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Original Article

Pellitorine, an extract of *Tetradium daniellii*, is an antagonist of the ion channel TRPV1

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ABSTRACT

Background: Transient Receptor Potential Vanilloid 1 (TRPV1) confers noxious heat and inflammatory pain signals in the peripheral nervous system. Clinical trial of resiniferatoxin from *Euphorbia* species is successfully aimed at TRPV1 in cancer pain management and heading toward new selective painkiller status that further validates this target for drug discovery efforts. *Evodia* species, used in traditional medicine for hundreds of years, are a recognised source of different TRPV1 agonists, but no antagonist has yet been reported.
Hypothesis/Purpose: In a search for painkiller leads, we noted for the first time a TRPV1 antagonist activity in the fresh fruits of *Tetradium daniellii* (Benn.) T.G. Hartley (syn. *Evodia hupehensis* Dode).
Methods: Through a combination of extraction and purification methods with functional TRPV1-specific Ca^{2+} uptake assays (bioactivity-guided fractionation/isolation/purification); we isolated a new painkiller candidate that is a distant structural homologue of capsiate exovanilloids and endovanilloids such as anandamide, but a putative competitive inhibitor of the TRPV1. Four additional inactive compounds (*N*-isobutyl-4,5-epoxy-2*E*-decadienamide, geranylpsoralen, 8-(7′,8′-epoxygeranyloxy)psoralen, and xanthotoxol) were also co-purified with pellitorine. Their structures were established by extensive 1D- and 2D-NMR spectroscopic analysis.
Results: ^{1}H- and ^{13}C NMR determination of the chemical structure revealed it to be pellitorine, (2*E*,4*E*)-*N*-(2-methylpropyl)deca-2,4-dienamide, which can compete structurally with algesics released in inflammation. In contrast to previous isolates from *Evodia* species, pellitorine blocked capsaicin-evoked Ca^{2+} uptake with an IC_{50} of 154 μg/ml (0.69 mM/l). *N*-Isobutyl-4,5-epoxy-2*E*-decadienamide and geranylpsoralen, 8-(7′,8′-epoxygeranyloxy)psoralen, and xanthotoxol did not affect the TRPV1.
Conclusion: This is the first evidence that pellitorine, an aliphatic alkylamide analogue of capsaicin, can serve as an antagonist of the TRPV1 and may inhibit exovanilloid-induced pain.

Introduction

Transient Receptor Potential Vanilloid 1 (TRPV1), one of 28 members of the transient receptor potential (TRP) family of ion channels, transduces pain signals in the peripheral nervous system (PNS) of mammals, including human. TRPV1 expressing nerve endings of C- and Aδ-type primary afferent nociceptive neurons are triggered by endo-, and exovanilloids, moderate heat (Tominaga et al., 1998), and acute or chronic inflammatory mediators, either lipid-like eicosanoids (Olah et al., 2001; Zygmunt et al., 1999; Hwang et al., 2000) or sensitized by peptides such as bradykinin (Pan and Chen, 2004; Di Marzo et al., 2002). Moreover, metabolic changes leading to tissue

Abbreviations: ANA, anandamide; CAPS, capsaicin; CapZ, capsazepine; HaCaT, human immortalized keratinocyte cell line; pMTH, plasmid containing metallothionein promoter; PNS, peripheral nervous system; PUFA, polyunsaturated fatty acids; RPC, rotation planar chromatography; RTX, resiniferatoxin; TRP, transient receptor potential; TRPV1, Transient Receptor Potential Vanilloid 1

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acidification potentiate the receptor for chronic pain signalling (Caterina et al., 1997; Tominaga et al., 1998).

Pain sensation can be reduced by the inhibition of TRPV1, and therefore identification of potent TRPV1 antagonists have been in the focus of research studies. Capsazepine, the first TRPV1 antagonist, was reported by Bevan et al. (1992). Since the identification of TRPV1, numerous TRPV1 antagonists have been synthetized. An efficient antagonist like SB-705,498 (Gunthorpe et al., 2007) showing IC_{50} values at nanomolar range has been demonstrated to block the activation of TRPV1 by capsaicin, heat, and decreased pH. However, potent antagonists of TRPV1 have exhibited undesirable chemical, pharmacological or pharmacokinetic properties such as poor solubility causing decreased absorption (Stec et al., 2008), short half-life (Tafesse et al., 2004), severe hyperthermia (Gavva et al., 2008; Tamayo et al., 2008) or low oral bioavailability (Tafesse et al., 2004). The most effective antagonists such as SB-705498, NEO6860 or JNJ-39439335 have been investigated in clinical trials to evaluate their safety and efficacy in the treatment of various pain-associated conditions. Phase I and II studies of the above mentioned compounds have been completed without publication of results or terminated because of insufficient number of study subjects.

It is perhaps not surprising that number of plant species have evolved secondary metabolites, which target the TRPV1 in mammals to defend themselves. Thus, RTX and analogous irritant vanilloid phytotoxins most likely have evolved to repel herbivorous mammals. From the *Evodia* genus evodiamine and rutaecarpine have been described as moderate agonists of the TRPV1 (Pearce et al., 2004; Deng and Li, 2005; Wang et al., 2005; Yi et al., 2004; Yu et al., 2005). However, there has as yet no report of any antagonist which may inhibit pain through competition with *bona fide* endovanilloids at binding site of the TRPV1. We have therefore investigated *Tetradium. daniellii* in a search for new vanilloid analogues targeting the TRPV1 either as agonists or antagonists.

Materials and methods

For vacuum liquid chromatography (VLC) TLC silica gel 60 G 15 μm (Merck, Darmstadt, Germany) was used. For open column chromatography (OCC) LiChroprep RP-18 (40–63 μm, Merck, Darmstadt, Germany) was applied. Preparative TLC was carried out on 20 × 20 cm silica gel (Silica gel 60 F_{254}, Merck, Darmstadt, Germany) plates. Rotation planar chromatography (RPC) was performed using Chromatotron (Model 8924, Harrison Research, Palo Alto, CA, USA) on manually prepared silica gel layer, thickness 2 mm (Silica gel 60 GF_{254}, Merck, Darmstadt, Germany). Chromatographic fractions were monitored by TLC on silica gel (Merck 5715), visualized by spraying with conc. H_2SO_4, followed by heating. NMR spectra were recorded in $CDCl_3$ on a Bruker Avance DRX 500 spectrometer at 500 MHz (^1H) or 125 MHz (^{13}C); the signals of the deuterated solvent were taken as the reference.

All chemicals and reagents were commercially available. Analytical grade reagents were used during the extraction of plant material. For HPLC separation, high purity solvents for HPLC were used. During *in vitro* and *in vivo* assays, tissue culture quality chemicals or solvents were used.

Plant material

The fruits of *T. daniellii* were collected in a public park at Hódmezővásárhely (Hungary) in September, and were stored at −15 °C until preparation. A voucher specimen (No. 760) has been deposited in the Herbarium of the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

Extraction and isolation

The frozen plant material (10 kg) was percolated with MeOH (60 l) at room temperature. After concentration to 1.5 l, the MeOH extract was partitioned between cyclohexane (4 × 1.5 l), $CHCl_3$ (4 × 1.5 l) and H_2O. The cyclohexane layer was dried in vacuum yielding a dark green oily residue (110 g), which was subjected to VLC (VLC-1) on silica gel, using a gradient system of cyclohexane – EtOAc – EtOH (100:0:0, 19:1:0, 9:1:0, 4:1:0, 7:3:0, 70:30:3, 15:10:1, and 1:1:1). Altogether 83 fractions, each of 250 ml, were collected and successively combined in 18 fractions (Evo1 – Evo18) after TLC monitoring. Combined fractions Evo10, eluted with cyclohexane – EtOAc – EtOH (4:1) exhibited capsaicin (CAPS)-induced Ca^{2+}-uptake inhibitory activity, and were further fractionated by means of VLC (VLC-2) on silica gel with *n*-hexane – acetone mixtures of increasing polarity (19:1, 9:1, 17:3, 4:1, 3:1, 7:3, and 3:2). The fractions obtained here were combined in nine main fractions (Evo10/1 – Evo10/9) depending on their compositions. Only one fraction (Evo10/1) was found to be effective, and was re-chromatographed by OCC on RP-18 silica gel with elution of 70, 75, 80, 85, 90, 95 and 100% MeOH. The combined fractions (Evo10/1/A – Evo10/1/H) from this separation were assayed for their efficacy, and the active fraction Evo10/1/B was selected for further chromatography. This fraction was purified by preparative TLC on silica gel using $CHCl_3$ – acetone (49:1) as developing system, to afford pellitorine (**1**) (2.4 mg) (Fig. 4). after three-step chromatographic separations using VLC (silica gel, *n*-hexane – acetone gradient), RPC (silica gel, toluene – ethyl acetate gradient) and preparative TLC (silica gel, $CHCl_3$ – acetone 19:1), combined fractions 29–33 from the VLC-1 separation yielded *N*-isobutyl-4,5-epoxy-2*E*-decaenamide (**3**) (1.2 mg), 8-geranyloxypsoralen (**5**) (150 mg), 8-(7′,8′-epoxygeranyloxy)psoralen (**7**) (2.3 mg) and xanthotoxol (**4**) (4.3 mg) (Fig. 4).

(2*E*,4*E*)-*N*-(2-methylpropyl)deca-2,4-dienamide (=pellitorine) (**1**): amorphous solid; ^1H NMR (500 MHz, $CDCl_3$, δ ppm): 5.74 (1H, d, J = 15.0 Hz, H-2), 7.18 (1H, dd, J = 15.0, 10.2 Hz, H-3), 6.12 (1H, dd, J = 15.2, 10.4 Hz, H-4), 6.07 (1H, dt, J = 15.1, 6.4 Hz, H-5), 2.13 (2H, dt, J = 7.2, 6.9 Hz, H-6), 1.39 (2H, m, H-7), 1,32 – 1,2 (4H, m, H-8, H-9), 0.87 (3H, t, J = 7.0 Hz, H-10), 3.15 (1H, t, J = 6.5 Hz, H-1′), 1.79 (1H, sept, J = 6.7 Hz, H-2′), 0.92 (6H, d, J = 6.7 Hz, H-3′,4′), 5.49 (1H, brs, NH). The data are identical with those published by Ley et al. (2004).

N-isobutyl-4,5-epoxy-2*E*-decaenamide (**2**): amorphous solid; ^1H NMR (500 MHz, $CDCl_3$,δ ppm): 6.06 (1H, d, J = 15.2 Hz, H-2), 6.65 (1H, dd, J = 15.2, 6.4 Hz, H-3), 3.19 (1H, brd, J = 6.4 Hz, H-4), 2.85 (1H, dt, J = 6.4, 1.9 Hz, H-5), 1.60 (2H, m, H-6), 1.43 (2H, m, H-7), 1.32 (4H, m, H-8, H-9), 0.90 (3H, t, J = 7.3 Hz, H-10), 3.16 (1H, t, J = 6.5 Hz, H-1′), 1.80 (1H, sept, J = 6.7 Hz, H-2′), 0.93 (6H, d, J = 6.7 Hz, H-3′,4′), 5.5 (1H, brs, NH). The data are in good agreement with those published by Wei et al. (2004).

8-Geranyloxypsoralen (**3**): white crystals; mp. 57–59 °C; ^1H NMR (500 MHz, $CDCl_3$,δ ppm): 6.35 (1H, d, J = 9.6 Hz, H-3), 7.75 (1H, d, J = 9.6 Hz, H-4), 7.35 (1H, s, H-5), 6.80 (1H, d, J = 2.1 Hz, H-6), 7.68 (1H, d, J = 2.1 Hz, H-7), 5.01 (3H, m, H-1′, H-7′), 5.59 (2H, t, J = 7.0 Hz, H-2′), 1.69 (3H, s, H-4′), 2.00 (4H, m, H-5′, H-6′), 1.56 (3H, s, H-9′), 1.64 (3H, s, H-10′); ^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 160.5 (C-2), 114.7 (C-3), 144.3 (C-4), 113.2 (C-5), 106.7 (C-6), 146.6 (C-7), 143.1 (C-8), 144.0 (C-9), 116.5 (C-10), 125.8 (C-11), 149.0 (C-12), 70.1 (C-1′), 119.4 (C-2′), 131.6 (C-3′), 16.5 (C-4′), 39.6 (C-5′), 26.3 (C-6′), 123.8 (C-7′), 131.7 (C-8′), 17.6 (C-9′), 25.6 (C-10′). NMR chemical shifts are in good agreement with the published values (Miyake et al., 1999).

8-(7′,8′-Epoxygeranyloxy)psoralen (**4**): amorphous solid; ^1H NMR (500 MHz, $CDCl_3$,δ ppm): 6.37 (1H, d, J = 9.6 Hz, H-3), 7.76 (1H, d, J = 9.6 Hz, H-4), 7.36 (1H, s, H-5), 6.81 (1H, d, J = 2.1 Hz, H-6), 7.69 (1H, d, J = 2.1 Hz, H-7), 5.03 (2H, d, J = 7.1 Hz, H-1′), 5.65 (2H, t, J = 7.1 Hz, H-2′), 1.73 (3H, s, H-4′), 2.17 (1H, m, H-5′), 2.14 (1H, m, H-5′), 1.59 (2H, m, H-6′), 2.64 t (1H, J = 6.2 Hz, H-7′), 1.24 (3H, s, H-9′), 1.28 (3H, s, H-10′). The data are in good agreement with those published by Ziegler and Spiteller (1992).

Xanthotoxol (**5**): white crystal; mp. 249–250 °C. Identification of **5**

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