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On the inhibition of capsaicin response in dorsal root ganglion neurons by nobilamide B and analogues: a structure–activity relationship study†

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Nobilamide B, a TRPV1 antagonist, and a series of Ala-substituted analogues were synthesized and their neuroactivity was assessed in a primary culture of dorsal root ganglion (DRG) neurons. Analogues 4, 6, and 7 showed comparable activity, affecting capsaicin response in neurons, in contrast to 2, 3, and 5 which showed minimal blocking. Compounds 2, 3, and 5 correspond to analogues in which D-Phe, D-Leu and D-allo-Thr have been substituted with Ala, respectively. The observed loss of bioactivity in these three analogues highlights the importance of D amino acids in the primary structure of nobilamide B.

Nobilamide B (1) belongs to a family of neuroactive peptides (*i.e.*, nobilamides) that were isolated from strains of bacteria cultured in tissues of the mollusk species *Chicoreus nobilis* and *Conus tribblei* found in the Philippines.¹ These short peptides, consisting of at most seven amino acid residues, exhibit long-acting antagonistic activity on the transient receptor vanilloid-1 (TRPV1) channel. TRPV1 is a receptor implicated in the sensation of pain and inflammation, expressed mostly in sensory neurons where it is activated by stimuli such as acidic pH, heat, and ligands both endogenous (*e.g.* bradykinin) and exogenous (*e.g.* capsaicin in chili peppers).² The activation of TRPV1 through direct and indirect mechanisms results in the sensation of pain, making it an attractive target for novel analgesics. The role of TRPV1 antagonists in the suppression of pain has been studied in mice models, and great interest in their potential as therapeutic agents for the treatment of chronic and disease-caused hyperalgesia has grown over the years, with a few drugs entering clinical trials.^{3–5}

The viability of developing nobilamide B as a TRPV1 antagonist-based drug was previously demonstrated in a fluorometric assay which measured Ca²⁺ influx in dorsal root ganglion (DRG) cells.^{1,6} These neuronal cells are known to include a substantial fraction of nociceptors (~30–50%), in-

cluding TRPV1. It was found to inhibit the activation of TRPV1 by capsaicin with an IC₅₀ of 275 μM – the highest potency among the nobilamides. Moreover, it was proposed that nobilamide B binds directly to the pore loop region of the channel, covalently inactivating TRPV1 in an “essentially irreversible” manner. This was based upon previous structure–activity relationship (SAR) studies where a loss of inhibitory activity was observed when (1) the electrophile dehydrobutyryne (Dhb) was reduced or absent and (2) nucleophilic Cys residues on the binding site were modified.¹

The total synthesis of nobilamide B was first reported by Yamashita *et al.* in 2014, employing Staudinger ligation to stereoselectively construct Z-enamide structures present in both linear (nobilamide B) and macrocyclic (nobilamide D) peptides, using their phosphinophenol and azide derivatives as precursors.⁷ As an alternative, the total synthesis of nobilamide B using Fmoc-Ala-(Z)Dhb-OH was reported by Jacinto *et al.* using a more facile approach, carrying out EDC/CuCl-induced dehydration of Thr to produce Dhb.⁶ To better understand the neuroactivity of nobilamide B, amino acid substitutions can be made using these synthetic techniques. Alanine scanning mutagenesis is a widely-used technique in studying proteins to identify amino acid residues important for the function, stability and overall conformation of proteins: as Ala is “chemically inert” the sequential substitution of amino acids in a peptide or protein allows the determination of which residues are essential for activity.⁸ By substituting each of the six amino acid residues of nobilamide B with Ala (Chart 1) and performing SAR studies on the resulting analogues, the individual effect of each amino acid on the peptide's activity can be observed.

Herein we report the synthesis of nobilamide B and six Ala-substituted analogues. The ¹H NMR data obtained for

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Chart 1 Nobilamide B and Ala-substituted analogues.

peptides 1 to 7 are summarized in Table 1, and the ^{13}C NMR data are in the ESI.† The chemical shifts of the proton and carbon at the alpha position of each amino acid in a peptide can serve as indicators of a secondary structure, which also

give insights into the shape of its peptide backbone. This is usually done by comparing the observed H_α and C_α chemical shifts to published random coil values, a method later developed as the chemical shift index in predicting the presence

Table 1 ^1H chemical shifts of 1–7 in $\text{DMSO}-d_6$

Residue	No.	Chemical shift, ppm (J in Hz)						
		1	2	3	4	5	6	7
Z-Dhb	3	6.53 q (6.9)	6.54 q (7.0)	6.54 q (6.8)	6.54 q (6.8)	6.54 q (6.6)	6.53 q (7.0)	
	4	1.59 d (6.9)	1.60 d (7.0)	1.60 d (6.9)	1.60 d (6.9)	1.60 d (6.8)	1.59 d (7.0)	
	NH	9.03 s	9.05 s	9.03 s	9.04 s	9.08 s	9.00 s	
L-Ala[6]	2	4.38 m	4.38 m	4.38 m	4.38 m	4.37 m	4.36 m	4.29 m
	3	1.26 d (6.9)	1.26 d (7.0)	1.26 d (6.7)	1.26 d (6.8)	1.25 d (6.6)	1.26 d (7.1)	1.20 d (6.7)
	NH	8.18 d (7.0)	8.19 d (7.0)	8.15 m	8.17 d (6.9)	8.06 d (6.9)	8.10 d (7.4)	8.12 d (6.5)
L-Val	2	4.26 m	4.25 m	4.24 m	4.24 m	4.21 m		4.21 m
	3	1.98 m	1.97 td	1.98 m	1.97 m	1.99 m		1.97 m
	4/5		(13.3, 6.6) 0.81 m/0.85 m 0.85 d (6.7)	0.80 d (6.4) 0.84 d (6.4)	0.81 m/0.84 m	0.81 m/0.85 m		0.81 m/0.85 m
D- α -Thr	NH	7.74 d (8.9)	7.77 d (8.9)	7.72 d (8.7)	7.73 d (8.8)	7.70 d (8.8)		7.73 d (8.7)
	2	4.33 m	4.32 m	4.24 m	4.29 m		4.20 m	4.29 m
	3	3.78 m	3.76 m	3.74 m	3.77 m		3.77 m	3.78 m
L-Phe	4	1.02 d (6.0)	1.00 d (6.2)	0.96 d (5.7)	1.02 d (5.9)		1.01 d (6.2)	1.01 d (5.9)
	NH	8.13 d (8.6)	8.14 d (8.6)	7.95 d (8.3)	7.91 d (8.5)		8.13 d (8.6)	8.12 d (8.5)
	2	4.60 m	4.56 m	4.52 m		4.51 m	4.56 m	4.59 m
D-Leu	3	2.68 m/3.06 m	2.71 m/3.04 m	2.81 m/2.98 m		2.69 m/3.05 m	2.70 m/3.05 m	2.68 m/3.06 m
	Ph	7.11–7.33 m	7.19–7.27 m	7.14–7.29 m		7.12–7.30 m	7.13–7.28 m	7.13–7.29 m
	NH	8.32 d (8.5)	8.34 m	8.05 d (7.8)		8.31 d (8.4)	8.34 d (8.4)	8.31 d (8.4)
D-Phe	2	4.20 m	4.16 m		4.26 m	4.21 m	4.20 m	4.21 m
	3	1.13 m	1.13 m		1.45 m	1.14 m	1.12 m	1.13 m
	4	1.15 m	1.13 m		1.55 m	1.14 m	1.14 m	1.13 m
Prop.	5/6	0.69 d (5.5) 0.72 d (5.4)	0.67 d (5.9) 0.71 d (5.9)		0.82 m/0.87 m	0.70 d (5.5) 0.72 d (5.5)	0.69 d (5.2) 0.72 d (5.2)	0.69 d (4.9) 0.72 d (4.9)
	NH	7.94 d (8.2)	7.98 d (7.7)		8.11 d (7.3)	7.94 d (7.0)	7.98 d (7.6)	7.95 d (8.0)
	2	4.47 m		4.47 m	4.50 m	4.46 m	4.47 m	4.47 m
L-Ala[x]	3	2.68 m/2.92 m		2.74 m/2.89 m	2.73 m/3.00 m	2.69 m/2.92 m	2.66 m/2.91 m	2.68 m/2.92 m
	Ph	7.11–7.33 m		7.14–7.29 m	7.14–7.25 m	7.12–7.30 m	7.13–7.28 m	7.13–7.29 m
	NH	7.94 d (8.2)		8.05 d (7.8)	8.02 m	7.94 d (7.0)	7.93 m	7.95 d (8.0)
L-Ala[x]	2	1.98 m	2.09 q (7.5)	2.07 m	1.99 m	1.99 m	1.98 m	1.97 m
	3	0.82 m	0.94 t (7.6)	0.87 m	0.85 m	0.83 m	0.82 t (7.6)	0.83 m
	2		4.28 m	4.16 m	4.31 m	4.37 m	4.31 m	4.15 m
L-Ala[x]	3		1.13 m	1.02 d (6.8)	1.18 d (6.8)	1.25 d (6.6)	1.20 d (7.0)	1.24 d (7.4)
	NH		7.93 d (7.3)	8.13 m	8.08 d (7.4)	8.27 d (7.0)	7.93 m	8.06 d (7.0)

of secondary structures (α -helix or β -sheet) in proteins.^{9,10} This method has also been applied to the study of conopeptides where chemical shift differences were used in determining structural changes brought upon by amino acid substitutions.^{11,12} Comparing the calculated values for each peptide in this study using random coil values reported by Wishart *et al.*,⁹ similarities and differences in their peptide backbone structures were identified (Fig. 1). The small differences and similar trends observed in the $\Delta\delta H_\alpha$ chemical shifts suggest that the peptides adopt similar backbone structures. Close values were observed for most peptides, except for the changes in the sign of $\Delta\delta H_\alpha$ for peptides 5 at position 4, 6 at position 5 and 7 at position 6. The $\Delta\delta C_\alpha$ chemical shifts, however, reveal that analogues 2 and 3 may possess a different peptide backbone structure as compared to the others due to large deviations observed at positions 2 and 4, respectively. Ala replacement was not made at these specific positions for each analogue, indicating that the shift in the structure could be significant. It must also be noted that the large discrepancies are observed at positions 2 and 4, which also suggest the role of residues D-Leu and D-*allo*-Thr in maintaining the native peptide's structure.

The effects of Ala substitution on the neuroactivity of nobilamide B were determined by observing capsaicin response in DRG neurons. Peptides 1 to 7 were tested for neuroactivity in the DRG assay at a concentration of 300 μ M, approximating the IC_{50} value of nobilamide B (275 μ M) reported by Lin *et al.*¹ Each experiment was done in a heterogeneous population of neurons, with a fraction of small-diameter neurons expressing TRPV1 that was sensitive to capsaicin. In two separate experiments, capsaicin-responsive

neurons were identified and synthetic nobilamide B (1) was found to affect the capsaicin response in 33.6 (± 4.3)% of neurons. Observed activity after application of the compound includes: (1) a loss of response to two capsaicin pulses (complete block) and (2) partial inhibition which was manifested in either a decrease in intensity of recorded capsaicin responses or a reversible block, wherein the capsaicin response was abolished in the first pulse but was recovered after the second pulse. The rest of the capsaicin-responsive neurons were either not affected by the compounds or continually depolarized by capsaicin, with the fluorescence intensity failing to return to the baseline, and therefore an assessment of the activity could not be made.

Analogues 4, 6 and 7 also exhibited inhibitory activity that is similar to the observed neuroactivity of 1. Peptides 4, 6 and 7 were found to inhibit capsaicin response in 23.5 (± 9.8)%, 27.8 (± 5.1)% and 35.6 (± 15.4)% of the population of capsaicin-sensitive neurons, respectively. This was in contrast to analogues 2, 3 and 5 which did not show a comparable activity: these peptides affected capsaicin response in only 6.47 (± 3.17)%, 3.27 (± 1.57)% and 5.38 (± 1.03)% of capsaicin-sensitive neurons, respectively. A substantial difference in the percentages of capsaicin-sensitive population affected by these peptides is indicative of loss of activity. These results are summarized in Fig. 2 which shows the activity of peptides 1 to 7 in capsaicin-sensitive neurons. Representative traces of observed inhibitory activity of peptides 4, 6 and 7 are shown in Fig. 3.

Changing an amino acid from the D to L enantiomer must also cause a change in the overall structure of the peptide, resulting in a decrease or loss of activity. It was also

Fig. 1 Differences in observed chemical shifts and random coil values for each amino acid at positions 1 to 6 of nobilamide B and Ala-analogues: (A) α -proton and (B) α -carbon. There are small differences seen in the $^1H_\alpha$ chemical shifts, whereas clear outliers are observed in the $^{13}C_\alpha$ chemical shifts in analogue [Ala¹]-nobilamide B (2) at position 2 and [Ala²]-nobilamide B (3) at position 4. No comparison was drawn for position 7 because Dhb is an unnatural amino acid, thus it has no available reference value for chemical shifts in a random coil.

Fig. 2 Neuroactivity of nobilamide B and Ala-substituted analogues tested at 300 μ M: percentage of the population of capsaicin-sensitive neurons affected by peptides 1 to 7 combined from two trials. Nobilamide B, 1, ($33.6 \pm 4.3\%$) and analogues 7 ($35.6 \pm 15.4\%$), 6 ($27.8 \pm 5.1\%$) and 4 ($23.5 \pm 9.8\%$) are found to inhibit capsaicin responses in capsaicin-sensitive neurons, whereas analogues 5 ($5.38 \pm 1.03\%$), 3 ($3.27 \pm 1.57\%$) and 2 ($6.47 \pm 3.17\%$) do not display similar activity.

previously noted using NMR data that D-Leu and D-*allo*-Thr are essential in keeping the structural integrity of the native peptide, thus peptides 2 and 4 which contain substitutions of these amino acids do not display activity. Peptide 7 is also remarkable due to its observed activity despite the absence of Dhb. It had been previously proposed that Dhb could be the

pharmacophore of nobilamide B.¹ Namely, the peptide is said to bind to TRPV1 covalently through the interaction of Dhb of the peptide and Cys residues of the protein. In particular, C578, C621 and F660 were found to be essential amino acids in TRPV1 with which nobilamide B possibly interacted; mutation of the protein in these amino acids resulted in a loss of antagonism by nobilamide B. However, it was also noted that removing Cys residues also results in a loss of antagonism by the peptide, defying simple models of alkylation. The authors also concluded that Dhb is a modestly active electrophile that requires appropriate steric directing by residues on TRPV1, particularly F660. The activity of a synthetic nobilamide B analogue which substitutes Ala for Dhb further highlights the greater importance of the peptide backbone structure for its interaction with TRPV1, which exceeds that of the particular functional group Dhb being present.

In conclusion, our study reveals through a classical L-Ala scan that D amino acids in the primary sequence of nobilamide B are essential to its inhibitory activity against capsaicin in DRG neurons. These results are important in the context of pursuing the synthesis of nobilamide B analogues of improved water solubility or bioactivity, in which D-Leu, D-Phe and D-*allo*-Thr should be preserved. In addition, these results highlight the importance of the peptide backbone structure in the investigation of the binding mechanism of nobilamide B to TRPV1. As for the specific chemical nature of the changes brought by replacement of D-amino acids with L-Ala, whether it is the loss of the functional group or stereochemistry that results in the reduced activity can be investigated by a tandem SAR study with a D-amino acid scan, with D-Ala, and conversion of D-amino acids to their L-stereoisomers.^{13,14} Such studies can also lead to the discovery of analogues with improved activity. As the Philippines is recognized as “the centre of the centre of marine shore fish biodiversity”¹⁵ and is therefore proposed to be a rich source for the discovery and development of novel pharmacological agents,^{16,17} our study represents early efforts in this regard.

Methods

Peptide synthesis

Nobilamide B and analogues were synthesized using an Fmoc strategy, employing solution-phase and solid-phase peptide synthesis. Fmoc-Ala-(Z)Dhb-OH was synthesized as the precursor for peptides 1 to 6, using methods previously described.⁶ Peptides were constructed on 2-chlorotriyl chloride (Barlos) resin with 1.0–1.2 meq g⁻¹ substitution and 100–200 mesh size (Chem-Impex International, Inc.; Wood Dale, IL). All standard N-Fmoc protected amino acids were purchased from Sigma-Aldrich (St. Louis, MO). Fmoc-D-Phe-OH, Fmoc-D-Leu-OH, Fmoc-O-*tert*-butyl-D-*allo*-Thr-OH and L-threonine *tert*-butyl ester hydrochloride were purchased from Chem-Impex, as well as O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and 3-(3-dimethylaminopropyl)-1-ethyl-carbodiimide hydrochloride (EDC HCl). Oxyma and N,N-diisopropylethylamine were purchased from Merck.

Fig. 3 Inhibition of capsaicin response by nobilamide B analogues: (A) A7-nobilamide B (7), (B) A5-nobilamide B (6) and (C) A3-nobilamide B (4). Ratiometric Ca²⁺ imaging (fluorescence at 340 nm/380 nm) was used to monitor the increase in intracellular Ca²⁺ concentration over time upon application of KCl, C (capsaicin), and test compounds. Each trace corresponds to a single neuron, showing complete (loss of response after two capsaicin pulses) and partial (decrease in response) or reversible block (response to capsaicin was initially abolished but recovered after the second pulse).

Reverse-phase HPLC profiling and purification were performed using a Shimadzu Prominence HPLC system. HPLC profiles were obtained using a C18 analytical column (Inertsil ODS-3, 5 μ m, 4.6 $\times$ 250 mm, GL Sciences, Inc.). Purification was carried out using a C18 semi-preparative column (Inertsil ODS-3, 5 μ m, 10 $\times$ 250 mm, GL Sciences, Inc.). Solvents used were (A) 0.1% TFA in H₂O and (B) 0.1% TFA in MeCN. NMR spectra were obtained using a 500 Agilent (Varian) NMR spectrometer (¹H 500 MHz, ¹³C 125 MHz). Chemical shifts were reported in ppm and coupling constants in Hz. Electrospray ionization mass spectrometry (ESI-MS) positive mode analyses were performed using a Thermo LTQ-Orbitrap XL mass spectrometer at the Salk Institute for Biological Studies (La Jolla, CA), a Shimadzu LCMS-8030 at the Marine Science Institute (UP Diliman) and a Waters Xevo G2-XS QToF mass spectrometer at the Institute of Chemistry (UP Diliman).

Manual solid-phase peptide synthesis was carried out in a small glass column with a stopcock and porous frit as described in the literature.^{6,18} Automated peptide synthesis was carried out in a Focus XC automated synthesizer (Aapptec, LLC). Fmoc-Ala-(Z)Dhb-OH (287.4 mg, 1.2 eq) was dissolved in 2 mL DMF, mixed with DIEA (210 μ L, 2 eq) and loaded onto Barlos resin (612 mg, 1 eq) for 90 minutes. After capping with MeOH and washing with DMF and DCM, sample beads were taken for initial loading calculation (Fmoc assay substitution = 0.286 mmol g⁻¹). Fmoc-Ala-(Z)Dhb-O-Barlos (~100 mg) was then placed in each of the six reaction vessels of the Focus XC to construct the peptide sequences. Standard L amino acids were dissolved in 0.15 M Oxyma/DMF and loaded into the instrument's amino acid chambers. Coupling reagents used in the machine were 0.15 M HBTU/DMF and 0.45 M DIEA/DMF. Fmoc deprotection was carried out using 20% v/v 4-methylpiperidine in DMF. DCM and DMF were used as swelling and washing solvents, respectively. Unnatural amino acids were dissolved in DMF (5 mL 0.18 M, 6 eq), mixed with 0.15 M HBTU/Oxyma (5 mL 0.15 M, 5 eq) and 0.45 M DIEA (5 mL 0.45 M, 15 eq), and added manually to each reaction vessel in their activated forms. Propionic acid (5 mL 0.18 M, 6 eq) was mixed with HBTU/Oxyma and DIEA and added last to each reaction. Each coupling step done using the instrument lasted for 30 minutes.

Cleavage of the peptide was carried out by mixing the resin in 9:1 TFA/DCM for 1 hour and 30 minutes. The mixture was filtered and washed with DCM. Filtrates and washings were pooled together and dried *in vacuo* by co-evaporation with DCM, MeOH and ether. The peptide product was dissolved in DMSO and then freeze-dried. Peptide products were purified by semi-preparative RP-HPLC using a solvent gradient of 5% to 100% B over 40 minutes, with a flow rate of 3 mL min⁻¹, detected at λ = 220 nm and 254 nm.

Nobilamide B (1) white powder; analytical HPLC (5% to 100% B over 40 minutes, λ = 220 nm, 254 nm) t_R = 25.70 min. ESI-MS [M + H]⁺: 836.50 m/z (C₄₃H₆₁N₇O₁₀ = 835.45 Da).

[Ala¹]-nobilamide B (2) white powder; analytical HPLC (5% to 100% B over 40 minutes, λ = 220 nm, 254 nm) t_R = 22.0 min. ESI-MS [M + H]⁺: 760.50 m/z (C₃₇H₅₇N₇O₁₀ = 759.42 Da).

[Ala²]-nobilamide B (3) white powder; analytical HPLC (5% to 100% B over 40 minutes, λ = 220 nm, 254 nm) t_R = 22.9 min. ESI-MS [M + H]⁺: 794.50 m/z (C₄₀H₅₅N₇O₁₀ = 793.40 Da).

[Ala³]-nobilamide B (4) white powder; analytical HPLC (5% to 100% B over 40 minutes, λ = 220 nm, 254 nm) t_R = 22.1 min. ESI-MS [M + H]⁺: 760.43 m/z (C₃₇H₅₇N₇O₁₀ = 759.42 Da).

[Ala⁴]-nobilamide B (5) white powder; analytical HPLC (5% to 100% B over 40 minutes, λ = 220 nm, 254 nm) t_R = 26.7 min. ESI-MS [M + H]⁺: 806.45 m/z (C₄₂H₅₉N₇O₉ = 805.44 Da).

[Ala⁵]-nobilamide B (6) white powder; analytical HPLC (5% to 100% B over 40 minutes, λ = 220 nm, 254 nm) t_R = 24.4 min. ESI-MS [M + H]⁺: 808.50 m/z (C₄₁H₅₇N₇O₁₀ = 807.42 Da).

[Ala⁷]-nobilamide B (7) white powder; analytical HPLC (5% to 100% B over 40 minutes, λ = 220 nm, 254 nm) t_R = 25.2 min. ESI-MS [M + H]⁺: 824.55 m/z (C₄₂H₆₁N₇O₁₀ = 823.45 Da).

DRG assay

The neuroactivity of nobilamide B and analogues was assessed using the DRG calcium imaging facility at the UP Marine Science Institute. The DRG assay was adapted from the work of Teichert *et al.*¹⁹ using a fluorescence microscope (Olympus BX41) with a Lambda LS light source (300 W xenon arc lamp) system. Images were captured using a digital camera (QICam Fast 1394) and Nikon Elements software. Briefly, DRG cells were harvested from the spinal column of a 21 day-old mouse and the cells were plated and cultured in 10% DMEM at 37 °C, 5% CO₂ overnight in a 24-well plate. The cells were incubated first with 2.5 μ M fluorescent ratiometric calcium probe Fura-2-AM at 37 °C, 5% CO₂ for 1 hour, followed by equilibration at room temperature for 45 minutes. DRG cells were pulsed with KCl (25 mM) and capsaicin (200 nM) for 15 seconds, each with 5 to 6 minutes interval and washing steps after every pulse. KCl was added to identify excitable neurons, and capsaicin was subsequently added to differentiate the neurons that are TRPV1-expressing. To identify blocking of the TRPV1 receptor, cells were incubated with the nobilamides (300 μ M) for 6 minutes (without washing) after the first capsaicin pulse. Fluorescence emission at 510 nm was recorded at 340 and 380 nm excitation wavelengths every 2 seconds. The ratio of fluorescence intensities at 340 nm and 380 nm was plotted over time to monitor the relative increase in calcium concentration within the cells. Approximately 60 to 100 capsaicin responsive neurons or regions of interest (ROIs) were selected per well.

Conflicts of interest

There are no conflicts to declare.

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