Bioorganic & Medicinal Chemistry Letters 20 (2010) 2729–2732

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# (−)-Menthylamine derivatives as potent and selective antagonists of transient receptor potential melastatin type-8 (TRPM8) channels

Giorgio Ortar [a,*], Luciano De Petrocellis [b,*], Ludovica Morera [a], Aniello Schiano Moriello [b], Pierangelo Orlando [c], Enrico Morera [a], Marianna Nalli [a], Vincenzo Di Marzo [d]

[a] Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, piazzale Aldo Moro 5, 00185 Roma, Italy
[b] Endocannabinoid Research Group, Istituto di Cibernetica, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, 80078, Pozzuoli (Napoli), Italy
[c] Endocannabinoid Research Group, Istituto di Biochimica delle Proteine, Consiglio Nazionale delle Ricerche, via P. Castellino 111, 80131 Napoli, Italy
[d] Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, via dei Campi Flegrei 34, 80078, Pozzuoli (Napoli), Italy

ARTICLE INFO

*Article history:*
Received 4 February 2010
Revised 17 March 2010
Accepted 19 March 2010
Available online 25 March 2010

*Keywords:*
Transient receptor potential channels
TRPA1
TRPV1
TRPM8
(−)-Menthylamine derivatives

ABSTRACT

A series of twenty-two (−)-menthylamine derivatives was synthesized and tested on TRPM8, TRPV1, and TRPA1 channels. Five of the novel compounds, that is, **1d**, **1f**, **2b**, **2c**, and **2e** behaved as potent TRPM8 antagonists with $IC_{50}$ values versus icilin and (−)-menthol between 20 nM and 0.7 μM, and were between 4- and ~150-fold selective versus TRPV1 and TRPA1 activation. Compound **1d** also induced caspase 3/7 release in TRPM8-expressing LNCaP prostate carcinoma cells, but not in non-TRPM8 expressing DU-145 cells. Five other derivatives, that is, **1a**, **1g**, **1h**, **2f**, and **2h** were slightly less potent than previous compounds but still relatively selective versus TRPV1 and TRPA1.



Ion channels belonging to the transient receptor potential (TRP) superfamily that are activated by distinct temperature thresholds are referred to as 'thermo-TRPs'[1] and their targeting represents a novel and promising strategy in pain relief.[2] Four of these channels (TRPV1–TRPV4) respond to heat and two others (TRPA1 and TRPM8) are sensitive to cold. One of the best-investigated thermo-TRPs is the transient receptor potential channel of melastatin type-8 (TRPM8), which is activated by moderately cool temperatures (<23–28 °C) and by compounds that evoke a sensation of coolness, such as (−)-menthol, the homomenthylamide WS-12 and icilin (Fig. 1).[3] Interestingly, both (−)-menthol and icilin stimulate human TRPA1 channels as well,[4] and a number of TRPV1 antagonists such as capsazepine, BCTC, and SB-452533 and the non-specific blocker of various calcium channels SKF96365 have been reported to be also potent TRPM8 antagonists,[5] indicating a significant degree of pharmacological overlap between TRPA1, TRPV1 and TRPM8 channels. In contrast, WS-12 elicits a robust response in TRPM8 expressing HEK cells ($EC_{50}$ = 30[6] and 193 nM[3]) and in *Xenopus* oocytes ($EC_{50}$ = 12 μM),[7] while none of related TRP channels like TRPM3 and TRPV6[3] and of the other thermo-TRPs[7] are activated at a concentration optimally effective for TRPM8 responses. The localization of TRPM8 in both Aδ and C-fibers may account for abnormal cold sensitivity in some pathologic states, thus providing a rationale for the design of TRPM8 modulators as novel antihyperalgesic or antiallodynic agents,[8] although the role of TRPM8 in cold allodynia in patients with cold injury was recently questioned.[9]

The expression of TRPM8 in tissues not subjected to temperature changes suggests, however, other important functions for this ion channel and indeed a series of benzyloxyphenylamides and carbamates,[10] and of phosphorus-containing benzothiophene and benzofuran derivatives[11] has been disclosed in the patent literature as TRPM8 antagonists for the treatment of urological and respiratory disorders. Last but not least, TRPM8 is overexpressed in a range of cancers including prostate, breast, lung, and colon, while, within normal tissues, it is predominantly expressed in the human prostate.[6,12] Its knock-down in human prostate carcinoma cells using mRNA silencing techniques was reported to inhibit cell proliferation.[12] Thus, TRPM8 is a tumor marker with potential use in cancer diagnosis as well as therapy.

With this background, TRPM8 agonists and antagonists based on the 3-substituted-*p*-menthane structure have been reported in a patent application to be effective at inhibiting growth of cells expressing TRPM8 and/or inducing their apoptosis and/or necrosis, but no evidence for their capability of modulating the functional

* Corresponding authors. Tel.: +39 6 49913612; fax: +39 6 49693268 (G.O.); tel.: +39 081 8675173 (L.D.P.).
*E-mail addresses:* giorgio.ortar@uniroma1.it (G. Ortar), l.depetrocellis@cib.na.cnr.it (L.D. Petrocellis).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.03.076

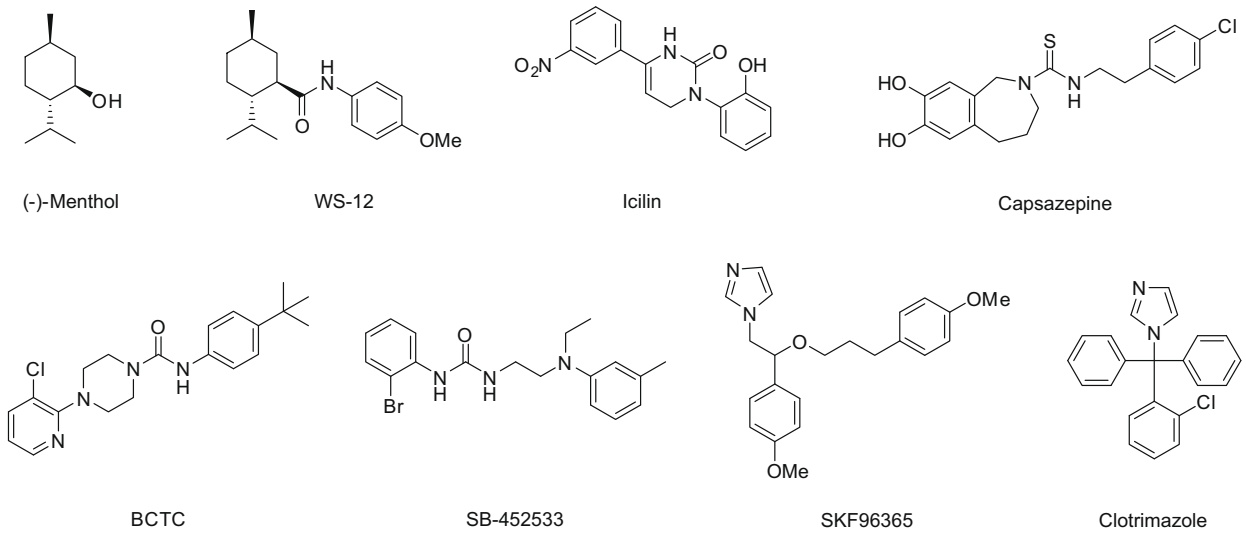



**Figure 1.** Structures of some TRPM8 activators and antagonists.

activity of recombinant TRPM8 selectively over other TRP channels was provided.[13] Recently, clotrimazole, an antimycotic drug widely used for the topical treatment of candidiasis and ringworm infections, was described as a potent TRPM8 antagonist ($IC_{50}$ ~200 nM for the inhibition of inward TRPM8 currents) and a useful tool to discriminate between TRPM8- and TRPA1-mediated responses, although this compound also activated TRPV1 channels.[14] Thus, lack of selectivity represents the most important problem that prevents the use of known TRPM8 antagonists, as it complicates the interpretation of their effects in vitro and in vivo. To the best of our knowledge, no truly selective TRPM8 antagonist has been reported to date.

With the aim of gaining further insight into the functional and pharmacological properties of thermo-TRPs and in an attempt to identify compounds with the ability to discriminate between TRPM8, TRPV1 and TRPA1, we prepared 22 new derivatives of (−)-menthylamine and examined their functional activity at these three channels (Table 1).

The synthesis of amides **1a–i** was carried out by condensation of (−)-menthylamine hydrochloride (**4**)[15] and the appropriate carboxylic acids **5a–i** using 1-hydroxybenzotriazole (HOBt)/*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as the carboxylate activator (Scheme 1).[16] Carbamates **2a–j** and ureas 3a–**c** were synthesized by condensation of (−)-menthylisocyanate (**6**)[15] with the appropriate phenols **7a–j** or amines **8a–c** (Scheme 2).[16]

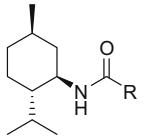
The choice of analogues was based on substituents found in other TRP modulators, with the reasoning that their presence on the (−)-menthylamine scaffold could boost their potency or selectivity toward TRPM8. Thus, for instance, the *t*-Bu-Ph, oleyl, Cl-Ph, $CF_3$-Ph, serotonin and vanillamine groups of **1b, 1c, 1g, 1h, 2b, 2c, 2d, 2e, 2h, 2i, 3b**, and **3c** could mimic the same groups of TRPV1 ligands BCTC, capsazepine, *N*-oleoylethanolamine, *N*-arachidonoylserotonin (AA-5-HT), capsaicin, and of piperazinyl carbamates and ureas recently found to act as fatty acid amide hydrolase (FAAH) and TRP ligands.[17]

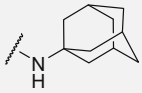
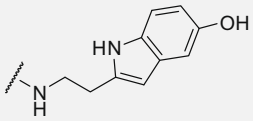
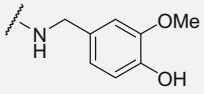
The novel 22 compounds synthesized here were tested for their ability to induce intracellular $Ca^{2+}$ elevation in HEK-293 cells stably transfected with either the human TRPV1, the rat TRPA1, or the rat TRPM8 cDNAs. Control experiments were carried out using non-transfected HEK-293 cells. The compounds that were found to be inactive at elevating intracellular $Ca^{2+}$ were then given to cells 5 min before known agonists of TRPM8, TRPV1, and TRPA1 channels, that is, menthol or icilin, capsaicin, and mustard oil isothiocyanate, respectively, to check whether or not they exhibited any functional antagonism. Most compounds were found to efficiently antagonize the agonist effect of either menthol or icilin on TRPM8-mediated intracellular $Ca^{2+}$ elevation in HEK-293 cells overexpressing rat TRPM8 channels, and, usually only at much higher concentrations, to stimulate intracellular $Ca^{2+}$ elevation in HEK-293 cells overexpressing rat TRPA1 or human TRPV1 channels, but not in wild-type HEK-293 cells.

The results of the pharmacological assays are shown in Table 1 and the salient aspects of the structure–activity relationships (SARs) can be summarized as follows. Five of the novel compounds examined, that is, **1d, 1f, 2b, 2c**, and **2e** behaved as potent TRPM8 antagonists with $IC_{50}$ values versus icilin and (−)-menthol between 20 nM and 0.7 μM, and were between 4- and ~150-fold selective versus TRPV1 and TRPA1 activation. Five other derivatives, that is, **1a, 1g, 1h, 2f**, and **2h**, although slightly less potent than the above mentioned compounds, were still relatively selective versus TRPV1 and TRPA1. Three other compounds, that is, **1e, 2a**, and **2j** elicited a potent TRPM8 antagonist activity but were not selective versus TRPA1 activation. Finally, three compounds, that is, **1b, 3a**, and **3c** exhibited a significant and rather selective TRPV1 agonist activity. With the exception of **1b, 1c**, and **1i**, all *N*-menthyl amides **1** and *N*-menthyl carbamates **2** showed potent TRPM8 antagonism with no clear-cut superiority of one type of functionality over the other. The inactivity of compound **1b** was therefore rather unexpected in view of the good antagonist activity of the carbamate congener **2e**. Thus, reversal of the amide linkage shifted the agonism of *p*-menthane-based TRPM8 ligands toward antagonism, whereas that of the carbamate function retained full antagonist activity.[13] The reverse amide **1i**, which directly compares with WS-12, did not exhibit however a particularly good TRPM8 antagonist potency. The selectivity towards TRPV1 and TRPA1 of compounds of the **1** and **2** series was appreciably influenced by the nature and position of the substituent on the aromatic moiety and by the length of the alkyl chain in the case of ω-phenylalkylamides. In this respect, compounds with meta substituted aromatic rings were generally more selective than the para substituted ones, irrespective of the nature of the substituent (see compounds **2b, 2f, 2h** and **2c, 2g, 2i**). Finally, the three urea derivatives **3** were uniformly inactive as TRPM8 antagonists.

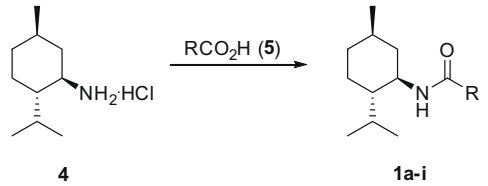
The most potent and highly selective (>80-fold) TRPM8 functional antagonist synthesized here, compound **1d**, was also tested

**Table 1**
Results of TRPM8, TRPV1, and TRPA1 assays of (−)-menthylamine derivatives **1–3**[a]



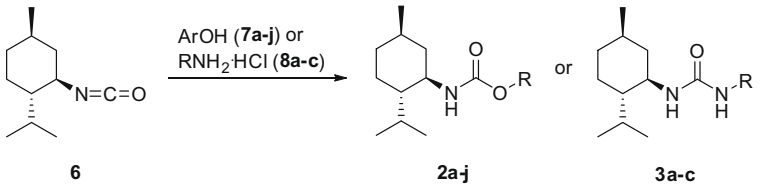
| Compound | R | TRPM8[b] ($IC_{50}$, μM) | TRPM8[c] ($IC_{50}$, μM) | TRPV1 (efficacy)[d] | TRPV1 ($EC_{50}$, μM) | TRPA1 (efficacy)[e] | TRPA1 ($EC_{50}$, μM) |
|---|---|---|---|---|---|---|---|
| **1a** | Ph-4-Me | 2.5 ± 0.1 | 2.2 ± 0.1 | 5.4 ± 2.1 | NM | NM | NM |
| **1b** | Ph-4-*t*-Bu | 60.8 ± 9.3 | 97.6 ± 11.9 | 27.4 ± 1.9 | 0.25 ± 0.10 | 137.6 ± 10.1 | 4.9 ± 1.7 |
| **1c** | Oleyl | >100 | 60.6 ± 4.4 | 4.4 ± 0.4 | NM | NM | NM |
| **1d** | Ph-4-Ph | 0.05 ± 0.01 | 0.02 ± 0.002 | 7.8 ± 0.3 | NM | 34.7 ± 0.01 | 4.1 ± 0.01 |
| **1e** | $(CH_2)_6Ph$ | 0.4 ± 0.01 | 0.4 ± 0.03 | 8.0 ± 0.01 | NM | 42.8 ± 1.9 | 0.4 ± 0.1 |
| **1f** | $(CH_2)_7Ph$ | 0.5 ± 0.1 | 0.4 ± 0.04 | 5.5 ± 0.01 | NM | 67.0 ± 2.2 | 26.7 ± 1.3 |
| **1g** | Ph-3-Cl | 2.1 ± 0.1 | 2.8 ± 0.2 | 41.9 ± 1.8 | 7.6 ± 0.6 | NM | NM |
| **1h** | Ph-4-Cl | 1.6 ± 0.1 | 0.7 ± 0.02 | 53.8 ± 0.8 | 4.1 ± 0.2 | 222.6 ± 20.7 | 40.2 ± 12.8 |
| **1i** | Ph-4-OMe | 9.6 ± 3.5 | 12.8 ± 0.6 | 9.6 ± 0.5 | NM | NM | NM |
| **2a** | OPh-4-Me | 0.7 ± 0.1 | 1.1 ± 0.04 | 3.4 ± 1.0 | NM | 73.9 ± 2.4 | 0.7 ± 0.1 |
| **2b** | OPh-3-$CF_3$ | 0.6 ± 0.03 | 0.3 ± 0.04 | 7.4 ± 1.5 | NM | 220.2 ± 20.7 | 13.8 ± 7.8 |
| **2c** | OPh-4-$CF_3$ | 0.7 ± 0.06 | 0.2 ± 0.01 | 3.3 ± 0.01 | NM | 88.9 ± 9.9 | 3.0 ± 1.4 |
| **2d** | OPh-3-*t*-Bu | 3.3 ± 0.6 | 5.9 ± 1.1 | 16.3 ± 0.6 | 5.8 ± 1.0 | 272.8 ± 35.2 | 36.7 ± 17.7 |
| **2e** | OPh-4-*t*-Bu | 0.08 ± 0.01 | 0.1 ± 0.02 | 23.8 ± 1.1 | 18.3 ± 2.4 | 76.0 ± 2.3 | 11.7 ± 1.4 |
| **2f** | OPh-3-Ph | 1.5 ± 0.4 | 0.05 ± 0.002 | 50.9 ± 1.0 | 7.1 ± 0.6 | 282.5 ± 16.1 | 31.0 ± 5.2 |
| **2g** | OPh-4-Ph | 7.4 ± 0.4 | 0.7 ± 0.06 | 4.4 ± 1.8 | NM | 87.2 ± 6.9 | 6.7 ± 2.6 |
| **2h** | OPh-3-Cl | 0.5 ± 0.04 | 1.5 ± 0.2 | 22.6 ± 3.1 | 19.2 ± 8.3 | 309.0 ± 52.1 | 30.0 ± 17.5 |
| **2i** | OPh-4-Cl | 4.1 ± 0.4 | 0.8 ± 0.03 | 15.1 ± 3.8 | 42.8 ± 23.8 | 181.2 ± 25.4 | 7.1 ± 3.7 |
| **2j** | OPh-4-OMe | 2.6 ± 0.2 | 2.2 ± 0.2 | 46.6 ± 3.0 | 53.8 ± 3.6 | 134.7 ± 30.3 | 3.2 ± 2.0 |
| **3a** |  | >100 | >100 | 24.4 ± 0.1 | 1.0 ± 0.06 | 158.8 ± 12.4 | 11.2 ± 3.4 |
| **3b** |  | 31.0 ± 0.7 | 15.8 ± 0.8 | 12.7 ± 0.01 | 52.2 ± 0.03 | NM | NM |
| **3c** |  | 19.8 ± 0.6 | 30.5 ± 2.9 | 63.2 ± 0.8 | 0.23 ± 0.02 | NM | NM |

NM, not measurable when efficacy is lower than 10%.
[a] Data are means ± SEM of $N = 3$ determinations.
[b] Determined against the effect of icilin (0.25 μM).
[c] Determined against the effect of menthol (20 μM).
[d] As percent of ionomycin (4 μM).
[e] As percent of allyl isothiocyanate (100 μM).




**Scheme 1.** Synthesis of compounds **1a–i**. Reagents and conditions: **5**, HOBt/EDC, rt, 1 h, then **4**, $Et_3N$, DMF, rt, 16 h.

on the human androgen-responsive prostate carcinoma cell line, LNCaP, which expresses high levels of TRPM8, and as a negative control, on the DU-145 cell line, which, like other androgen-unresponsive prostate carcinoma cell lines, does not express appreciable levels of TRPM8 channels.[18] As expected, and as previously shown for other TRPM8 antagonists,[13] compound **1d** (1 μM) induced the apoptosis of LNCaP, but not DU-145, cells, as measured by the release of caspase 3/7 (from 16521 ± 3779 to 58576 ± 29921, in LNCaP cells, $P$ <0.01; and from 9281 ± 247 to 9768 ± 1496, not significant, in DU-145 cells; arbitrary units,




**Scheme 2.** Synthesis of compounds **2a–j** and **3a–c**. Reagents and conditions: $Et_3N$, DMF or AcOEt, rt, 16 h.

means ± SD of $N = 3$ experiments). In LNCaP cells, the compound was as efficacious as the standard pro-apoptotic mixture of anti-FAS antibody and camptothecin[19] (from 16521 ± 3779 to 58954 ± 5130 in LNCaP cells, $P < 0.01$; and from 9281 ± 247 to 165595 ± 15965, in DU-145 cells; arbitrary units, means ± SD of $N = 3$ experiments).

In conclusion, in the present work we have disclosed a series of derivatives of (−)-menthylamine that act as potent TRPM8 antagonists with $IC_{50}$ values similar or lower than those of previously reported unselective antagonists. Special attention should be paid to compounds **1d, 1f, 2b, 2c**, and **2e**, the excellent selectivity of which may allow for their use as pharmacological tools, thus aiding future biological studies aimed at deciphering the multiple roles of TRPM8 in mammalian species. The pro-apoptotic effect of **1d** also warrants further studies aiming at investigating its potential therapeutic use against prostate carcinoma.

## Acknowledgment

The authors are grateful to Roberta Verde for valuable technical help.

## Supplementary data

Supplementary data (detailed experimental procedures and characterization data for all products and biochemical assays) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.076.

## References and notes

1. Venkatachalam, K.; Montell, C. *Annu. Rev. Biochem.* **2007**, *76*, 387.
2. Cortright, D. N.; Szallasi, A. *Curr. Pharm. Des.* **2009**, *15*, 1736.
3. Bödding, M.; Wissenbach, U.; Flockerzi, V. *Cell Calcium* **2007**, *42*, 618.
4. Kühn, F. J. P.; Kühn, C.; Lückhoff, A. *J. Biol. Chem.* **2009**, *284*, 4102.
5. Wiel, A.; Moore, S. E.; Waite, N. J.; Randall, A.; Gunthorpe, M. J. *Mol. Pharmacol.* **2005**, *68*, 518.
6. Beck, B.; Bidaux, G.; Bavencoffe, A.; Lemonnier, L.; Thebault, S.; Shuba, Y.; Barritt, G.; Skryma, R.; Prevarskaya, N. *Cell Calcium* **2007**, *41*, 285.
7. Sherkheli, M. A.; Gisselmann, G.; Vogt-Eisele, A. K.; Doerner, J. F.; Hatt, H. *Pak. J. Pharm. Sci.* **2008**, *21*, 370.
8. (a) Fleetwood-Walker, S.; Mitchell, R.; Proudfoot, C. W. J. PCT Int. Appl. WO2008015403, 2008.; (b) Xing, H.; Chen, M.; Ling, J.; Tan, W.; Gu, J. G. *J. Neurosci.* **2007**, *27*, 13680; (c) Proudfoot, C. J.; Garry, E. M.; Cottrell, D. F.; Rosie, R.; Anderson, H.; Robertson, D. C.; Fleetwood-Walker, S. M.; Mitchell, R. *Curr. Biol.* **2006**, *16*, 1591.
9. Namer, B.; Kleggetveit, I. P.; Handwerker, H.; Schmelz, M.; Jorum, E. *Pain* **2008**, *139*, 63.
10. (a) Lampe, T.; Alonso-Alija, C.; Beck, H.; Rosentreter, U.; Sandner, P.; Stahl, E.; Stelte-Ludwig, B. PCT Int. Appl. WO2007017094, 2007.; (b) Lampe, T.; Alonso-Alija, C.; Beck, H.; Rosentreter, U.; Sandner, P.; Stahl, E.; Stelte-Ludwig, B. PCT Int. Appl. WO2007017093, 2007.; (c) Lampe, T.; Alonso-Alija, C.; Bauser, M.; Beck, H.; Rosentreter, U.; Sandner, P.; Stahl, E.; Stelte-Ludwig, B. PCT Int. Appl. WO2007017092, 2007.; (d) Lampe, T.; Alonso-Alija, C.; Stelte-Ludwig, B.; Sandner, P.; Bauser, M.; Beck, H.; Lustig, K.; Rosentreter, U.; Stahl, E.; Takagi, H. PCT Int. Appl. WO2006040136, 2006.
11. Colburn, R. W.; Dax, S. L.; Flores, C.; Matthews, J.; Qin, N.; Youngman, M. A.; Teleha, C.; Reany, L. PCT Int. Appl. WO2007134107, 2007.
12. (a) Kim, S.-H.; Nam, J.-H.; Park, E.-J.; Kim, B.-J.; Kim, S.-J.; So, I.; Jeon, J.-H. *Biochim. Biophys. Acta, Mol. Bas. Dis.* **2009**, *1792*, 33; (b) Prevarskaya, N.; Zhang, L.; Barritt, G. *Biochim. Biophys. Acta, Mol. Bas. Dis.* **2007**, *1772*, 937; (c) Flockerzi, V.; Wissenbach, U. *BIOspektrum* **2005**, *11*, 620.
13. Natarajan, S. K.; Moreno, O.; Graddis, T. J.; Duncan, D.; Laus, R.; Chen, F. PCT Int. Appl. WO2005020897, 2005.
14. Meseguer, V.; Karashima, Y.; Talavera, K.; D'Hoedt, D.; Donovan-Rodriguez, T.; Viana, F.; Nilius, B.; Voets, T. *J. Neurosci.* **2008**, *28*, 576.
15. Schopohl, M. C.; Bergander, K.; Kataeva, O.; Fröhlich, R.; Waldvogel, S. R. *Synthesis* **2003**, 2689.
16. General procedure for the synthesis of compounds **1a**–**i**. To a stirred solution of **5a**–**i** (0.25 mmol) in DMF (1 mL) were added at 0 °C HOBt (0.26 mmol) and EDC (0.26 mmol). The mixture was stirred for 15 min at 0 °C and for 1 h at room temperature. Then (−)-menthylamine hydrochloride (**4**) (0.30 mmol) and $Et_3N$ (0.30 mmol) were added, and the mixture was stirred overnight at room temperature. The mixture was diluted with brine and extracted with AcOEt. The organic phase was washed with 2 N HCl solution, saturated $NaHCO_3$, and brine, dried ($Na_2SO_4$), and evaporated under vacuum. The residue was purified by column chromatography or crystallized from $CH_2Cl_2$/hexane. General procedure for the synthesis of compounds **2a**–**j** and **3a**–**c**. A solution of (−)-menthylisocyanate (**6**) (0.47 mmol), **7a**–**j** or **8a**–**c** (0.47 mmol), and $Et_3N$ (0.56 mmol) in dry DMF or AcOEt (1 mL) was stirred overnight at room temperature. The mixture was diluted with brine and extracted with AcOEt. The organic phase was washed twice with brine, dried ($Na_2SO_4$), and evaporated under vacuum. The residue was purified by column chromatography or crystallized from MeOH.
17. Morera, E.; De Petrocellis, L.; Morera, L.; Schiano Moriello, A.; Ligresti, A.; Nalli, M.; Woodward, D. F.; Di Marzo, V.; Ortar, G. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6806.
18. Prevarskaya, N.; Flourakis, M.; Bidaux, G.; Thebault, S.; Skryma, R. *Biochem. Soc. Trans.* **2007**, *35*, 133.
19. Costa-Pereira, A. P.; Cotter, T. G. *Br. J. Cancer* **1999**, *80*, 371.