Discovery of a Remarkable Methyl Shift Effect in the Vanilloid Activity of Triterpene Amides

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ABSTRACT: As part of a study on triterpenoid conjugates, the dietary pentacyclic triterpenoids oleanolic (2a) and ursolic acids (3a) were coupled with vanillamine, and the resulting amides (2b and 3b, respectively) were assayed for activity on the vanilloid receptor TRPV1. Despite a structural difference limited to the location of a methyl group in their conformationally rigid pentacyclic core, oleanoloyl vanillamide dramatically outperformed ursoloyl vanillamide in terms of potency (EC50 = 35 ± 2 nM for 2b and 5.4 ± 2.3 μM for 3b). Using molecular docking and dynamics, this difference was translated into distinct accommodation modes at the TRPV1 vanillyl ligand pocket, suggesting a critical role of a C–H π–π interaction between the triterpenoid C-29 methyl and Phe591 of TRPV1. Because the molecular mechanisms underlying the activation process of transient receptor channels (TRPs) remain to be fully elucidated, the observation of spatially restricted structure–activity information is of significant relevance to identify the molecular detail of TRPV1 ligand gating.
The pentacyclic triterpenoid scaffold of oleanolic and ursolic acid is devoid of conformational mobility, and the two compounds only differ in the location of a methyl group, making it possible that the presence of a substitution at C-19 interferes, by steric hindrance, with the fitting of 3b into the ligand binding site of TRPV1. However, a docking study by using the available structure of TRPV1 in its activated state (PDB id: 5IRX) suggested a more complex and different scenario. In fact, both compounds docked into the vanilloid-binding pocket, as defined by the S3–S4 helices, S4–S5 linker of one subunit, and the S5–S6 helices of the adjacent subunit. The two best not-redundant poses in terms of binding energy value for each compound (Figures 1 and 2, panel B) only differed in the orientation of the vanillyl moiety (hereinafter referred to as OMe-in when the methoxy group points toward the cleft between helices S3 and S4 and OMe-out when it is rotated by 180°).

Table 1. TRPV1 Activity Data for the Vanillamides 2b and 3b Compared to the Activity of Capsaicin (1)

| compound               | efficacy (relative to ionomycin 4 μM) | potency EC50 (capsaicin 4 nM) | IC50 (capsaicin 0.1 μM) |
|------------------------|---------------------------------------|-------------------------------|------------------------|
| 2b (oleanoyl vanillamide) | 72 ± 1                                 | 35 ± 2 nM                     | 50 ± 2 nM              |
| 3b (ursoloyl vanillamide) | 16 ± 1                                 | 5.4 ± 2.3 μM                  | 7.5 ± 0.7 μM           |
| 1 (capsaicin)          | 79 ± 1                                 | 5.3 ± 0.4 nM                  | 8.0 ± 0.3 nM           |

aData were obtained in HEK-293 cells, stably transfected with recombinant human TRPV1 (hTRPV1).

Figure 1. Representative energy-minimized OMe-in docking poses of 2b and 3b (tan and olive drab, respectively, panel A) and capsaicin (salmon, panel B) after best fit of the protein backbone. Ligands are shown in ball-and-stick representation, whereas protein residues within 4.5 Å from the ligand are shown in stick representation. Ribbons and selected side chain stick bonds of TRPV1 monomers A and B are colored in dark gray and sky blue, respectively. Oxygen, nitrogen, and sulfur atoms are colored in red, blue, and yellow, respectively. Only polar hydrogens are shown and colored white.
At odds with the starting hypothesis, in the emerging scenario the $\beta$-oriented methyl on C-19 does not prevent accommodation of 3b in the binding site, but its translocation on C-20 rather induces a better fit of 2b in the binding site. In fact, in both poses, the vanillyl group of 2b is deeper inside the pocket than in 3b, and this arrangement is promoted by a C–H $\pi$phenyl interaction\(^{16,17}\) between the C-29 methyl group and Phe591 (S5 helix-B monomer) side chain, which pushes down the terpenoid scaffold. Conversely, the lack of this methyl in 3b induces a shift of the terpenoid scaffold toward Phe591, resulting in a looser binding of the vanillyl group in the ligand pocket. In the OMe-in orientation, both vanillamides are engaged in H-bonds between the hydroxyl group and both the Ser512 (S3) and Arg557 (S4) side chains, whereas in the OMe-out orientation only the phenolic hydroxy of 2b can form a H-bond with the Ser512 side chain, whereas the methoxy groups of both isomers are H-bonded to the Arg557 side chain. The C-3 hydroxy of the cyclic scaffold of both isomers is close to the sulfur atom of the Met581 (S4–S5 linker) side chain in both the OMe-in and OMe-out orientation. Because in rigid systems the effect of substitution can be directly translated into the occupancy of a specific area of the ligand-binding space, it was interesting to investigate if the site of the C–H $\pi$phenyl interaction of the C-29 oleaeryl methyl was also occupied by capsaicin. When this archetypal vanilloid ligand was docked into the vanilloid-binding pocket, one of the $\omega$-methyls was indeed spatially close to Phe591 (S5-B), with two orientations of the vanillyl group of the same OMe-in and OMe-out type being observed, in accordance to the binding mode of 2b and 3b (Figures 1 and 2, panel B). On account of a major conformational mobility and a slender carbon–carbon connectivity, the branched acyl tail of capsaicin allows two H-bonds of its amide group with either both the Thr550 (S4) and the Tyr511 (S3) side chains (OMe-out orientation) or, alternatively, Tyr511 (S3) (OMe-in orientation), rationalizing the higher potency of capsaicin compared to 2b (EC\(_{50}\) = 5.3 and 35 nM, respectively). For comparison purposes, we also evaluated the activity of the corresponding acidic parent triterpenoids (oleanolic and ursolic acids, 2a and 3a, respectively), previously reported to act as weak antagonists at TRPV1.\(^{13,14}\) We confirmed that both compounds behave as weak antagonists, inhibiting the capsaicin response by 20 ± 3% and 30 ± 1% at 25 $\mu$M, respectively. The corresponding docking complexes are reported in Figure S1. Ursolic acid (2a) engages a H-bond between its carboxylate and the Thr550 side chain, while the arrangement of the polycyclic moiety is substantially preserved in comparison to its vanillamide-conjugated derivative. Conversely, oleanolic acid adopts a completely different orientation, engaging Ser512 with a H-bond with its hydroxy group. A hypothetical corresponding pose of ursolic acid, with the carboxylate group forming a H-bond with the Thr550 side chain, is prevented by a steric clash between the C-29 methyl group and Phe591. Thus, since both acidic precursors are endowed with a weak and comparable inhibitory activity, the dramatic difference in the activity profile between 2b and 3b can be ascribed to the introduction of a vanillamide group. To confirm and further explore the better accommodation of 2b vs 3b within the site emerging from the docking, we carried out 100 ns of molecular dynamics in the membrane environment for both OMe-in complexes. The root mean square deviation (rmsd) of both protein and ligands, shown in Figure 3, shows smaller fluctuations in both protein and ligands for the 2b complex in comparison with those of 3b. In fact, the latter is characterized by both a drift in protein backbone and a higher mobility of the ligand in the four binding sites of the tetramer. Thus, MD calculations show a relative structural destabilization on going from 2b to 3b of the active form of TRPV1 used to derive the theoretical complexes, corresponding to the cryo-EM structure in complex with resiniferatoxin. The greater structural stability of 2b is also
confirmed by the network of H-bonds engaged within each binding site in comparison to that of 3b, as shown in Table 2, reporting H-bond occurrences greater than 10% over the simulated 100 ns of production run. In fact, while only one H-bond with Ser152 with an occurrence of ~40% in three sites out of four, 2b forms additional H-bonds with Arg557 and/or Glu570, with an overall occurrence of H-bonds well above 50%, up to ~74%. Moreover, methyl C29 forms stable hydrophobic interactions with both Phe591 and Ala549 during the whole simulated period, as shown in Figure S2. The representative frames from molecular dynamics are shown in Figure 4. The greater capability of 2b to stabilize the active form of TRPV1 is fully consistent with the higher agonist efficacy observed for this compound in comparison with 3b.

In conclusion, a comparative analysis of bioactivity data, docking experiments, and MD simulations has highlighted the critical role of the C-29 methyl of triterpenoids for significant and effective binding to TRPV1, with only the oleanane skeleton having this methyl in the correct location for the interaction. As the rigid ring system of both triterpenoid vanillamides encompasses conformationally constrained versions of the side chain of capsaicin, it is not unrealistic that a similar interaction may occur between Phe591 and one of the \( \omega \)-methyls of capsaicin, thus disclosing a role for this residue in agonist binding and receptor activation.

**EXPERIMENTAL SECTION**

**General Experimental Procedures.** IR spectra were obtained on an Avatar 370 FT-IR Thermo Nicolet. \(^1\)H (300 MHz) and \(^13\)C (75 MHz) NMR spectra were measured on a Bruker spectrometer. \(^1\)H (500 MHz) and \(^13\)C (126 MHz) NMR spectra were measured on an Agilent spectrometer. Chemical shifts were referenced to the residual solvent signal (CDCl\(_3\), \( \delta \_H = 7.26, \delta \_C = 77.16 \), or DMSO-\( d_6 \), \( \delta \_H = 2.50, \delta \_C = 39.52 \), hept). Low- and high-resolution ESIMS spectra were obtained on an LTQ OrbitrapXL (Thermo Scientific) mass spectrometer. Silica gel 60 (63–200 mesh) used for gravity column chromatography was purchased from Merck. Reactions were monitored by TLC on silica gel Merck 60 F254 (0.25 mm) plates and neutral alumina Macherey-Nagel ALUGRAM (0.20 mm) plates that were visualized by UV inspection (254 and 365 nm) and/or staining with 5% H\(_2\)SO\(_4\) in EtOH and heating. Organic phases were dried with anhydrous Na\(_2\)SO\(_4\) before evaporation. Chemical reagents and solvents were from Sigma-Aldrich.

**Synthesis of Triterpenoid Vanillamides.** Synthesis of Oleanoyl Vanillamide (2b) as Representative. (a) Carboxylate activation: To a stirred solution of N-hydroxysuccinimide (1.51 g; 13.1 mmol) in EtOAc (50 mL) were added oleanolic acid (4.5 mmol) and dicyclohexylcarbodiimde (DCC, 22.2 mmol). The mixture was stirred at room temperature (rt) for 16 h and then worked up by filtration and evaporation. The residue was purified by gravity column chromatography using petroleum ether/EtOAc (8:2) as mobile phase, to give the hydroxysuccinimide ester as a white powder (1.32 g, 52% yield). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta \) 5.31 (1H, brt), 3.46 (1H, m), 3.20 (1H, m), 2.79 (4H, m), 1.15 (3H, s), 0.98 (3H, s), 0.92 (3H, s), 0.91 (3H, s), 0.90 (3H, s), 0.80 (3H, s), 0.76 (3H, s). (b) Amidation: To a stirred solution of oleanoyl

![Table 2. Occurrence of Ligand–Protein H-Bonds for TRPV1 in Complex with Compounds 3b and 2b during 100 ns of MD](image)

| protein binding site | compound 3b (HB occurrence) | compound 3b (frames with ≥1 HB) | compound 2b (HB occurrence) | compound 2b (frames with ≥1 HB) |
|----------------------|-----------------------------|-------------------------------|-----------------------------|-------------------------------|
| 1                    | OH–Ser512 (36.8%)           | 42.42%                        | OH–Ser512 (51.2%)           | 52.80%                        |
| 2                    | OH–Ser512 (34.8%)           | 44.28%                        | OH–Ser512 (64.2%); OH–Glu570 (50%) | 73.8%                        |
| 3                    | OH–Ser512 (40.8%)           | 40.99%                        | OH–Ser512 (41.0%); OH–Arg557 (39.7%); CO–Tyr511 (33%) | 66.80%                        |
| 4                    | OH–Ser512 (58.7%)           | 58.7%                         | OH–Ser512 (57.6%)           | 57.6%                         |
hydroxysuccinimide (300 mg, 0.54 mmol) in CH2Cl2 (4 mL) was added vanillic acid (150 mg, 1.1 mmol). The mixture was stirred at rt for 24 h and then worked up by dilution with brine and extraction with CH2Cl2. The organic phase was treated with Na2SO4 and filtered, and the solvent evaporated. The residue was purified by gravity column chromatography using petroleum ether/EtOAc (3:7) for 24 h and then worked up by dilution with brine and extraction with CH2Cl2.

### Oleanoyl vanillamide (2b)

White powder; IR αmax (KBr) 3544, 3465, 3158, 1770, 1653, 1515, 1457, 1379, 1235, 1025, 834, 816, 739, cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 6.76 (1H, d, J = 8.0 Hz, H-5), 6.68 (1H, dd, J = 8.0, 2.0 Hz, H-6), 5.24 (1H, t, J = 3.6 Hz, H-12), 4.24 (2H, s, H-7), 3.81 (3H, s, H-8), 3.13 (1H, dd, J = 10.7, 5.3 Hz, H-3), 2.81 (1H, dd, J = 14.0, 4.6 Hz, H-18), 2.00 (1H, td, J = 14.7, 5.4 Hz, H-16a), 1.82 (1H, m, H-11), 1.81 (1H, m, H-22a), 1.70 (1H, m, H-15a), 1.68 (1H, m, H-16b), 1.64 (1H, m, H-22b), 1.62 (1H, m, H-19a), 1.56 (1H, m, H-1a), 1.53 (2H, m, H-2), 1.48 (1H, m, H-6a), 1.47 (1H, m, H-9), 1.38 (1H, dd, J = 12.4, 3.3 Hz, H-7a), 1.31 (2H, m, H-6b and H-21a), 1.26 (1H, m, H-7b), 1.19 (1H, m, H-21b), 1.11 (1H, m, H-19b), 1.09 (3H, s, H-27), 1.07 (1H, m, H-15b), 0.91 (3H, s, H-23), 0.89 (1H, m, H-1b), 0.87 (3H, s, H-30), 0.85 (3H, s, H-29), 0.83 (3H, s, H-25), 0.71 (3H, s, H-24), 0.69 (3H, s, H-26), 0.66 (1H, dd, J = 11.7, 6.6 Hz, H-5); ¹³C NMR (126 MHz, CDCl₃) δ 175.6 (C-28), 147.1 (C-3'), 145.1 (C-4'), 143.0 (C-13), 129.6 (C-1'), 122.9 (CH-12), 120.5 (CH-C-6), 114.7 (CH-C-5), 110.9 (CH-C-7), 78.8 (CH-C-3), 55.8 (CH-C-20), 55.2 (CH-C-26), 47.5 (CH-C-30), 45.7 (CH₂-C-19), 43.4 (CH₂-C-7), 42.1 (CH-C-18), 39.3 (C-C-8), 38.7 (C-C-4), 38.5 (CH₂-C-1), 37.0 (C-C-10), 33.7 (CH₂-C-21), 32.9 (CH₂-C-29), 32.7 (CH₂-C-7), 32.0 (CH₂-C-22), 30.6 (C-C-20), 28.0 (CH₂-C-23), 27.7 (CH₂-C-15), 26.7 (CH₂-C-25), 25.6 (CH₂-C-27), 23.4 (CH₃-C-30), 23.4 (CH₃-C-21), 23.0 (CH₃-C-16), 18.3 (CH₃-C-6), 16.8 (CH₂-C-26), 15.6 (CH₃-C-14), 15.3 (CH₃-C-25); HR-ESIMS m/z 591.4273 [M+H⁺] (calc for C₃₈H₅₇NO₄, 591.4288).

Molecular Docking and Molecular Dynamics Studies. The starting ligand geometry of the ligands was built with Ghemical 2.99 and energy minimized at molecular mechanics level first, followed by a single-point HF energy evaluation at the STO-3G level to derive the partial atomic charges for the ligand by the RESP procedure. Two docking studies were performed with AutoDock 4.2. The TRPVI (PDB id: 5IRX) and the ligands were processed with AutoDock Tools (ADT) package version 1.5.6.1 to merge nonpolar hydrogens, calculate Gasteiger charges, and select rotatable side chain bonds. Grid dimensions of 60 × 50 × 60, respectively, centered in the binding pocket, were generated with the program AutoGrid 4.2 included in the Autodock 4.2 distribution, with a spacing of 0.375 Å. A total of 100 molecular docking runs for each docking calculation were performed adopting a Lamarckian Genetic Algorithm (LGA) and the protocol already published. Flexibility was used for all rotatable bonds of the docked ligands. For each docking run, the best not-redundant poses in terms of binding energy values were selected as representatives and underwent energy minimization with the Amber16 package using the ff14SB version of AMBER ff14SB force field for the protein and gaff parameters for the ligand. UCSF Chimera 1.4.5 was used for figures of the molecular complexes. The energy-minimized complexes were embedded in a POPC bilayer using the charm++ web-interface, and then MD simulations in the membrane environment were carried out with the pmdem.cuda module of the Amber16 package, using lipid 14 (lips), ff14SB force field, and gaff (ligand) force field parameterization. MD production runs were carried out for 100 ns. The Cpttraj module of AmberTools16 was used for trajectory analysis. The full MD protocol has been published elsewhere.

TRPVI Channel Assay. Compound effects on intracellular Ca²⁺ concentration ([Ca²⁺]) were determined using the selective intra-cellular fluorescent probe for Ca²⁺ Fura-4, and assays were performed as described. Briefly, HEK-293 cell lines stably transfected with recombinant human TRPV1 (selected by Geneticin 600 μg mL⁻¹) or not transfected were cultured in DMEM containing 0.02% Pluronic F-127. After that, the cells were rinsed and resuspended in Tyrode's solution (145 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4), then transferred to a quartz cuvette of a spectrophluorimeter (PerkinElmer LS50B, excitation λ = 488 nm, emission λ = 516 nm) under continuous stirring. Cell fluorescence before and after the addition of various concentrations of test compounds was measured normalizing the effects against the response to ionomycin (4 μM). The potency of the compounds (EC₅₀ values) is determined as the concentration required to produce half-maximal increases in [Ca²⁺]. Antagonist behavior is evaluated against the agonist of the TRPVI capsain (100 nM) and analyzed by adding the compounds directly in the quartz cuvette 5 min before stimulation of cells with the agonist. IC₅₀ is expressed as the concentration exerting a half-maximal inhibition of agonist effect, taking as 100% the effect on [Ca²⁺⁺], exerted by capsain (100 nM) alone. Dose–response curve fitting (sigmoidal dose–response variable slope) and parameter estimation were performed with Graph-Pad Prism8 (GraphPad Software Inc.). All determinations were performed at least in triplicate.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://doi.org/10.1021/acs.jnatprod.0c00639.

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Notes
The authors declare no competing financial interest.

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