); The human TNF-α Elisa kit (Advanced Medical Science, Bangkok, Thailand).

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker NMR spectrometer operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR in appropriate deuterated solvents. Chemical shifts (δ) were reported in part per million (ppm) relative to either tetramethylsiline (TMS) or the residual protonated solvent signal as a reference. Coupling constants (*J*) were reported in hertz (Hz). Peak multiplicities were reported in singlet (s), doublet (d), triplet (t), multiplet (m), doublet of doublets (dd) and triplet of doublets (td). FT-IR spectra were recorded on a Perkin-Elmer Model 1600 Series FT-IR spectrometer in the wavenumber range of 4000-400 cm^{-1}. Mass spectra were measured in positive ion mode with a static accelerating voltage of +20 kV on a Bruker Daltonics Microflex microTOF mass spectrometer using sodium formate for calibration.

Experiment

1. Synthesis of benzylamine derivatives 3 and 6

1.1 Synthesis of 4-hydroxy-3-methoxybenzylamine hydrochloride (3)

The mixture of vanillin (1) (1.00 eq.), hydroxylamine hydrochloride (1.20 eq.), and sodium acetate (1.20 eq.) were dissolved in water and stirred at 80 °C to produce oxime 2. After that, the oxime 2 (1.00 eq.) was reduced using 10% palladium on carbon (400 mg) in ethanolic hydrochloride solution.

The reaction mixture was filtered, dried under reduced pressure to obtain **3** in 91% yield. R_f = 0.31 (25% MeOH:CH_2Cl_2); melting point (mp): 225-226 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.21 (s, 1H), 8.44 (s, 3H), 7.19 (d, *J* = 2.0 Hz, 1H), 6.86 (dd, J_1 = 8.1, J_2 = 2.0 Hz, 1H), 6.79 (d, *J* = 8.1 Hz, 1H), 3.86 (s, 2H), 3.77 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 147.5, 146.8, 124.6, 121.7, 115.3, 113.9, 55.7, 41.9; FT-IR (ATR) cm^{-1}; 3163 (N-H stretching) and 1611 (N-H bending)

1.2 Synthesis of 4-hydroxy-3-nitrobenzylamine hydrochloride (**6**)

4-hydroxybenzaldehyde (**4**) (1.00 eq.) was dissolved in sulfuric acid and cooled in an ice bath. The mixture of ice-cold nitrating agent (Icke et al., 2003) 0.43 mL was slowly added. After that, the reaction mixture was stirred at room temperature. The mixture was poured into ice and stirred until yellow solid was precipitated out, then filtered, washed, and then crystallized from ethanol to obtain **5**. Next, compound **5** (1.00 eq.) and ammonium formate (5.00 eq.) were mixed and refluxed at 190 °C. After that, the mixture was cooled and 37% HCl (2.00 mL) was added and further refluxed at 100 °C. The crude **6** was slowly precipitated out, filtered, washed and recrystallized with absolute ethanol to give pure **6** as light yellow crystal in 64% yield. R_f = 0.44 (25% MeOH/CH_2Cl_2); mp: 265-266 °C; ^{1}H NMR (400 MHz, D_2O) δ 8.25 (d, *J* = 2.3 Hz, 1H), 7.74 (dd, J_1 = 8.7, J_2 = 2.3 Hz, 1H), 7.26 (d, *J* = 8.7 Hz, 1H), 4.23 (s, 2H); ^{13}C NMR (100 MHz, D_2O) δ 153.8, 138.0, 127.4, 126.3, 125.1, 120.7, 42.0; FT-IR (ATR) cm^{-1}: 3281 (N-H stretching), 1631 (N-H bending), 1534 and 1332 (NO_2 stretching); HRMS : calcd. for $C_7H_9N_2O_3$: m/z 169.0608, found m/z 169.0605 $[M]^+$

2. Synthesis of indole and 5-nitroindole acetic acid derivative 11-14

Indole derivatives **7** or **8** (1.00 eq.) were reacted with NaH (3.00 eq.) in anh. DMF and stirred at room temperature. The mixture was cooled to 0 °C and esters **9** or **10** (1.20 eq.) were added. The reaction mixture was stirred at 0 °C and then at room temperature. The reaction mixture was hydrolyzed with 4M KOH and adjusted to pH 2 with 1M HCl to obtain crude product. The compounds **11-14** were subjected to column chromatography.

Compound **11** brown oil (34 %); R_f = 0.44 (50% EtOAc/Hexane); ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.02 (s, 1H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.46 (d, J_1 = 8.3 Hz, 1H), 7.35 (d, *J* = 3.2 Hz, 1H), 7.12 (td, J_1 = 7.7, J_2 = 1.0 Hz, 1H), 7.01 (td, J_1 = 7.5, J_2 = 1.0 Hz, 1H), 6.42 (dd, J_1 = 3.0, J_2 = 0.9 Hz, 1H), 4.17 (t, *J* = 7.0 Hz, 2H), 2.23 (t, *J* = 7.4 Hz, 2H), 1.82 – 1.70 (m, 2H), 1.52-1.40 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.2, 135.6, 128.5, 128.1, 120.9, 120.4, 118.8, 109.7, 100.4, 45.1, 33.2, 29.3, 21.9; FT-IR (ATR) cm^{-1}; 1699 (C=O stretching), 1312 (C-O stretching)

Compound **12** brown oil (66 %); R_f = 0.25 (25% EtOAc/Hexane); ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.00 (s, 1H), 7.53 (d, *J* = 8.0 Hz, 1H), 7.44 (d, *J* = 8.1 Hz, 1H), 7.34 (d, *J* = 3.1 Hz, 1H), 7.12 (td, J_1 = 7.5, J_2 = 1.0 Hz, 1H), 7.00 (td, J_1 = 7.4, J_2 = 1.0 Hz, 1H), 6.41 (dd, J_1 = 3.0, J_2 = 0.8 Hz, 1H), 4.14 (t, *J* = 7.1 Hz, 2H), 2.17 (t, *J* = 7.3 Hz, 2H), 1.80 – 1.68 (m, 2H), 1.58 – 1.45 (m, 2H), 1.31 – 1.17 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.3, 135.6, 128.5, 128.1, 120.9, 120.4, 118.7, 109.7, 100.3, 45.3, 33.6, 29.6, 25.8, 24.1; FT-IR (ATR) cm^{-1}; 1700 (C=O stretching), 1312 (C-O stretching)

Compound **13** yellow solid (96%); R_f = 0.56 (10% MeOH/CH_2Cl_2); mp: 142-143 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (d, *J* = 2.2 Hz, 1H), 8.11 (dd, J_1 = 9.1, J_2 =2.3 Hz, 1H), 7.34 (d, *J* = 9.0 Hz, 1H), 7.24 (d, *J* = 3.2 Hz, 1H), 6.68 (d, J_1 = 3.2 Hz, 1H), 4.20 (t, *J* = 7.0 Hz, 2H), 2.38 (t, *J* = 7.2

Hz, 2H), 1.99 – 1.86 (m, 2H), 1.70 – 1.59 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.0, 141.8, 138.9, 131.0, 127.9, 118.5, 117.4, 109.3, 104.3, 46.7, 33.3, 29.6, 22.1; FT-IR (ATR) cm^{-1}; 1698 (C=O stretching), 1503 and 1329 (NO_2 stretching)

Compound **14** yellow solid (94%); R_f = 0.56 (12.5% MeOH/CH_2Cl_2); mp: 130-132 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (d, *J* = 2.3 Hz, 1H), 8.11 (dd, J_1 = 9.1, J_2 =2.2 Hz, 1H), 7.34 (d, *J* = 9.1 Hz, 1H), 7.24 (t, *J* = 3.3 Hz, 1H), 6.68 (d, *J* = 3.2 Hz, 1H), 4.18 (t, *J* = 7.0 Hz, 2H), 2.35 (t, *J* = 7.3 Hz, 2H), 1.95 – 1.85 (m, 2H), 1.74 – 1.61 (m, 2H), 1.44 – 1.31 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.6, 141.7, 138.9, 131.0, 127.9, 118.4, 117.4, 109.3, 104.2, 46.7, 33.6, 30.0, 26.4, 24.2; FT-IR (ATR) cm^{-1}; 1699 (C=O stretching), 1506 and 1320 (NO_2 stretching)

3. Synthesis of capsaicin derivatives 15-20

Compounds **11-14** (1.00 eq.) were dissolved in anh. DMF and then cooled to 0 °C with ice bath. EDC.HCl (1.20 eq.) and HOBt (1.20 eq.) were added and the mixtures were stirred at 0 °C. and then at room temperature. After that, the mixture of **3** or **6** (1.20 eq.) and triethylamine (3.00 eq.) was added, and the solution was at room temperature. Next, the reaction mixture was diluted with brine, extracted with ethyl acetate. The organic layer was washed with a citric acid solution (10% w/v), 0.5 M $NaHCO_3$ and dried over anh. sodium sulfate. The solution was then concentrated to obtain crude products. The desired capsaicin derivatives **15-20** were purified by column chromatography.

Compound **15** white solid (45%), R_f = 0.25 (50%EtOAc:Hexane); mp: 122-123 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 7.52 (d, *J* = 7.9 Hz, 1H), 7.35 (d, *J* = 8.3 Hz, 1H), 7.14 (d, *J* = 3.1 Hz, 1H), 7.11 (td, J_1 = 7.7, J_2 = 1.0 Hz, 1H), 7.00 (td, J_1 = 7.5, J_2 = 1.0 Hz, 1H), 6.80 (d, *J* = 1.9 Hz, 1H), 6.73-6.66 (m, 2H), 6.40 (dd, J_1 = 7.6, J_2 = 0.9 Hz, 1H), 4.22 (s, 2H), 4.16 (t, *J* = 7.0 Hz, 2H), 3.73 (s, 3H), 2.22 (t, *J* = 7.3 Hz, 2H), 1.89 – 1.77 (m, 2H), 1.68 – 1.55 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 175.5, 149.0, 146.8, 137.4, 131.5, 130.1, 128.9, 122.2, 121.6, 121.4, 120.0, 116.1, 112.5, 110.4, 101.8, 56.3, 46.7, 44.0, 36.6, 30.9, 24.4; FT-IR (ATR) cm^{-1}; 3278 (N-H stretching), 1645 (C=O stretching), 1622 (N-H bending), 1241 and 1118 (C-O stretching); HRMS : calcd. for $C_{21}H_{24}N_2O_3Na$: m/z 375.1678, found m/z 375.1679 $[M+Na]^+$

Compound **16** white solid (41%); R_f = 0.26 (2% MeOH:CH_2Cl_2); mp: 105-106 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 7.52 (d, *J* = 7.9 Hz, 1H), 7.34 (d, *J* = 8.3 Hz, 1H), 7.15 (d, *J* = 3.2 Hz, 1H), 7.11 (td, J_1 = 7.6, J_2 = 1.0 Hz, 1H), 6.99 (J_1 = 7.6, J_2 = 0.9 Hz, 1H), 6.84 (d, *J* = 1.9 Hz, 1H), 6.76-6.67 (m, 2H), 6.39 (d, *J* = 3.8 Hz, 1H), 4.22 (s, 2H), 4.14 (t, *J* = 6.9 Hz, 2H), 3.81 (s, 3H), 2.17 (t, *J* = 7.4 Hz, 2H), 1.87 – 1.78 (m, 2H), 1.68 – 1.57 (m, 2H), 1.35 – 1.22 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 175.7, 149.0, 146.9, 137.5, 131.5, 130.1, 129.0, 122.2, 121.6, 121.5, 120.0, 116.1, 112.6, 110.4, 101.7, 56.4, 46.8, 44.0, 36.9, 31.0, 27.4, 26.6; FT-IR (ATR) cm^{-1}; 3316 (N-H stretching), 1651 (C=O stretching), 1626 (N-H bending), 1239 and 1121 (C-O stretching); HRMS : calcd. for $C_{22}H_{26}N_2O_3Na$: m/z 389.1844, found m/z 389.1836 $[M+Na]^+$

Compound **17** yellow solid (73%); R_f = 0.48 (100% EtOAc); mp: 134-135 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 8.53 (d, *J* = 2.2 Hz, 1H), 8.04 (dd, J_1 = 9.1, J_2 = 2.2 Hz, 1H), 7.50 (d, *J* = 9.1 Hz, 1H), 7.40 (d, *J* = 3.2 Hz, 1H), 6.82 (d, *J* = 1.7 Hz, 1H), 6.74 – 6.64 (m, 3H), 4.28 – 4.20 (m, 4H), 3.75 (s, 3H), 2.24 (t, *J* = 7.3 Hz, 2H), 1.91 – 1.79 (m, 2H), 1.69 – 1.56 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 175.3, 149.0, 146.9, 142.6, 140.3, 132.8, 131.5, 129.2, 121.5, 118.7, 117.7,

116.1, 112.5, 110.7, 104.7, 56.3, 47.2, 44.0, 36.4, 30.8, 24.2; FT-IR (ATR) cm^{-1}; 3273 (N-H stretching), 1646 (C=O stretching), 1623 (N-H bending), 1509 and 1328 (NO_2 stretching), 1282 (C-O stretching); HRMS : calcd. for $C_{21}H_{23}N_3O_5Na$: m/z 420.1539, found m/z 420.1530, $[M+Na]^+$

Compound **18** yellow solid (72%); R_f = 0.46 (100% EtOAc); mp: 115-116 °C; ^1H NMR (400 MHz, CD_3OD) δ 8.52 (d, *J* = 2.2 Hz, 1H), 8.04 (dd, J_1 = 9.1, J_2 = 2.3 Hz, 1H), 7.48 (d, *J* = 9.1 Hz, 1H), 7.40 (d, *J* = 3.2 Hz, 1H), 6.84 (d, *J* = 1.8 Hz, 1H), 6.75-6.67 (m, 3H), 4.23 - 4.18 (m, 4H), 3.80 (s, 3H), 2.18 (t, *J* = 7.3 Hz, 2H), 1.90 - 1.78 (m, 2H), 1.71 - 1.58 (m, 2H), 1.35 - 1.21 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 175.6, 149.0, 146.8, 142.6, 140.3, 132.8, 131.5, 129.2, 121.5, 118.7, 117.7, 116.1, 112.6, 110.7, 104.7, 56.4, 47.3, 44.0, 36.8, 31.0, 27.3, 26.5; FT-IR (ATR) cm^{-1}; 3277 (N-H stretching), 1634 (C=O stretching), 1611 (N-H bending), 1507 and 1336 (NO_2 stretching), 1272 (C-O stretching); HRMS : calcd. for $C_{22}H_{25}N_3O_5Na$: m/z 434.1683, found m/z 434.1686, $[M+Na]^+$

Compound **19** yellow solid (35%); R_f = 0.43 (100% EtOAc); mp: 139-140 °C; ^1H NMR (400 MHz, CD_3COCD_3) δ 8.56 (d, *J* = 2.3 Hz, 1H), 8.04 (dd, J_1 = 9.1, J_2 = 2.3 Hz, 1H), 7.99 (d, *J* = 2.2 Hz, 1H), 7.63 (d, *J* = 9.1 Hz, 1H), 7.60 (dd, J_1 = 8.6, J_2 =2.2 Hz, 1H), 7.56 (d, *J* = 3.2 Hz, 1H), 7.10 (d, *J* = 8.6 Hz, 1H), 6.75 (d, *J* = 2.5 Hz, 1H), 4.39-4.31 (m, 4H), 2.29 (t, *J* = 7.3 Hz, 2H), 1.99 - 1.84 (m, 2H), 1.72 - 1.60 (m, 2H); ^{13}C NMR (100 MHz, CD_3COCD_3) δ 172.9, 154.2, 142.2, 139.8, 137.7, 133.3, 132.8, 128.7, 124.2, 120.6, 118.4, 117.2, 110.7, 104.4, 100.8, 47.0, 42.2, 35.9, 30.5, 23.6; FT-IR (ATR) cm^{-1}; 3302 (N-H stretching), 3036 (N-H stretching), 1641 (C=O stretching), 1629 (N-H bending), 1536 and 1330 (NO_2 stretching), 1261 (C-O stretching); HRMS : calcd. for $C_{20}H_{20}N_4O_6Na$: m/z 435.1277, found m/z 435.1275, $[M+Na]^+$

Compound **20** yellow solid (35%); R_f = 0.46 (100% EtOAc); mp: 124-125 °C; ^1H NMR (400 MHz, CD_3COCD_3) δ 8.56 (d, *J* = 2.2 Hz, 1H), 8.04 (dd, J_1 = 9.1, J_2 = 2.3 Hz, 1H), 7.99 (d, *J* = 2.2 Hz, 1H), 7.64 (d, *J* = 9.1 Hz, 1H), 7.60 (dd, J_1 = 8.6, J_2 =2.3 Hz, 1H), 7.56 (d, *J* = 3.2 Hz, 1H), 7.12 (d, *J* = 8.6 Hz, 1H), 6.74 (d, *J* = 3.1 Hz, 1H), 4.37 (d, *J* = 6.1 Hz, 2H), 4.32 (t, *J* = 7.2 Hz, 2H), 2.22 (t, *J* = 7.3 Hz, 2H), 1.94 - 1.83 (m, 2H), 1.74 - 1.61 (m, 2H), 1.37 - 1.33 (m, 2H); ^{13}C NMR (100 MHz, CD_3COCD_3) δ 173.1, 154.2, 142.1, 139.8, 137.7, 133.4, 132.8, 128.7, 124.1, 120.6, 118.4, 117.2, 110.7, 104.4, 100.8, 47.1, 42.1, 36.3, 30.7, 27.0, 25.8; FT-IR (ATR) cm^{-1}; 3262 (N-H stretching), 3099 (N-H stretching), 1640 (C=O stretching), 1629 (N-H bending), 1532 and 1325 (NO_2 stretching), 1251 (C-O stretching); HRMS : calcd. for $C_{21}H_{22}N_4O_6Na$: m/z 449.1435, found m/z 449.1432, $[M+Na]^+$

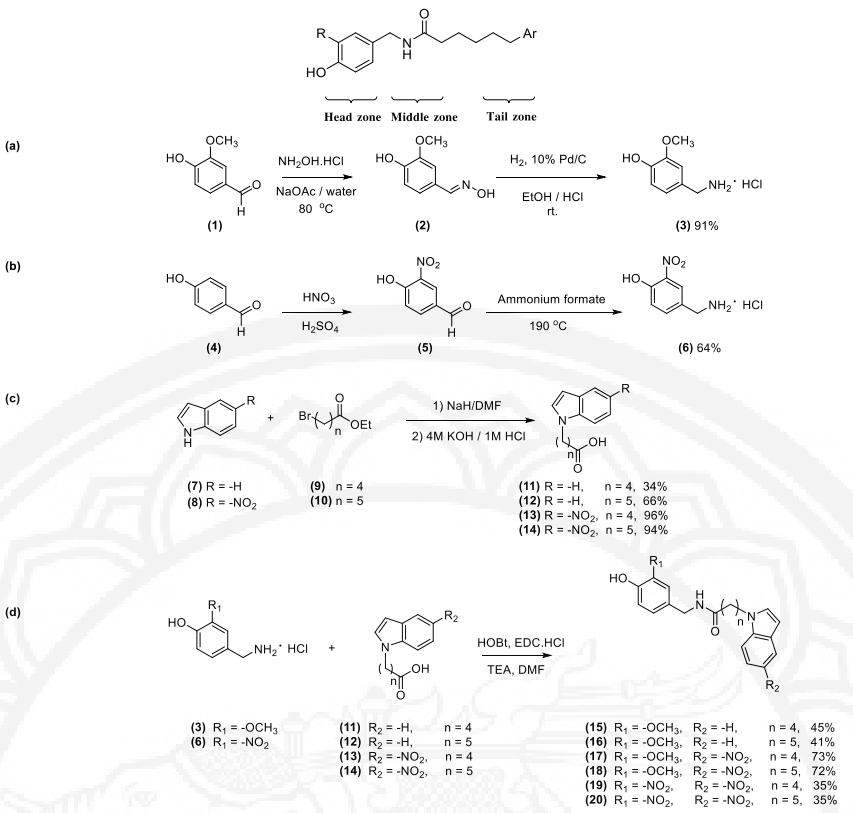

Figure 3 Overview of the syntheses of capsaicin derivatives **15-20** divided into three steps: i) preparation of benzylamine derivatives (a) and (b) ii) construction of indole and 5-nitroindole acetic acid derivatives (c) iii) formation of capsaicin derivatives via peptide coupling reagents (d)

Biological activity

1. Peripheral blood mononuclear cells (PBMCs) procedure

Buffy coats were diluted with phosphate buffer saline (PBS) and gently mixed to give homogeneous buffy coat solution. After that, the buffy coats solution (30 mL) was gently added on the top layer of lymphoprep (15 mL) solution without spreading of blood. The mixture was then centrifuged at 800 x g with no break at approximately 20 °C in a swing-out rotor. After centrifugation, the PBMCs were removed from the mixture, washed with PBS, then centrifuged at 600 x g with break at approximately 5 °C in swing-out rotor. Finally, the PBMCs were washed with PBS and centrifuged at 200 x g with break at approximately 5 °C in swing-out rotor to give the desired PBMCs.

2. Effect of capsaicin and capsaicin derivatives on cell viability

PBMCs in RPMI solution were added into 96-well plate at a concentration of $1x10^5$ cell/well. The volume in each well was 100 µL. Capsaicin and capsaicin derivatives in RPMI containing 1 %PEG (100 µL) were added to achieve concentrations of 10, 100 and 200 µM and plates were incubated for 18 and 24 hours. After that, PBMCs suspension was mixed with trypan blue and then visually examined via microscope. A viable cell was a clear cytoplasm whereas a nonviable cell was a blue cytoplasm.

3. Effect of capsaicin and capsaicin derivatives **15-20** TNF-α production by LPS-stimulated PBMCs (modification of protocol from Hougee et al. (Hougee et al., 2005)

3.1 Sample preparation

PBMCs in RPMI solution were added into 96-well microplate at $1x10^5$ cells/well in 100 μL of media. After that, a mixture of 20 μM of compounds **15-20** in 1% of PEG/RPMI (50 μL) and 100 nM of LPS in RPMI (50 μL) was added to the test wells. The plates containing the cell suspension were incubated for 16 hours in 5% CO_2 at 37 °C. After that, the culture supernatant was collected.

3.2 Human TNF-α assay procedure

The concentration of TNF-α in culture supernatants was assessed by enzyme-linked immune sorbent assay (ELISA). The assays were conducted utilizing the LEGEND MAX™ Human TNF-α ELISA Kit with pre-coated plates according to the manufacturer instructions. The supernatant solution was thawed, diluted properly in assay diluents and assayed for TNF-α concentration with duplicate run. The absorbance of each well was read at 450 nm on a microplate reader (PerkinElmer Life Sciences, Downers Grove, IL, USA). TNF-α was measured from standard curve using Synergy H1 Hybrid Reader (Bio-Tek) and relative inhibition (%) was calculated as shown in the equation below:

$$\text{Relative inhibition increase (\%)} = \frac{(\text{Average conc. of TNF-}\alpha\text{ of capsaicin} - \text{Average conc. of TNF-}\alpha\text{ of sample})}{\text{Average conc. of TNF-}\alpha\text{ of capsaicin}} \times 100$$

Results

In this investigation, the studied capsaicin derivatives **15-20** were inspired by mimicking the benzyl group of the daphnane diterpenoid found in the terminal region of RTX, which might assist its extraordinary potency against TRPV1 that was almost 6 orders of magnitudes greater than that of capsaicin. Moreover, the position of phenylalanine (F544) located close to HC chain of capsaicin, as described the study by Yang et al. (Yang et al., 2015), could be an interesting residue for the introduction of additional interactions. This led us to modify the HC chain of capsaicin by placing either an indole or a nitroindole residue at its tail part and placing either vanillyl or 4-hydroxy-3-nitrobenzyl residues at the head part (Figure 3). Additionally, the length of the linker in the novel capsaicin derivatives was varied to contain 4 or 5 atoms in order to observe the effect on the interaction of the potential ligands in the focus area of TRPV1.

Synthesis Part

Synthesis of benzylamine derivatives 3 and 6

Compound **3** was easily prepared via oximination and hydrogenation. Hydrogen gas was used at atmospheric pressure to avoid over reduction of the aromatic residue. This reaction gave benzylamine **3** in high yield (91%). Next, compound **6** was synthesized via nitration and reductive amination. Leuckart reaction (Crossley & Moore, 1944) for chemoselective reduction to give the desired amine **6** in moderate yield (64%).

Synthesis of indole and 5-nitroindole acetic acid derivatives 11-14

Indole derivatives **11** and **12** and 5-nitroindole derivatives **13** and **14** alkylated on the nitrogen atom with carboxy terminated alkyl chains were prepared via *N*-alkylation reaction (Fan et al., 1998) of indole (**7**) with brominated alkyl ethyl esters **9** and **10** to obtain carboxylic acids **11** and **12**, respectively in moderate yield (34-66%). Carboxylic acids **13** and **14** were obtained in high yield (94-96%) by reacting 5-nitroidole (**8**) with similar reaction and reagents as those used in the synthesis of **11** and **12**.

Synthesis of capsaicin derivatives 15-20

Capsaicin derivatives **15-18** were readily prepared by reaction between carboxylic acids **11-14** and benzylamine **3** while capsaicin derivatives **19-20** were synthesized from reaction between carboxylic acids **13-14** and benzylamine **6** using EDC.HCl and HOBt as coupling reagent. Compounds **15-20** were obtained in satisfactory yield (35-73%)

Biological activity

Determination of anti-inflammatory activity was performed using the inhibition of TNF-α production assay, which was selected as preliminary screening on PBMCs, which express TRPV1 and TRPV2; however, TRPV2 does not respond on capsaicin or proton, but only to extreme heat (Montell, 2001). Therefore, the reduction of TNF-α production, which could originate from the interaction of novel capsaicin derivatives with TRPV1, was used for the simple and quick screening of the novel capsaicin derivatives in this investigation (Engler et al., 2007).

First, the suitable concentration of capsaicin and novel capsaicin derivatives needed to be identified prior to the inflammation testing. Therefore, the cell viability test was initially conducted with the PBMCs by using the trypan blue; it was found that PBMCs were more than 90% viable at 10 μM for both capsaicin and novel capsaicin derivatives. The effect of capsaicin and all capsaicin derivatives on TNF-α production was studied at 5 μM, which is approximately 2 times lower than the concentration at which PMBCs are more than 90 % viable and thus should ensure viability of the cells during the testing. Positive control of TNF-α production by the PBMCs was obtained with their stimulation with LPS in the absence of capsaicin and novel derivatives **15-20**. The test runs were then performed with co-administration of LPS and the test compounds. The production of TNF-α was evaluated using a commercial ELISA based kit with a absorbance readout at 450 nm. The relative increment of inhibition of TNF-α caused by the administration derivatives **15-20** in comparison to capsaicin was also calculated and is shown in Table 1.

Table 1 Preliminary data of concentration of TNF-α production and relative increase inhibition of TNF-α production comparing with capsaicin after dose 5 μM of capsaicin and capsaicin derivatives **15-20** on LPS-stimulated PBMCs

Sample	Conc. of TNF-α (pg/ml)	Relative increase of inhibition of TNF-α production compared with capsaicin (%)
PBMCs	-	-
PBMCs+LPS	426.55±9.35	-
PBMCs+LPS+**15**	45.10±1.82	29.15
PBMCs+LPS+**16**	49.13±0.68	22.81
PBMCs+LPS+**17**	42.35±0.23	33.46
PBMCs+LPS+**18**	43.81±0.46	31.18
PBMCs+LPS+**19**	33.32±0.23	47.65
PBMCs+LPS+**20**	30.58±0.91	51.95
PBMCs+LPS+Capsaicin	63.65±2.51	-

As can be seen in Table 1, PBMCs were stimulated with LPS in the absence of the tested compounds formed 426.55 pg/ml of TNF-α the content of TNF-α released into the media dropped to 63.65 pg/ml upon the co-administration of capsaicin. Furthermore, administrations of **15** and **16**, which contain indole at the tail

region, exhibited content of TNF-α significant lower in comparison to that obtained with capsaicin administration. The observed TNF-α concentrations were 45.10 pg/ml and 49.13 pg/ml for **15** and **16**, respectively. Approximately, similar contents of TNF-α as with **15** and **16** were also observed after the administration of **17** and **18** that bear nitroindole at the tail region. The concentrations of TNF-α in this case were 42.35 pg/ml and 43.81 pg/ml for compounds **17** and **18**, respectively. Interestingly, **19** and **20**, possessing nitroaromatic at the head part and nitroindole at the tail part, showed the lowest TNF-α production with concentration of 33.32 pg/ml and 30.58 pg/ml, respectively. Surprisingly, increasing the length of the HC chain between the amide bond and the heterocyclic residues from 4 to 5 carbon atoms did not show any significant effect on the inhibition of TNF-α production. However, addition of nitro group at both sides of capsaicin in **19** and **20** significantly improved the inhibition of TNF-α production.

Discussion

In this study, we aim to demonstrate the relationship of increasing the anti-inflammatory activity and binding interaction of ligands with TRPV1 with an indirect, easy, and simple approach. First, the mechanism of interactions of TRPV1 signaling pathways and anti-inflammatory effects was previously discussed by Tsuhi et al. (Tsuji & Aono, 2012). Second, the PBMCs that were employed in this investigation were also reported to express TRPV1 (Engler et al., 2007). Therefore, the PMBCs were considered as a good choice for this study as there were expected to provide results utilizing experiments that would be both cost and time effective.

Comparison of **15-18** with capsaicin has shown that the novel derivatives show increased relative inhibition of TNF-α productions with moderate increases in effectiveness (22.81%-33.46%) in comparison to capsaicin. This improved performance probably originates from the replacement of the HC chain in capsaicin with indole and nitroindole moieties that can interact with other molecules by hydrogen bonding via the NH moiety. (Shimazaki et al., 2009) Additionally, nitroindole residue was previously incorporated into ribose or 2-deoxyribose as a universal base in DNA and PNA (Loakes & Brown, 1994; Loakes et al., 1995; Wheaton et al., 2006) due to its non-ambiguous complementary base pairing with strong binding interaction via π-π stacking interactions. Therefore, it is reasonable to assume that the π-π stacking interactions at the tail part of **15-18** would probably enable the indole and nitroindole moieties to freely rotate inside the terminal part of the S1-S4 domain, which has relatively large content of aromatic residues, to attain the decent binding interaction with TRPV1. This in turn probably causes better inhibition of TNF-α production than that seen for capsaicin, which contains only HC chain at the tail part possess, which devoid of π-π stacking potential.

Furthermore, **17** and **18** exhibited improved inhibition of TNF-α production and this probably might originate from the nitro group on nitroindole ring that strongly enhances dipole moment on aromatic rings, which is one of the key parameters that contributes to π-π stacking interactions by polarizing the aromatic ring and thereby promoting electrostatic interactions (Loakes & Brown, 1994; Wheaton et al., 2006; Wichai, 2003). Moreover, the effect of nitro group was even clearly revealed that when two nitro groups were attached on both the nitrobenzylic and nitroindole moieties; consequently, **19** and **20** exhibited even more significantly improved inhibition of TNF-α production due to the dual-stacking interactions at both ends. The relative increase of inhibition of TNF-α for **19** and **20** comparing with capsaicin reached 47.65% and 51.95%, respectively.

The preliminary data of improved anti-inflammation effects of capsaicin derivatives **15-20** presented herein, suggest that **15-20** could exhibit better binding with TRPV1 on PMBCs than capsaicin as a result of their improved π-π properties. Clearly, **19** and **20** constitute the most promising compounds for further study of π-π stacking and binding affinity in TRPV1

Conclusion

In conclusion, new capsaicin derivatives were designed with the aim to mimic the terminal part of RTX. The ability to manipulate the binding interaction and the biological activity of the capsaicin derivatives was demonstrated. The length of HC chain between the amide bond and heterocyclic residues showed no effect on the inhibition of TNF-α production. However, the effect of the nitro groups present on aromatic residues at both ends of capsaicin derivatives **19** and **20** resulted in the most promising biological activity. These novel capsaicin derivatives with enhanced stacking interactions at both ends should be one of the criteria for better design of capsaicin derivatives for pharmaceutical applications in the future. However, the identification of exact position of indole and nitroindole moieties during their interaction with TRPV1 receptor still needs further study by docking and simulation methods. Additionally, the standard testing of compounds with HEK-293 cells expressing rTRPV1 and hTRPV1 is currently being performed.

Acknowledgement

This project was financially supported by Thailand Research Fund (TRF) RDG6020016. Mukthung, C. was financially supported by Center of Excellence for Innovation in Chemistry (PERCH-CIC) and Naresuan University. The authors would like to thank Department of Chemistry and Science Lab Centre (SLC), Faculty of Science, Naresuan University and Naresuan University Hospital. The authors sincerely thank Emeritus Prof. Nuntavan Bunyapraphatsara for comments and capsaicinoids extracts.

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