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Pain Relief Drugs

The Engineering of an Orally Active Conotoxin for the Treatment of Neuropathic Pain**

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Neuropathic pain results from damage to, or disruption of, function in the nervous system and is associated with a wide range of conditions, including diabetes, shingles, leprosy, multiple sclerosis, HIV/AIDS, stroke, cancer, and nerve damage due to trauma or surgery.[1] Treatment of neuropathic pain still presents a significant challenge because current therapeutics, such as morphine, gabapentin and antidepressants are either ineffective in many patients, decline in efficacy over time due to the development of tolerance, or produce severe side-effects.[2]

The venom of marine cone snails has become a valuable source of novel peptidic ligands for a wide variety of membrane receptors and ion channels, and several have potential for the treatment of neuropathic pain, stroke and other neurological conditions. One of these, ω-conotoxin MVIIA, is approved in the U.S. and Europe for the treatment of chronic pain and others are in various stages of clinical or preclinical investigation.[3] However, despite this promise, their potential as therapeutics is limited by their susceptibility to degradation within the body[4] and lack of activity when administered orally. Despite attempts to improve the oral delivery of conotoxins,[5] all current candidate conotoxins require invasive intrathecal delivery, for example, which severely limits their widespread application, increases susceptibility to infection, and is associated with increased morbidity over a preferred oral route of delivery.

We recently showed that the synthetic cyclization of a model conotoxin, MII, stabilized the structure and reduced susceptibility to proteolysis, thus potentially enhancing the therapeutic potential of conotoxins.[4b] In the current study we applied this approach to re-engineer the α-conotoxin Vc1.1, from the cone snail *Conus victoriae*,[6] and demonstrate that the cyclic peptide is orally active, a surprising and exciting finding for a peptide-based molecule. Vc1.1 is a 16-residue peptide with an amidated C-terminus, two disulfide bonds in a Cys^{I}–Cys^{III} and Cys^{II}–Cys^{IV} arrangement, and an α-helical structure typical of all α-conotoxins.[7] It is an effective analgesic in several rat models of neuropathic pain[8] and therefore has attracted considerable interest as a potential treatment for this condition.[3a,9]

The sequence of our engineered cyclic Vc1.1 analogue (cVc1.1) is shown in Figure 1. A linker comprising six residues (GGAAGG) was designed (see Supporting Information) and

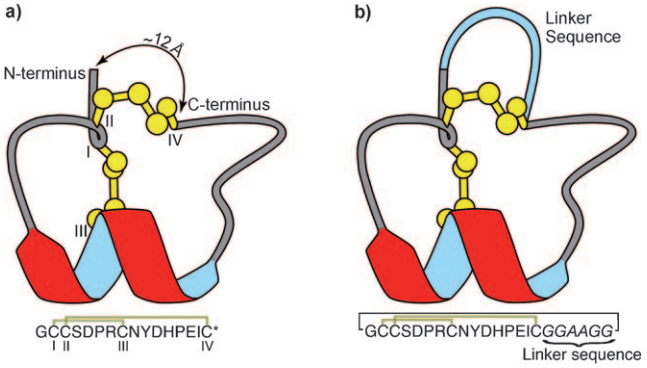

Figure 1. Strategy for the cyclization of the α-conotoxin Vc1.1. a) The sequence and schematic diagram of the three-dimensional structure of Vc1.1, which exhibits the classical α-conotoxin fold, consisting of a helical region and two disulfide bonds in a I–III, II–IV arrangement. In Vc1.1 the C-terminus is amidated, as indicated by the asterisk. The N and C termini of Vc1.1 are separated by ca. 12 Å. b) The sequence and proposed cyclization strategy for cVc1.1. The N and C termini are joined by a linker sequence of six residues. This linker length is sufficiently long so as not to introduce strain into the peptide and hence alter the structure of the conotoxin.

incorporated into the native sequence to join the N and C termini, without affecting the three-dimensional structure or biological activity as assessed by a preliminary screen that tested the peptide's ability to inhibit nicotine-induced catecholamine release (see Supporting Information; Figure S8). The peptide was synthesized by solid phase peptide synthesis using the *tert*-butoxycarbonyl(Boc)/in situ neutralization methodology[10] and cyclized by intramolecular native chemical ligation.[11] A selective disulfide bond strategy was employed, utilizing acetamidomethyl (Acm) protecting groups to produce the same disulfide connectivity as the linear peptide, Cys^{I}–Cys^{III} and Cys^{II}–Cys^{IV}.

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The structural scaffold of α-conotoxins is highly conserved and is thought to be crucial in presenting the relevant side-chains for interaction with receptors.[12] Therefore, when modifying conotoxins it is important to ensure that the native structure is retained. We used NMR spectroscopy to compare the structures of Vc1.1[7] and cVc1.1. An initial indication of a lack of structure perturbation was obtained from Hα secondary shifts, which were almost identical for Vc1.1 and cVc1.1. Negative secondary shifts for residues P6 to D11 indicated helicity in this region of cVc1.1, consistent with the structure of Vc1.1. Conservation of this structural motif is considered to be important for retaining biological activity.[13]

The high resolution structure of cVc1.1 was calculated using distance and angle restraints obtained from TOCSY, NOESY and DQF-COSY NMR spectra (Tables S1 and S2). Figure 2a shows the ensemble of the 20 lowest-energy structures of cVc1.1 superimposed over residues G1 to C16. The structure of cVc1.1 is well defined with a mean rmsd (residues 1 to 16) (Figure 2a) of 0.37 ± 0.17 Å for the backbone atoms. Figure 2b shows that, as predicted from the chemical shift data, cVc1.1 has an α-helical region from residues P6 to D11 and also contains a helical region from P13 to C16, and a type I β turn from residues G1 to S4. Figure 2c shows an overlay of Vc1.1 and its cyclic analogue. The mean structures closely overlay, with a backbone rmsd of 0.55 Å. Interestingly, although the structures are very similar, a comparison of amide temperature coefficient data (Table S4) suggests that the structure of cVc1.1 is somewhat more rigid than Vc1.1.

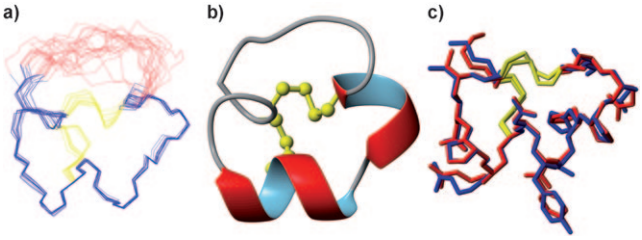

Figure 2. Experimentally determined three-dimensional structure of cVc1.1. a) The ensemble of 20 low-energy structures of cVc1.1 superimposed over the backbone N, Cα, and C atoms from Gly1 to Cys16. b) Ribbon representation of the average structure of cVc1.1 showing the helical region between residues P6 and D11. Disulfide bonds are shown in yellow as ball and sticks. c) A structural comparison of cVc1.1 (red) with Vc1.1 (blue), illustrating the similarities in backbone conformation and sidechain orientation. The linker region in cVc1.1 is omitted for clarity.

Although Vc1.1 has been shown to be an effective analgesic in several rat models of human neuropathic pain,[8] its specific receptor target has not been unequivocally identified. We, and others, have shown that Vc1.1 is a potent antagonist of recombinant α9α10 nicotinic acetylcholine receptors (nAChRs) expressed in *Xenopus* oocytes[9b,14] and we also recently showed that Vc1.1 indirectly inhibits N-type calcium channel currents in rodent dorsal root ganglion (DRG) neurons through activation of $GABA_B$ receptors.[15] Agonists of the $GABA_B$ receptor are known to produce relief of neuropathic pain.[16] The role of α9α10 nAChRs in pain remains unclear and controversial; but targeting of this receptor might cause deleterious side effects as small molecule antagonists of the α9α10 nAChR have been implicated in causing hearing impairment.[17] In the current study, we compared the potency of Vc1.1 and cVc1.1 on α9α10 nAChRs expressed in *Xenopus* oocytes and on high voltage-activated (HVA) calcium channels in rat DRG neurons.

Linear Vc1.1 reversibly inhibited ACh-evoked α9α10 nAChR-mediated currents in a concentration-dependent manner with an IC_{50} of 64.2 ± 15.0 nM and a Hill coefficient of 1.1 ± 0.2 ($n = 12$), similar to that reported previously,[14] whereas the inhibition of ACh-evoked currents by cVc1.1 exhibited an IC_{50} of 765.6 ± 98.2 nM and a Hill coefficient of 1.2 ± 0.1 ($n = 13$) (Figure 3a,b). The inhibition of HVA Ca^{2+} channel currents in rat DRG neurons by linear Vc1.1 was concentration-dependent and the concentration–response relationship exhibited an IC_{50} value of 1.7 nM ($n = 8$–25 cells per data point) with maximum inhibition occurring at 1 μM, as reported previously[15a] (Figure 3c,d). In contrast, cVc1.1 exhibited greater potency and the concentration–response relationship for inhibition of HVA Ca^{2+} channel currents gave an IC_{50} of 0.3 nM ($n = 2$–13 cells per data point). We have proposed that linear Vc1.1 inhibits HVA Ca^{2+} channel currents via activation of $GABA_B$ receptors.[15] It appears likely that the cyclic derivative also acts through a $GABA_B$ receptor dependant pathway; in the presence of the selective $GABA_B$ receptor antagonist CGP 55845 (1 μM), cVc1.1 failed to inhibit the HVA calcium channel currents (Figure S10). Therefore, not only is cVc1.1 a more potent inhibitor of the $GABA_B$-modulated N-type ($Ca_V2.2$) channel, the proposed target for analgesia, than linear Vc1.1, it is also more selective for the inhibition of HVA Ca^{2+} channel currents over the α9α10 nAChR subtype when compared to linear Vc1.1. The extra rigidity of cVc1.1, as suggested by amide temperature coefficient data, might be the reason for this increased selectivity for the inhibition of N-type Ca^{2+} currents.

Although peptides in general display exquisite potency and selectivity for many therapeutic targets, a major limitation to their use as drugs is their ineffectiveness when delivered orally. This limitation is caused by a combination of low uptake and poor stability of peptides within the digestive system. For example, no activity was observed in rat models of neuropathic pain when Vc1.1 was administered orally, even though the peptide exhibited analgesic activity when delivered through subcutaneous injection.[8] An initial comparison of the stability of Vc1.1 and cVc1.1 in simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and human serum revealed that the cyclic analogue was more stable than its linear counterpart (Figure S11). cVc1.1 was slightly more stable when incubated for 24 h in SGF than linear Vc1.1. Interestingly, in SIF and human serum a substantial proportion (42 ± 2% and 46 ± 6%, respectively) of Vc1.1 was observed to disulfide shuffle to an inactive isomer whereas this was not observed for cVc1.1 with 93 ± 7% and 92 ± 4% of the active peptide remaining after 24 h in SIF and serum, respectively. To investigate if this stabilization effect by cyclization translated into oral activity, we administered

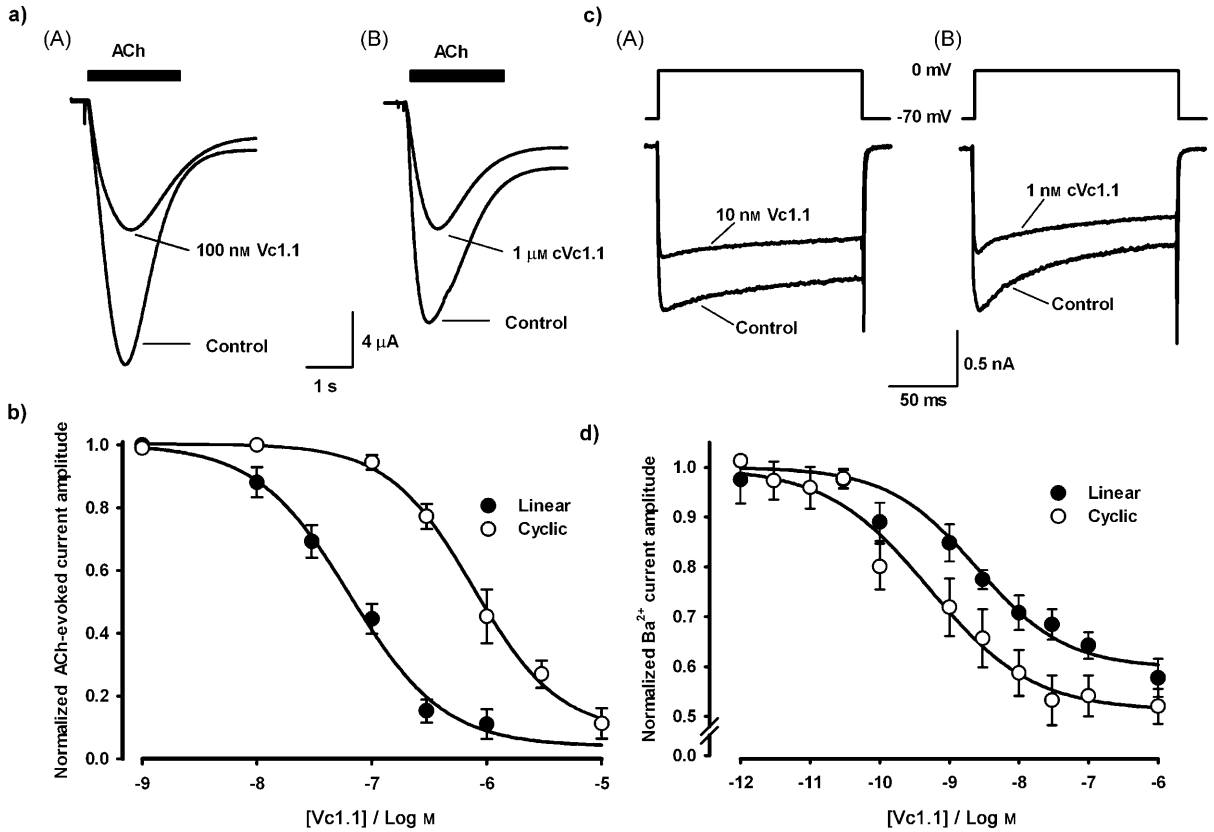

Figure 3. Linear and cyclic Vc1.1 inhibition of α9α10 nAChRs expressed in *Xenopus* oocytes and Ca^{2+} channel currents in rat DRG neurons. a) Superimposed traces of ACh-evoked currents obtained in the absence (control) and presence of 100 nM Vc1.1 (A) and 1 μM cyclic Vc1.1 (B), respectively. α9α10 nAChRs were expressed in *Xenopus* oocytes, voltage clamped at −80 mV and currents were evoked by 30 μM ACh in the absence and presence of the peptides. b) Concentration–response relationships for inhibition of α9α10 nAChR-mediated currents by Vc1.1 and cyclic Vc1.1. Peak current amplitudes were measured before and after 300 s incubation of the peptides. Fits of concentration–response relationships for Vc1.1 (●) and cVc1.1 (○) inhibition gave IC_{50} values of 64.2 ± 15.0 nM ($n_H = 1.1 \pm 0.2$; $n = 12$) and 765.6 ± 98.2 nM ($n_H = 1.2 \pm 0.1$; $n = 13$), respectively. Each data point represents the average of 3–7 oocytes for curve fitting and represents arithmetic means ± standard error of the fit using the logistic equation given in the Supporting Information. c) Superimposed depolarization-activated whole-cell Ba^{2+} currents elicited by a voltage step from a holding potential of −70 mV to 0 mV in the absence (control) and presence of 10 nM Vc1.1 (A) and 1 nM cyclic Vc1.1 (B), respectively. d) Concentration–response relationship for inhibition of high-voltage-activated Ca^{2+} channel currents in DRG neurons by linear Vc1.1 (●) ($n = 25$ cells per data point) and cyclic Vc1.1 (○) ($n = 13$ cells per data point). Data points represent means ± standard error of normalized peak current amplitude. The IC_{50} values for inhibition of Ca^{2+} channel currents by linear Vc1.1 was 1.7 nM and 0.3 nM for cyclic Vc1.1.

cVc1.1 orally to rats in the chronic constriction injury model (CCI) of neuropathic pain.[18] Single oral doses of cVc1.1 at 0.3 and 3.0 mg kg^{-1} produced significant dose-dependent relief of mechanical allodynia, reaching a maximal effect approximately 1 h after treatment (Figure 4a). The mean pain relief, measured as change in paw withdrawal threshold (PWT) area under curve values, produced by single oral doses of cVc1.1 at 0.3 mg kg^{-1} (24 ± 6.8 g, $n = 10$) and 3.0 mg kg^{-1} (58.9 ± 6.9 g, $n = 10$) were significantly larger ($p < 0.05$ and $p < 0.01$, respectively) than that for untreated animals (8.3 ± 1.3 g, $n = 10$). Single oral doses of cVc1.1 at 0.3 and 3.0 mg kg^{-1} did not significantly ($p > 0.05$) alter baseline PWT responses in the uninjured (contralateral) hindpaw of CCI-rats (Figure S12). A second assay was performed with a single dose of cVc1.1 (3 mg kg^{-1}, $n = 10$) over a longer time period, and cVc1.1 was still providing significant pain relief after 4 h (Figure S13).

Gabapentin is a widely used drug for the treatment of neuropathic pain. To assess the relative effectiveness of cVc1.1 as an oral analgesic, we compared the activity of cVc1.1 with gabapentin. The analgesic effect of cVc1.1 in the CCI-rat model was found to be comparable to that of gabapentin (72 ± 7.0 g min, $n = 10$) administered at 30 mg kg^{-1}, approximately 120 times the dosage of cVc1.1 (Figure 4). Thus cVc1.1 is more than two orders of magnitude more potent than the current leading drug for neuropathic pain, and we are continuing development towards clinical trials in humans.

In summary, we have developed an orally active peptide, based on a natural conotoxin, with exciting potential for the treatment of neuropathic pain. Our orally active peptide exhibits higher selectivity and potency for $GABA_B$ receptor-mediated N-type Ca^{2+} channel currents, the proposed target for analgesia, than the parent linear peptide. Current treatments for neuropathic pain are effective for only 40–60% of patients and are associated with a range of unwanted side effects.[2b] Given that conotoxins act on a range of receptors involved in pain pathways, members of this group offer a

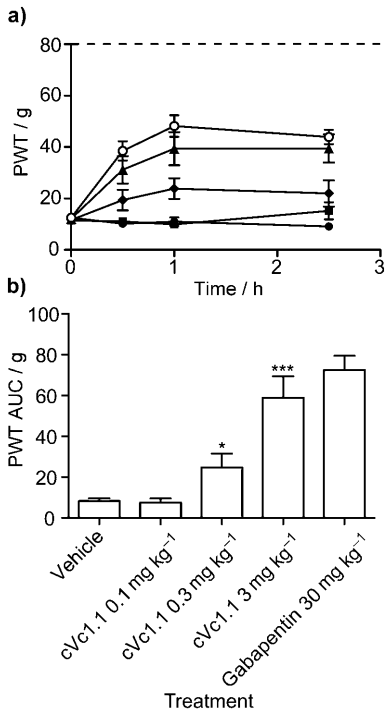

Figure 4. Oral dosing of cVc1.1 produces significant pain relief in the CCI rat model of neuropathic pain. a) Time course of paw withdrawal thresholds (PWT) in CCI-rats after single oral doses of 0.1 mg kg^{-1} (■), 0.3 mg kg^{-1} (◆), and 3 mg kg^{-1} (▲) ($n=10$) of cVc1.1; and of 30 mg kg^{-1} of gabapentin (○) compared to vehicle (●). b) Total area under curve values showing that a single dose of cVc1.1 at either 0.3 mg kg^{-1} or 3 mg kg^{-1} provides significant pain relief compared to the control ($p<0.1$ (*) and 0.01 (***), respectively). A single oral dose of cVc1.1 at 3 mg kg^{-1} produced similar pain relief of tactile allodynia in CCI-rats to that of gabapentin at 30 mg kg^{-1} ($n=10$).

potential source of novel analgesics. By developing an orally active analogue of Vc1.1, we have substantially enhanced its potential as a new therapy for neuropathic pain. More generally, the high potency and selectivity of peptides and their applicability to an enormous range of medical conditions make them attractive leads for drug development. However, for their potential to be fully realized, approaches for improving their pharmaceutical properties, including improved stability and oral activity, must be developed. In this study, we have demonstrated that backbone cyclization can be used to achieve this goal.

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[1] World Health Organization in *Neurological Disorders: Public Health Challenges* (Ed.: D. C. Bergen), World Health Organization, Geneva, **2006**.
[2] a) G. Cruccu, *Curr. Opin. Neurobiol.* **2007**, *20*, 531; b) R. H. Dworkin, A. B. O'Connor, M. Backonja, J. T. Farrar, N. B. Finnerup, T. S. Jensen, E. A. Kalso, J. D. Loeser, C. Miaskowski, T. J. Nurmikko, R. K. Portenoy, A. S. Rice, B. R. Stacey, R. D. Treede, D. C. Turk, M. S. Wallace, *Pain* **2007**, *132*, 237.
[3] a) B. G. Livett, K. R. Gayler, Z. Khalil, *Curr. Med. Chem.* **2004**, *11*, 1715; b) H. Terlau, B. M. Olivera, *Physiol. Rev.* **2004**, *84*, 41; c) R. J. Lewis, *Prog. Mol. Subcell. Biol.* **2009**, *46*, 45.
[4] a) C. J. Armishaw, N. L. Daly, S. T. Nevin, D. J. Adams, D. J. Craik, P. F. Alewood, *J. Biol. Chem.* **2006**, *281*, 14136; b) R. J. Clark, H. Fischer, L. Dempster, N. L. Daly, K. J. Rosengren, S. T. Nevin, F. A. Meunier, D. J. Adams, D. J. Craik, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 13767; c) E. S. Lovelace, C. J. Armishaw, M. L. Colgrave, M. E. Wahlstrom, P. F. Alewood, N. L. Daly, D. J. Craik, *J. Med. Chem.* **2006**, *49*, 6561.
[5] J. T. Blanchfield, O. P. Gallagher, C. Cros, R. J. Lewis, P. F. Alewood, I. Toth, *Biochem. Biophys. Res. Commun.* **2007**, *361*, 97.
[6] D. W. Sandall, N. Satkunanathan, D. A. Keays, M. A. Polidano, X. Liping, V. Pham, J. G. Down, Z. Khalil, B. G. Livett, K. R. Gayler, *Biochemistry* **2003**, *42*, 6904.
[7] R. J. Clark, H. Fischer, S. T. Nevin, D. J. Adams, D. J. Craik, *J. Biol. Chem.* **2006**, *281*, 23254.
[8] N. Satkunanathan, B. Livett, K. Gayler, D. Sandall, J. Down, Z. Khalil, *Brain Res.* **2005**, *1059*, 149.
[9] a) M. Vincler, J. M. McIntosh, *Expert Opin. Ther. Targets* **2007**, *11*, 891; b) M. Vincler, S. Wittenauer, R. Parker, M. Ellison, B. M. Olivera, J. M. McIntosh, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 17880.
[10] M. Schnölzer, P. Alewood, A. Jones, D. Alewood, S. B. H. Kent, *Int. J. Pept. Protein Res.* **1992**, *40*, 180.
[11] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776.
[12] S. H. Hu, J. Gehrmann, P. F. Alewood, D. J. Craik, J. L. Martin, *Biochemistry* **1997**, *36*, 11323.
[13] E. L. Millard, N. L. Daly, D. J. Craik, *Eur. J. Biochem.* **2004**, *271*, 2320.
[14] S. T. Nevin, R. J. Clark, H. Klimis, M. J. Christie, D. J. Craik, D. J. Adams, *Mol. Pharmacol.* **2007**, *72*, 1406.
[15] a) B. Callaghan, A. Haythornthwaite, G. Berecki, R. J. Clark, D. J. Craik, D. J. Adams, *J. Neurosci.* **2008**, *28*, 10943; b) B. Callaghan, D. J. Adams, *Channels* **2010**, *4*, 1.
[16] H. Pan, Z. Wu, H. Zhou, S. H. Chen, H. Zhang, D. Li, *Pharmacol. Ther.* **2008**, *117*, 141.
[17] a) J. A. Ballestero, P. V. Plazas, S. Kracun, M. E. Gomez-Casati, J. Taranda, C. V. Rothlin, E. Katz, N. S. Millar, A. B. Elgoyhen, *Mol. Pharmacol.* **2005**, *68*, 822; b) R. G. van Kleef, H. P. Vijverberg, R. H. Westerink, *Toxicol. In Vitro* **2008**, *22*, 1568.
[18] G. J. Bennett, Y. K. Xie, *Pain* **1988**, *33*, 87.

