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Themed Section: The pharmacology of TRP channels

RESEARCH PAPER

Effect of chirality and lipophilicity in the functional activity of evodiamine and its analogues at TRPV1 channels

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BACKGROUND AND PURPOSE

Evodiamine, a racemic quinazolinocarboline alkaloid isolated from the traditional Chinese medicine *Evodiae fructus*, has been reported to act as an agonist of the transient receptor potential vanilloid type-1 (TRPV1) cation channel both *in vitro* and *in vivo*. Evodiamine is structurally different from all known TRPV1 activators, and has significant clinical potential as a thermogenic agent. Nevertheless, the molecular bases for its actions are still poorly understood.

EXPERIMENTAL APPROACH

To investigate the structure-activity relationships of evodiamine, the natural racemate was resolved, and a series of 23 synthetic analogues was prepared, using as the end point the intracellular Ca^{2+} elevation in HEK-293 cells stably overexpressing either the human or the rat recombinant TRPV1.

KEY RESULTS

S-(+) evodiamine was more efficacious and potent than *R*-(–) evodiamine, and a new potent lead (Evo30) was identified, more potent than the reference TRPV1 agonist, capsaicin. In general, potency and efficacy correlated with the lipophilicity of the analogues. Like other TRPV1 agonists, several synthetic analogues could efficiently desensitize TRPV1 to activation by capsaicin.

CONCLUSIONS AND IMPLICATIONS

Evodiamine qualifies as structurally unique lead structure to develop new potent TRPV1 agonists/desensitizers.

LINKED ARTICLES

This article is part of a themed section on the pharmacology of TRP channels. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2014.171.issue-10

Abbreviations

DMSO, dimethyl sulfoxide; IRTX, 5-iodo-resiniferatoxin; PI3K, phosphoinositite 3-kinase

Introduction

Transient receptor potential (TRP) channels are a large family of non-selective cation channels variously regulated (De Petrocellis and Di Marzo, 2005). They respond to physical and chemical stimuli such as temperature, pH, light, osmolarity, touch, pheromones, oxidative stress and lipids (Venkatachalam and Montell, 2007). Mutations in many of the genes that encode TRP channels cause pathological conditions known as 'TRP channelopathies' (Nilius and Owsianik, 2010). Members of the TRP vanilloid-(TRPV1-V4), melastatin-(TRPM2, M3, M5 and M8) and ankiryn-type (TRPA1) subfamilies are gated by temperature changes (<15 to >53°C), and are known as 'thermoTRPs'. ThermoTRPs are expressed in sensory nerve terminals and initiate sensory nerve impulses following the detection of chemical and thermal stimuli (Patapoutian *et al.*, 2009).

TRPV1 (Caterina *et al.*, 1997) is a unique pain-sensing transducer playing a pivotal role in the maintenance of inflammatory conditions secondary to tissue injury by trauma, infection, surgery, burns or diseases with an inflammatory component (Jara-Oseguera *et al.*, 2008; Basbaum *et al.*, 2009). It is also known as the 'capsaicin receptor', and was initially described in dorsal root, trigeminal and nodose ganglia neurons (Caterina *et al.*, 1997). TRPV1 displays wide tissue and cellular expression in both peripheral and central nervous systems (Cristino *et al.*, 2006; Moran *et al.*, 2011), and in both neuronal and non-neuronal cells, its highest expression being in sensory neurons (Sanchez *et al.*, 2001). It is activated by pungent compounds such as capsaicin and resiniferatoxin, spider and tarantula toxins, noxious temperatures (>42°C) and low pH (<5.9) (Caterina *et al.*, 1997; Siemens *et al.*, 2006; Bohlen *et al.*, 2010). Endogenous mediators, like the endocannabinoid anandamide (Zygmunt *et al.*, 1999) and some eicosanoids (Hwang *et al.*, 2000; De Petrocellis *et al.*, 2012; Wen *et al.*, 2012), also activate TRPV1 channels. Molecular-modelling techniques demonstrated that the preferential conformations of these compounds in solution substantially overlap with those of capsaicin (Movahed *et al.*, 2005), although none of these endogenous compounds is as potent as this natural product. TRPV1 undergoes desensitization in the continuous presence of an activating stimulus, explaining why TRPV1 agonists can produce paradoxical analgesic and anti-inflammatory actions. Receptor desensitization is Ca^{2+}-dependent and implies dephosphorylation of the receptor (whereas phosphorylation usually causes its sensitization) and its endocytosis and degradation in lysosomes (Sanz-Salvador *et al.*, 2012). The validation of TRPV1 as a therapeutic target for pain prompted the development of several TRPV1 antagonists that have entered clinical trials for the treatment of acute, chronic and neuropathic pain (Basbaum *et al.*, 2009; Ferrer-Montiel *et al.*, 2012). Additionally, in the nose, stimulation of TRPV1 in nasal fibres elicits release of proinflammatory mediators, which contribute to rhinitic symptoms, so that blockade of the channel with SB-705498 counteracts such symptoms (Changani *et al.*, 2013). The TRPV1 antagonists SB-705498 and PF-04065463 also inhibited evoked-airway hyper-responsiveness to histamine. Indeed, TRPV1 is present on peripheral terminals of airway sensory nerves and modulation of its activity represents a potential target for the pharmacological treatment of airway hyper-responsiveness (Delescluse *et al.*, 2012). Functional TRPV1 channels are also expressed in rat peripheral arteries where they appear to exhibit different pharmacological properties from those located in sensory neurons (Czikora *et al.*, 2012). Thus, vascular TRPV1 in activation may represent an unwanted effect of TRPV1 antagonists when used as analgesics *in vivo*. On the other hand, vascular TRPV1 may be a new therapeutic target for the regulation of tissue blood distribution (Czikora *et al.*, 2012). Finally, TRPV1 and the neuropeptide substance P located on sensory neurones and non-neuronal cells were recently suggested to be important targets in sepsis and in this context TRPV1 seems to play a protective role (Bodkin and Fernandes, 2013).

The pharmacophore of TRPV1 antagonists fits within the pore region of a TRPV1 receptor homology model, with critical hydrogen bond interactions proposed between the antagonist and Tyr 667 on helix six. The molecular determinants that are required for activation or inhibition of TRPV1 by chemical ligands have been largely identified (Jordt and Julius, 2002; Gavva *et al.*, 2004; Phillips *et al.*, 2004), with the pore helix playing an important role in channel functionality and activation (Myers *et al.*, 2008).

TRPV1 can be activated by evodiamine (Figure 1), a racemic quinazolinocarboline alkaloid isolated from the fruits of the traditional Chinese medicine *Evodiae fructus* (Pearce *et al.*, 2004). This is a remedy known as Wu-Chu-Yu and used for the management of a diverse series of conditions (angina pectoris, hypertension, impotence, postpartum haemorrhage, pain, vomiting, pyresis, gastrointestinal disorders and headache), some of which are apparently unrelated

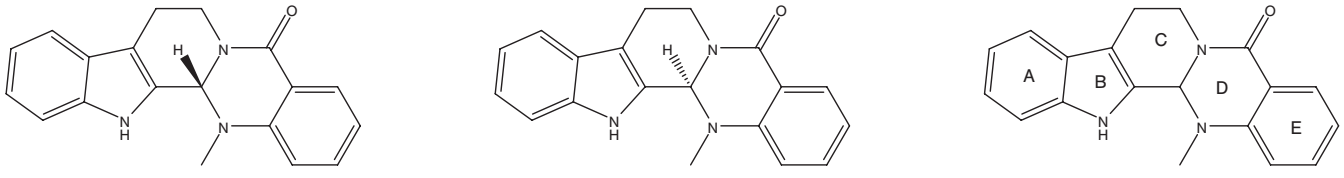


Figure 1
Structure of *S*(+) Evodiamine; *R*(–) Evodiamine and (±) Evodiamine.

to TRPV1 activity (Kobayashi, 2003). Evodiamine activates guinea pig, rat and mouse TRPV1 (Kobayashi *et al.*, 2000; 2001a,b; Kobayashi, 2003; Pearce *et al.*, 2004; Rang *et al.*, 2004) and shows anti-anxiety (Lu *et al.*, 2012), anti-obesity (Kobayashi *et al.*, 2001b; Wang *et al.*, 2008; Bak *et al.*, 2010), antinociceptive (Kobayashi, 2003), anti-inflammatory (Ko *et al.*, 2007), anti-allergic (Rang *et al.*, 2003) and anticancer (Kan *et al.*, 2004; 2007; Liao *et al.*, 2005; Yang *et al.*, 2008; 2009; 2013; Jiang and Hu, 2009; Chen *et al.*, 2010; Wang *et al.*, 2010; Dong *et al.*, 2012) properties, which are often attenuated by TRPV1 antagonists. Evodiamine inhibits the proliferation of a wide variety of tumour cells by inducing their apoptosis (Lee *et al.*, 2006; Zhang *et al.*, 2010; Tu *et al.*, 2013) and shows anti-angiogenesis effects (Shyu *et al.*, 2006). In endothelial cells, evodiamine and capsaicin induce nitric oxide production and endothelial nitric oxide synthase phosphorylation, by interacting with intracellular proteins important for the regulation of vascular tone, like calmodulin and PI3K/Akt (Chiou *et al.*, 1996; Domenicali *et al.*, 2005; Wang and Wang, 2005; Yao and Garland, 2005; Ching *et al.*, 2011). Administered chronically, evodiamine also produces TRPV1-dependent protection against atherosclerosis in mice (Wei *et al.*, 2013). The thermogenic potential of evodiamine has made it popular in the health food market as a non-pungent slimming agent, even though this activity has never been conclusively demonstrated in the clinic, and only relies on animal and cellular experiments (Wang *et al.*, 2008).

The interaction between evodiamine and TRPV1 is not fully understood. Previous mutation studies have suggested that the affinity of agonists with rat TRPV1 is affected by Tyr511, Ser512, Leu515, Phe543, Met547 and Lys571 (Lee *et al.*, 2011). With the aim of understanding the molecular basis for the recognition of evodiamine by TRPV1, a homology model has been constructed and the specific interaction between the two molecules has been studied using computational approaches. According to this model, ring A of evodiamine (Figure 1) establishes a hydrophobic interaction with Tyr511, while ring E forms π-π interactions with Tyr555 of human TRPV1. In addition, evodiamine makes two H-bonds between the carbonyl oxygen and the side chains of Lys571 and between the indole nitrogen and the side chains of Ile569 (Wang *et al.*, 2012). Capsaicin and evodiamine share certain pharmacophoric elements, but their lipophilic moiety is different, encompassing a saturated isononenyl group in capsaicin, and two phenyl rings in evodiamine. Animal species differences in the capability of activating TRPV1 have been reported for capsaicin but not evodiamine.

Apart from any structural difference between capsaicin and evodiamine, a critical, and surprisingly so far overlooked, issue is that capsaicin is achiral, while evodiamine is chiral. The recognition model for evodiamine is chiral, and a large difference in bioactivity is therefore to be expected between its enantiomers. In this context, the identification of the active enantiomeric form should provide the basis for any future structure-activity study on this interesting lead compound. To address these issues, we have compared the activity of the two enantiomers of evodiamine, and a series of synthetic enantiomerically pure analogues at both human and rat TRPV1 stably overexpressed in HEK-293 cells.

Methods

Synthesis, purification, resolution and characterization of evodiamine analogues

The synthesis of racemic evodiamine was carried out according to the related literature procedure (Danieli and Palmisano, 1978). Racemic evodiamine was condensed with (1*S*)-10-camphorsulfonyl chloride to provide diastereomer derivatives, the hydrolysis of which by NaOH (10%) provided the pure enantiomers (*S*)-evodiamine and (*R*)-evodiamine in almost quantitative yields (90%). The synthesis of evodiamine derivatives was accomplished in a synthetic sequence starting from L-tryptophan methyl ester hydrochloride, which in a two-step sequence provided Evo 05. Then, two cyclizations furnished its epimer at position 3, Evo 06, in a diastereomeric ratio of 8:2. Hydrolysis of the ester with LiOH and treatment of the corresponding acid or acyl chloride with various amines or hydroxyl derivatives afforded the desired compounds. The enantiomeric compounds were obtained starting from D-tryptophan methyl ester hydrochloride (D. Passarella, A. Sacchetti, M. Christodoulou, in preparation).

Assays of TRPV1-mediated elevation of intracellular [Ca^{2+}]

HEK-293 cells stably overexpressing recombinant human TRPV1 were selected by G-418 (Geneticin, Invitrogen Life Technologies, Grand Island, NY, USA; 600 μg·mL^{-1}), grown on 100-mm diameter Petri dishes as monolayers in minimum essential medium supplemented with non-essential amino acids, 10% FBS, and 2 mM glutamine, and maintained under 5% CO_2 at 37°C. On the day of the experiment, the cells were loaded for 1 h at 25°C with the cytoplasmic calcium indicator Fluo-4AM (Invitrogen Life Technologies) 4 μM in DMSO containing 0.02% Pluronic F-127 (Invitrogen). After loading, cells were washed twice in Tyrode's buffer (145 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose and 10 mM HEPES, pH 7.4), resuspended in the same buffer, and transferred to a quartz cuvette of the spectrofluorimeter (Perkin-Elmer LS50B; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) under continuous stirring (about 100 000 cells for assay). $[Ca^{2+}]_i$ was determined before and after the addition of various concentrations of test compounds by measuring cell fluorescence (excitation λ = 488 nm; emission λ = 516 nm). The potency (EC_{50} values) was determined as the concentration of test compound required to produce half-maximal increases in $[Ca^{2+}]_i$. The efficacy of the agonists was determined at 10 μM by comparing their effect with the analogous effect observed with 4 μM ionomycin (LKT Laboratories, Inc. St. Paul, MN, USA). Dose-response curves were fitted by a sigmoidal regression with variable slope. Curve fitting and parameter estimation were performed with GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA). All determinations were conducted at least in triplicate, and the compounds were tested also on wild-type HEK293 cells (i.e. not transfected with any TRP construct): when significant, the values of the effect on $[Ca^{2+}]_i$ in wild-type HEK293 cells were taken as baselines and subtracted from the values obtained from transfected cells. Statistical analysis of the data was performed by analysis of

variance at each point using ANOVA followed by the Bonferroni's test. LogP was calculated according to Tetko *et al*. (2005).

All molecular target nomenclature conformed with the BJP's Concise Guide to PHARMACOLOGY (Alexander *et al*., 2013).

Results

In agreement with previous literature, natural and racemic evodiamine produced a dose-dependent increase in intracellular calcium in HEK-293 cells stably transfected with the human recombinant TRPV1, with an EC_{50} = 155.20 ± 8.2 nM. When tested on the rat recombinant TRPV1, the EC_{50} was about fourfold higher (652.2 ± 26.7). Resolution of the racemate afforded the two enantiomers, and *S*-(+) evodiamine resulted more efficacious and almost more than fourfold more potent than *R*-(–) evodiamine at both human (EC_{50} = 113.4 ± 8.9 and 546.0 ± 24.2 nM, respectively) and rat (391.5 ± 3.3 and 1491 ± 46 nM, respectively) TRPV1 (Table 1, Figure 2). The specificity of the receptor response was verified by pretreating the cells for 5 min with the selective TRPV1 antagonist 5-iodo-resiniferatoxin (Wahl *et al*., 2001) at a concentration of 10 nM before the addition of the compounds. The effects of 1 μM (±)-, (+)- and (–)-evodiamine were all inhibited by 100% (Figure 2 and data not shown). Likewise, (±)-, (+)- and (–)-evodiamine were all inactive in untransfected HEK-293 cells (Figure 2 and data not shown).

In the light of these observations, a series of synthetic analogues of evodiamine were prepared, resolved and independently assayed. In all cases, the analogues of *S*(+)-evodiamine were more potent than those of *R*(–)-evodiamine (Table 2), with the following rank of potency:

VR002 < Evo 44 < Evo 09 < Evo 23 < Evo 28 < Evo 06 < Evo 46 < Evo 42 < Evo 38 < Evo 34 < Evo 30. The corresponding EC_{50} values ranked between ~5 μM and ~2 nM for both human and rat TRPV1, with VR002 being inactive.

Table 1

Effect of racemic and resolved evodiamine enantiomers on elevation of intracellular [Ca^{2+}] in HEK-293 cells overexpressing either the human or rat (r) TRPV1 channel

Compound	Efficacy (at 10 μM ± SE)	Potency EC_{50} ± SE, nM	Desensitization of 0.1 μM capsaicin response IC_{50} ± SE, nM	LogP ± SD
S(+) Evodiamine	82.9 ± 1.5	113.4 ± 8.9	176.9 ± 3.7	2.95 ± 0.37
	r 66.3 ± 0.1	r 391.5 ± 3.3	r 260.9 ± 17.6	
R(–) Evodiamine	76.0 ± 0.8	546.0 ± 24.2	674.9 ± 99.1	2.95 ± 0.37
	r 55.4 ± 0.1	r 1491 ± 46	r 1580 ± 40	
(+/–) Evodiamine	79.4 ± 0.9	155.2 ± 8.2	215.8 ± 7.3	2.95 ± 0.37
	r 71.9 ± 0.4	r 652.2 ± 26.7	r 523.4 ± 17.0	

All tests were carried out six times, and the compounds were tested also on HEK-293 cells not transfected with the TRPV1 receptor: none produced a significant elevation of intracellular [Ca^{2+}] (Figure 2). The specificity of the receptor response was also verified by pretreating the cells transfected with human or rat TRPV1 for 5 min with the specific antagonist 5-iodo-resiniferatoxin (Wahl *et al*., 2001) at a concentration of 10 nM before the addition of the compound at 1 μM: this resulted in a complete inhibition of evodiamine activity at TRPV1 (Figure 2).

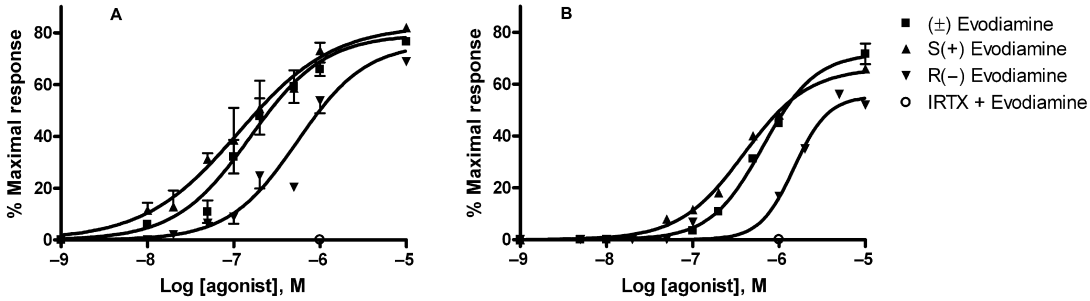


Figure 2

Dose-dependent effects of (±) evodiamine; *S*(+) evodiamine and *R*(–) evodiamine on TRPV1-mediated elevation of intracellular calcium in HEK-293 cells stably overexpressing the human (A) or the rat (B) recombinant TRPV1 channel. Data are means of $n = 6$ separate experiments. The specificity of the receptor response was also verified by pretreating the cells transfected with TRPV1 for 5 min with the specific antagonist 5-iodo-resiniferatoxin (IRTX) (Wahl *et al*., 2001) at a concentration of 10 nM before the addition of the compound at the concentration of 1 μM. This resulted in a complete inhibition of the activity of all three evodiamines at TRPV1 (empty circle).

Table 2

Effect of synthetic evodiamine analogues and their enantiomers on elevation of intracellular calcium in HEK-293 cells overexpressing either the human or rat (r) TRPV1 channel

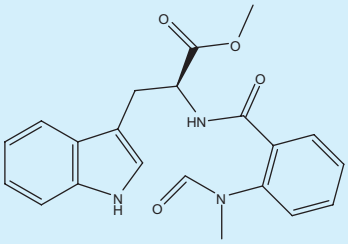
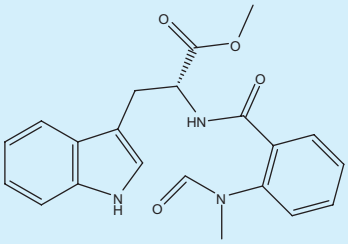
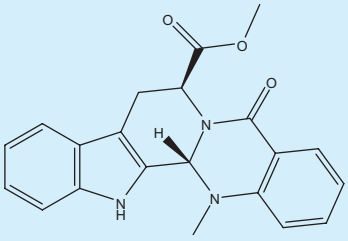
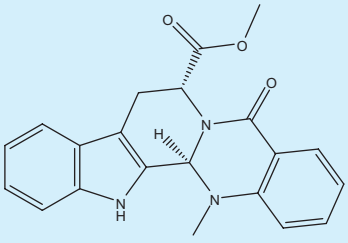
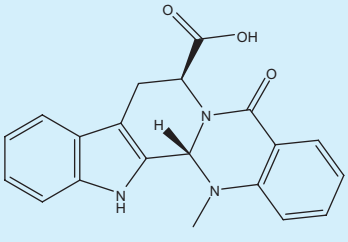
Compound	Structure	Efficacy (at 10 μM ± SE)	Potency EC_{50} ± SE	Desensitization of 0.1 μM capsaicin response IC_{50} ± SE	LogP ± SD
Evo 05		<10 0	NA NA	NA NA	1.48 ± 0.46
Evo 15		0 r < 10 (8.5 ± 0.1)	NA NA	NA NA	1.48 ± 0.46
Evo 06		70.0 ± 0.9 r 69.0 ± 3.2	1.18 ± 0.08 μM r 2.21 ± 0.40 μM	1.13 ± 0.04 μM r 1.68 ± 0.17 μM	2.77 ± 0.50
Evo 21		73.2 ± 1.7 r 62.2 ± 1.0	3.06 ± 0.37 μM r 5.42 ± 0.33 μM	4.42 ± 0.09 μM r 5.26 ± 0.19 μM	2.77 ± 0.50
Evo 09		10.6 ± 0.6 r < 10 (4.9 ± 0.1)	4.56 ± 1.16 μM NA	NA NA	2.45 ± 0.51

Table 2

Continued

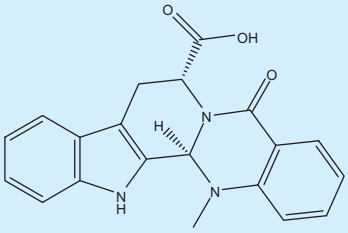
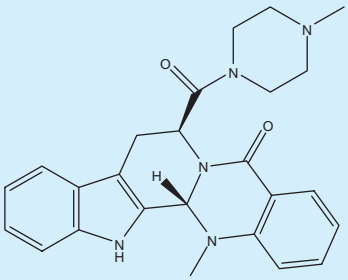
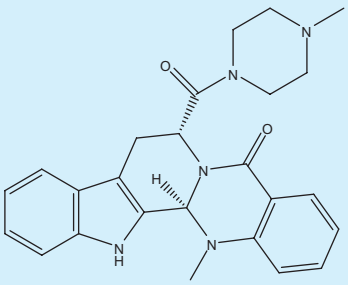
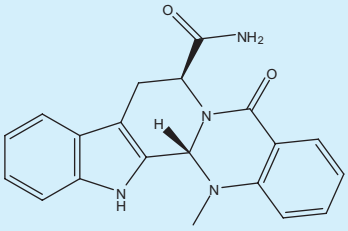
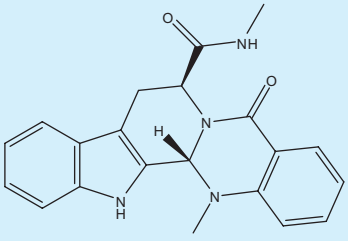
Compound	Structure	Efficacy (at 10 μM ± SE)	Potency EC_{50} ± SE	Desensitization of 0.1 μM capsaicin response IC_{50} ± SE	LogP ± SD
Evo 22		<10 (4.4 ± 0.6) r < 10 (6.5 ± 0.1)	NA NA	NA NA	2.45 ± 0.51
Evo 23		41.7 ± 1.0 r 25.1 ± 0.6	4.25 ± 0.50 μM r 3.12 ± 0.37 μM	7.31 ± 0.60 μM r 12.32 ± 0.74 μM	2.10 ± 0.72
Evo 29		12.9 ± 1.0 r < 10 (9.5 ± 0.1)	9.76 ± 2.17 μM NA	NA NA	2.10 ± 0.72
Evo 28		17.8 ± 0.7 r 16.1 ± 0.5	4.00 ± 0.83 μM r 5.74 ± 0.1 μM	34.06 ± 0.06 μM r 27.24 ± 7.10 μM	1.54 ± 0.85
VR002		<10 (3.9 ± 0.1) r < 10 (7.4 ± 0.1)	NA NA	NA NA	1.91 ± 0.80

Table 2

Continued

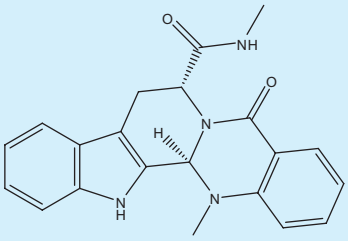
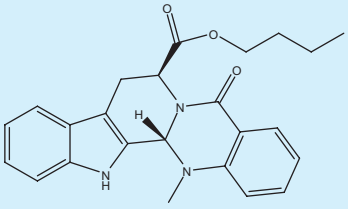
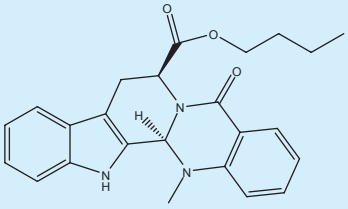
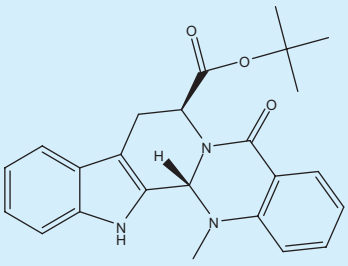
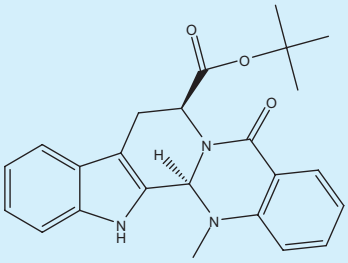
Compound	Structure	Efficacy (at 10 μM ± SE)	Potency EC_{50} ± SE	Desensitization of 0.1 μM capsaicin response IC_{50} ± SE	LogP ± SD
VR001		<10 (1.0 ± 0.1) r < 10 (1.2 ± 0.1)	NA NA	NA NA	1.91 ± 0.80
Evo 30		67.1 ± 0.9 r 72.1 ± 1.0	2.01 ± 0.11 nM r 2.45 ± 0.20 nM	1.65 ± 0.15 nM r 1.61 ± 0.04 nM	3.96 ± 0.57
Evo 31		51.2 ± 1.2 r 38.3 ± 0.8	89.6 ± 14.5 nM r 0.20 ± 0.02 μM	0.18 ± 0.01 μM r 0.28 ± 0.01 μM	3.96 ± 0.57
Evo 34		69.4 ± 0.8 r 69.5 ± 2.4	25.9 ± 1.2 nM r 89.1 ± 17.7 nM	25.95 ± 0.44 nM r 31.5 ± 1.4 nM	3.79 ± 0.49
Evo 35		41.8 ± 1.0 r 46.2 ± 1.9	0.33 ± 0.04 μM r 0.76 ± 0.16 μM	0.36 ± 0.02 μM r 1.05 ± 0.13 μM	3.79 ± 0.49

Table 2

Continued

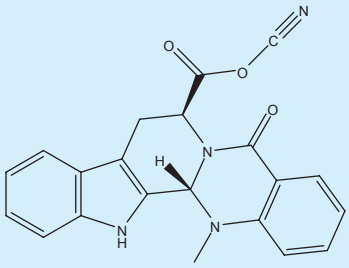
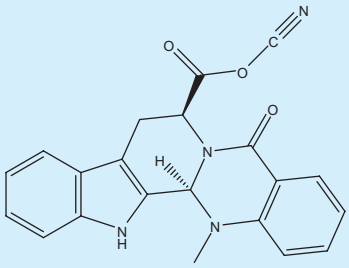
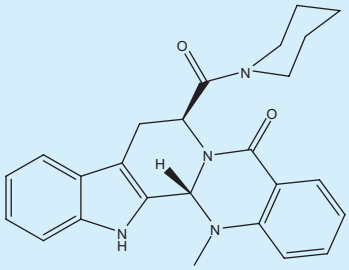
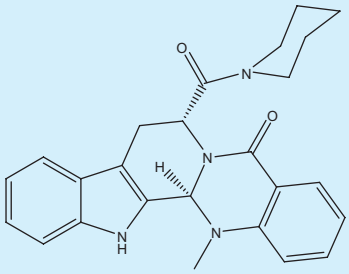
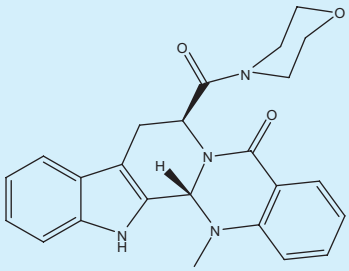
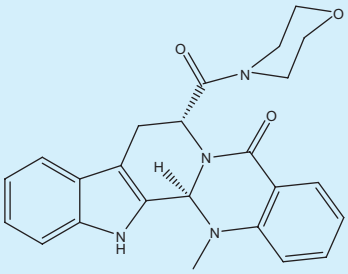
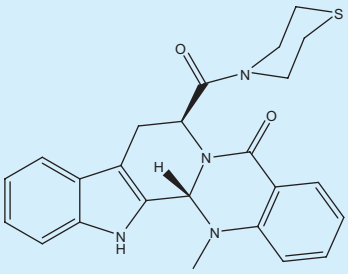
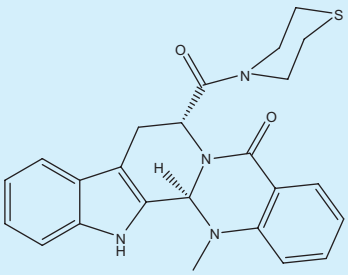
Compound	Structure	Efficacy (at 10 μM ± SE)	Potency EC_{50} ± SE	Desensitization of 0.1 μM capsaicin response IC_{50} ± SE	LogP ± SD
Evo 38		68.4 ± 0.6 r 50.2 ± 0.2	59.2 ± 2.4 nM r 76.1 ± 1.5 nM	45.1 ± 2.0 nM r 80.1 ± 2.4 nM	2.59 ± 1.02
Evo 39		58.3 ± 0.8 r 48.4 ± 1.1	96.5 ± 6.1 nM r 0.12 ± 0.02 μM	0.12 ± 0.01 μM r 0.22 ± 0.03 μM	2.59 ± 1.02
Evo 42		58.4 ± 0.6 r 48.9 ± 0.2	0.21 ± 0.01 μM r 0.87 ± 0.02 μM	0.38 ± 0.01 μM r 0.64 ± 0.03 μM	3.26 ± 0.48
VR007		60.4 ± 1.1 r 54.3 ± 2.1	3.08 ± 0.19 μM r 6.69 ± 0.94 μM	2.67 ± 0.10 μM r 6.94 ± 0.58 μM	3.26 ± 0.48
Evo 44		29.1 ± 1.9 r 34.3 ± 0.6	5.43 ± 1.23 μM r 5.43 ± 0.85 μM	5.35 ± 0.20 μM r 6.24 ± 0.40 μM	2.01 ± 0.63

Table 2

Continued

Compound	Structure	Efficacy (at 10 μM ± SE)	Potency EC_{50} ± SE	Desensitization of 0.1 μM capsaicin response IC_{50} ± SE	LogP ± SD
VR003		<10 (4.3 ± 0.1) r < 10 (5.6 ± 0.1)	10 μM NA	57.76 ± 6.63 μM NA	2.01 ± 0.63
Evo 46		55.5 ± 0.8 r 44.5 ± 0.6	0.27 ± 0.01 μM r 0.74 ± 0.03 μM	0.47 ± 0.01 μM r 0.98 ± 0.04 μM	2.83 ± 0.64
VR005		66.7 ± 0.5 r 51.7 ± 1.0	6.99 ± 0.14 μM r 6.85 ± 0.42 μM	5.91 ± 0.09 μM r 10.33 ± 0.63 μM	2.83 ± 0.64

All tests were carried out at least in triplicate, and the compounds were tested also on HEK-293 cells not transfected with the TRPV1 receptor: none produced a significant elevation of intracellular [Ca^{2+}] (not shown). The specificity of the receptor response for the substances that showed a substantial effect on intracellular [Ca^{2+}], was verified also by pretreating the human or rat TRPV1 transfected cells for 5 min with the specific antagonist 5-iodo-resiniferatoxin at the concentration of 10 nM before the addition of the compound (1 μM), and full antagonism was observed in each case (not shown). NA = not active.

The rank of potency of the synthetic *R*(–)-enantiomers was considerably different (VR001 = VR003 = Evo 22 < Evo 29 < VR005 < Evo 21 ~ VR007 < Evo 35 < Evo 31 ~ Evo 39), and the corresponding EC_{50} values ranked between ~10 and 0.1 μM, with VR001, VR003 and Evo 22 being inactive. The lipophilicity of these compounds is reported in Table 2, with LogP values ranking between ~1.9 and ~4. The rank, excluding compound Evo 28, which had a very low logP value (logP = 1.5), was as follows: VR002 = VR001 < Evo 44 = VR003 < Evo 23 = Evo 29 < Evo 09 = Evo 22 < Evo 38 = Evo 39 < Evo 46 = VR005 = Evo 06 = Evo 21 < Evo 42 = VR007 < Evo 34 = Evo 35 < Evo 30 = Evo 31. The Spearman's rank order correlation index between the EC_{50} at human and rat TRPV1 and the logP values of the synthetic *S*(+)-evodiamine enantiomers compounds was calculated and was r = –0.85 ($P < 0.001$) and –0.82 ($P < 0.001$), respectively; for the synthetic *R*(–)-evodiamine enantiomers it was r = –0.80 ($P = 0.003$) and –0.74 ($P = 0.01$) respectively. Therefore, a strong positive correlation exists between lipophilicity and potency of evodiamine analogues at both human and rat TRPV1.

Finally, the compounds, given to cells with a 5 min pre-incubation, were also found to desensitize the human recombinant TRPV1 to the intracellular Ca^{2+}-elevating effects of 100 nM capsaicin (Tables 1 and 2).

Discussion

The natural indoloquinazolone alkaloid evodiamine has a pentacyclic U-shaped structure, with a peripheral lipophilic core, the basic amino group in the concavity of the molecular structure, and the polar amide group on the central part of the outer rim. The evodiamine chemotype is unique within TRPV1 agonists, and, in general, very few heterocyclic chemotypes of natural activators are known, the best examples being the acyl amides of salsolinol (*N*-arachidonoylsalsolinol), an endogenous tetrahydroisoquinoline formed by a Pictet–Spengler condensation of dopamine with acetaldehyde (O'Dell *et al.*, 2007), and a number of tetrahydro-β-carbolines (Ortar *et al.*, 2013). Among TRPV1 agonists, little chiral discrimination had been observed so far within vanillamides of ricinoleic acid (Appendino *et al.*, 2006) and analogues of anandamide (Appendino *et al.*, 2009). Instead, within TRPV1 antagonists, enantiomeric pairs of indazole derivatives showed chiral discrimination, with the (*R*)-enantiomers being up to 30-fold more potent than their (*S*)-counterparts (Gomtsyan *et al.*, 2007). Within antagonists of another TRP channel, TRPA1, enantiomeric discrimination was demonstrated in dihydropyrimidones (Gijsen *et al.*, 2012) and for a *N*-1-Alkyl-2-oxo-2-chlorophenyl amide (Vallin *et al.*, 2012) ligand. Within antagonists of TRPM8, arylglycine derivatives were very recently identified, the two diastereomers of which were separated and the absolute configurations determined by Vibrational Circular Dichroism: the (*S,S*)-isomer [(*S*)-1-((*S*)-2-(2-fluorophenyl)pyrrolidin-1-yl)-2-(2-fluorophenylamino)-2-(4-(trifluoromethyl)phenyl)ethanone] and the corresponding (*R,S*)-isomer. The former diastereomer was more potent at inhibiting icilin-induced canine TRPM8 functional activity *in vitro* (IC_{50} = 0.006 μM) than the latter (IC_{50} = 0.045 μM) (Zhu *et al.*, 2013).

Chirality is often an important feature of drug efficacy; remarkably, the chiral discrimination observed with evodiamine could also be confirmed in a series of synthetic analogues, with compounds from the *S*-series being more potent than those from the *R*-series (Table 2). A similar level of enantiodifferentiation was also observed when a selection of enantiomeric pairs was assayed here on rat TRPV1.

Regarding the structure-activity relationships of evodiamine analogues, most chemical modifications were detrimental for activity. Thus, the opening of the five-membered ring (Evo 05 vs. Evo 15), the introduction of an acetamide- (Evo 28), an *N*-methyl-acetamide- (VR001), a methoxycarbonyl- (Evo 06 and Evo 21), a carboxyl- (Evo 09 and Evo 22) or a piperazylamido group- (Evo 23) on ring C, all caused a decrease of potency. The introduction of an oxycyano carbonyl group in the same position resulted in compound Evo 38 with a potency similar to *S*(+)-evodiamine, and compound Evo 39 with a potency higher than *R*(–)-evodiamine. Particularly interesting appeared to be the butoxycarbonyl derivatives Evo 30 and Evo 34, which showed activity in the low nanomolar range. Recent investigations have been devoted to the identification of TRPV1 regions involved in the recognition by some ligands and in the prediction of their binding modes (Kym *et al.*, 2009; Lee *et al.*, 2011; Wang *et al.*, 2012). The presence of a *tert*-butylphenyl group is frequently found in TRPV1 ligands, presumably because of the potentiation of π-π stacking and hydrophobic interactions (Vriens *et al.*, 2009). Finally, amidation with morpholine (Evo 44) (EC_{50} = 5.43 μM), piperidine (Evo 42) (EC_{50} = 210 nM) and thiomorpholine (Evo 46) (EC_{50} = 270 nM) produced compounds less potent than *S*(+)-evodiamine (EC_{50} = 113 nM).

With the exception of Evo28, exhibiting a very low LogP value (1.5), the rank of potency of the synthetic (*S*) enantiomers correlated nicely with lipophilicity. Recently, a strong correlation was reported, within TRPV1 agonists, between the kinetics of induction of calcium influx, pungency and lipophilicity, although the correlation was not linear for the extreme values of LogP (Ursu *et al.*, 2010). Other recent studies have shown that the lipophilicity of capsaicinoids can strongly influence both their efficacy at, and kinetics of activation of, TRPV1. We previously described the activity at the human TRPV1 of several capsaicin analogues, and found that *N*-arachidonyl-vanillamide (arvanil) (De Petrocellis *et al.*, 2000) and *N*-retinoyl-vanillamide (retvanil) (Appendino *et al.*, 2005), are among the most potent 'capsaicinoid' TRPV1 agonists. It was shown that the ligand (capsaicin, resiniferatoxin and anandamide) binding site of TRPV1 lies on the inner face of the plasma membrane and that much of TRPV1 itself is localized to internal membranes (Jung *et al.*, 1999; 2002; Zygmunt *et al.*, 1999; De Petrocellis *et al.*, 2001). Thus, lipophilicity of the agonist plays an important role in its ability to penetrate the cell membrane and interact with TRPV1 binding site, and influences both the kinetics and potency of vanilloids at the channel (Lazar *et al.*, 2006). *N*-palmitoyl-vanillamide (palvanil) (Melck *et al.*, 1999) exhibited a kinetics of activation of human recombinant TRPV1-mediated intracellular calcium elevation significantly slower than that of capsaicin (logP = 7.15 ± 0.79 and 3.74 ± 0.52, respectively) and exhibited no pungency in the eye-wiping assay in mice, as well as stronger desensitizing effects on TRPV1 and anti-hyperalgesic activity (De Petrocellis *et al.*, 2011). Two other capsaicin analogues with decreased pungency, arvanil and *N*-oleoyl-vanillamide (olvanil), are highly lipophilic due to the increased length of the fatty acid chain (logP = 7.20 ± 1.14 and 7.55 ± 0.91, respectively) and exhibit an EC_{50} of about 0.5 nM in a calcium assay, much lower than that of capsaicin (De Petrocellis *et al.*, 2000).

In conclusion, we present evidence that chiral discrimination exists in the vanilloid activity of evodiamine, and that lipophilicity is critical for this activity. These results indicate that evodiamine may represent a useful starting point for the development of new potent TRPV1 agonists/desensitizers, and confirm the pharmacological potential of the combination of natural building blocks to provide new bioactive compounds.

Conflict of interest

GF is an employee of Indena S.p.a.

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